Resistance of Environmental Bacteria to Heavy Metals and Antibiotics in Selected New Zealand Soils

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Abstract

The usage of superphosphate fertilizers, animal remedies and other material containing heavy metals (HMs) in agriculture and horticulture is a problematic issue resulting in accumulation of HMs in the soil. The presence of these HMs in soil leads to the induction of resistance of environmental bacteria to these heavy metals, and may co-select for resistance against a broad range of antibiotics (Abs). This co-selection may increase the health risk for both human and livestock because of resistance to the wide range of Abs. As well as direct health effects, an increase in Ab resistance may impose a significant burden on the livestock industry and primary production, leading to potential for immense social and financial losses. The current project was aimed to investigate resistance of soil-borne bacteria sampled from selected regions of New Zealand. Genetic diversity of these bacteria and molecular aspects of horizontal transfer of HM and Ab resistance genes to other bacterial hosts was also investigated.

Soil samples with different history of usage, including pastoral and arable with high levels of HMs (e.g. cadmium (Cd) and zinc (Zn)) were collected from the Waikato region (WR), as well as soil from an area of native bush (background) as control. Waikato Region (WR) is one of the regions in New Zealand with high levels of HMs in soil, due to the regular use of HM-containing superphosphate fertilizers and animal remedies. Belmont Regional Park (BRP) airstrip soil was used as a novel site to explore bacterial communities' resistance to HMs, and any co-selected Ab resistance. A comprehensive investigation was performed to simulate the soil environment contaminated with various levels of HMs to interrogate induced resistance to HMs and Abs in soil bacterial communities using microcosms with 6 week and 6 month incubation.

The experiments carried out to investigate soil bacterial resistance to HMs and Abs were divided into two different categories, including physiological and molecular experiments. The physiological tests included plate culturing with a range of HMs and Abs concentrations and Pollution Induced Community Tolerance (PICT) analysis. Molecular investigations using Terminal Restricted Fragments Lengths Polymorphism (TRFLP) and Next Generation 16s rDNA were

conducted to determine the probable changes in bacterial community structures induced by selection pressure of HMs presence in soil samples. Cd resistance genes were detected in individual bacterial isolates using specific oligomeric DNA primers via the polymerase chain reaction (PCR). Horizontal transfer of these genes to new bacterial recipients was investigated. Finally, Cd resistant bacterial isolates involved in Horizontal Gene Transfer (HGT) were identified using 16s rDNA Sanger Sequencing.

Results clearly showed that there were significant differences between the levels of resistance to HMs and Abs in bacterial isolates from WR's pastoral and arable soils compared to background soil (native bush). Differences between BRP soil samples with higher levels of HMs compared to those with lower HMs concentrations, and also microcosms' with a range of HM levels showed there were significantly greater number of bacterial isolates resistant to HMs and Abs in soils with the higher initial levels of HMs. Pollution Induced Community Tolerance (PICT) analysis provided complementary results in concordance with the results of plate culturing experiments and showed the higher levels of bacterial resistance to HMs and Abs in soils with the higher initial levels of HMs.

Terminal Restriction Fragment Length Polymorphism (TRFLP) and 16s rDNA Next Generation Sequencing experiments investigated HM-induced bacterial communities structure changes and revealed significant differences among the bacterial community structures in the selected BRP and microcosms soil samples.

The HGT experiments revealed the horizontally transfer of Cd resistance genes from donor isolates (from WR, BRP and microcosms soils) to a characterised recipient bacterial strain in vitro, suggesting these genes were carried by mobile genetic elements.

Overall, the result of the current project showed that there were higher levels of bacterial resistance to HM and also to Ab occured while different levels of HMs were present in soil. In addition, higher levels of HM and Ab resistance induction occurred in presence of specific concentrations of HMs in microcosms' soils. The bacterial community structures were changed

in the presence of various levels of HM in soil. The investigation of bacterial community structures changes in microcosms containing background soil samples were greater compared to the microcosms containing pastoral soils; it is concluded in higher changes in bacterial communities in soils in presence of selection pressure of HMs. Cd resistance genes located on mobile genetic elements were able to be transferred horizontally form donor bacterial starins to recipients and the transconjugants showed resistance not only to Cd, Zn and/or Hg, but also to a range of Abs; it showed the possibility of spread of these HM resistance genes to the new bacteria in soil and conferring HM and subsequent Ab resistance in recepients.

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Yours Faithfully

Ali Heydari

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Abbreviations:

6WCdM: 6 Weeks incubation Cd-spiked Microcosm
6WHgM: 6 Weeks incubation Hg-spiked Microcosm
6WM: 6 Weeks incubation Microcosm
6WZnM: 6 Weeks incubation Zn-spiked Microcosm
Ab: Antibiotic
AbR: Antibiotic Resistant
Amp: Ampicillin
AmpR: Ampicillin Resistant
B6MCdM: Background Soil-contained 6 Months incubation Cd-spiked Microcosms
B6MHgM: Background Soil-contained 6 Months incubation Hg-spiked Microcosms
B6MM: Background Soil-contained 6 Months incubation Microcosms
B6MZnM: Background Soil-contained 6 Months incubation Zn-spiked Microcosms
BM: Broth Microdilution
BRP: Belmont Regional Park
Cb: Carbenicillin
CbR: Carbenicillin Resistant
Cd: Cadmium
CdR: Cadmium Resistant
Cm: Chloramphenicol
CmR: Chloramphenicol Resistant
EC50: Half Maximal Effective Concentration
Ery: Erythromycin
EryR: Erythromycin Resistant
Hg: Mercury
HgR: Mercury Resistant
HGT: Horizontal Gene Transfer
HM: Heavy Metal
HMR: Heavy Metal Resistant
IZ: Inhibition Zone
MIC: Minimum Inhibitory Concentration
NGS: Next Generation Sequencing
NMS: Non-metric Multidimensional Scaling
P6MCdM: Pasture Soil-contained 6 Months incubation Cd-spiked Microcosms
P6MHgM: Pasture Soil-contained 6 Months incubation Hg-spiked Microcosms
P6MM: Pasture Soil-contained 6 Months incubation Microcosms
P6MZnM: Pasture Soil-contained 6 Months incubation Zn-spiked Microcosms
PCR: Polymerase Chain reaction
PICT: Pollution Induced Community Tolerance
RPM: Replica Plate Mating
SD: Standard Deviation
SmR: Streptomycin Resistant
Tc: Tetracycline
TcR: Tetracycline Resistant
TRFLP: Terminal Restriction Fragment Length Polymorphism
WR: Waikato Region
WRSS: Waikato Region Soil Set
Zn: Zinc
ZnR: Zinc Resistant

Chapter 1, Introduction

A range of human-related activities can result in the progressive accumulation of HMs in specific environmental compartments including the biosphere, and agricultural and horticultural soils, which sit at the base of the human food chain [1]. Permeation of HMs into the soil causes selection for bacterial HM resistance. It is now known that HM resistance frequently occurs together with co-selection toward antibiotic resistance, through the same or parallel mechanisms [2]. This co-selection for HM and Ab resistances introduces the potential for an increase in resistant microbial infections in both humans and livestock, which is one of the biggest threats to human health according to World Health Organisation (WHO) [3]. As well as direct health effects, an increase in Ab resistance may impose a significant burden on the livestock industry and primary production, leading to potential for immense social and financial losses [4].

In New Zealand and several other countries (including Australia and the United Kingdom), the long-term annual and widespread use of phosphate fertilisers since the mid-1940s has caused significant increases in the concentrations of the contaminant element Cd in productive soils [5]. This is in addition to increases in the nutrient elements (P, S and Ca). Accumulation is highest in the long-standing dairying regions of NZ, e.g. Waikato dairy farms, where the current average concentration of Cd in these soils is ~0.7 mg kg^{-1} compared with a background average of 0.16 mg kg^{-1}.

More recently, the substantial use of zinc (oxide and sulphate) in sheep and cattle as a preventative for facial eczema (pithomycotoxicosis disease) has resulted in a rapid accumulation of zinc in soils of many farms in the upper North Island of New Zealand [6].

Mercury is another naturally occurring metal with a high toxicity for biota. The mercury cycle in New Zealand is influenced equally by both natural (e.g. volcanic activities and soil mercury volatilization) and human-related activities (e.g. cement and steel manufacturing, fuel

consumption, waste deposition) and it is estimated that its emission is about 3000 kg/year over the whole country.

Ab resistance is a growing issue. Even though New Zealand is not using many Abs directly, ongoing use of various other substances in farming is causing the problem to get progressively worse.

For the current PhD project, environmental soil samples were collected from specific sites in two regions.

- In the Waikato region (WR), sites were selected from the Waikato Regional Soil Monitoring Programme (with the assistance of the Waikato Regional Council). Sampling targeted three sites that are near to each other and share the same soil type, but have different land-use histories: a native (or background) soil, a property used for cropping (horticulture), and a property used for pastoral agriculture. Total phosphorus, cadmium and zinc concentrations in these soils followed the order background < cropping < pastoral, corresponding to their individual histories.
- In the Wellington region, a pastoral farm property located in Belmont Regional Park (BRP), which is owned and leased for farming by the Greater Wellington Regional Council was sampled. This site features a fertiliser storage bin and farm airstrip, and is of interest because it shows both substantial cadmium enrichment, and a distinct concentration gradient. Both of these features reflect the extent of spillage of superphosphate fertiliser during loading and take-off of topdressing aircraft. Near to the fertiliser storage bin and aircraft loading area, cadmium concentrations are very high, and these progressively decrease with distance down the airstrip (Kim, N. unpublished results). This contamination gradient allowed investigation of how bacterial population and diversity might differ with Cd concentration at a real site.

This research also used spiking of soil samples (from the WR) with Cd, Zn or Hg in small laboratory microcosms. Spiking experiments had the advantage of allowing control over a single variable (e.g. the Cd concentration).

The annual antibiotic usage in New Zealand is about 75 tons and about half of this amount is used in farms and livestock husbandries. In New Zealand, antibiotics themselves are not used as general growth promotants, and a strong emphasis is placed on ensuring that antibiotic residues do not enter food products such as milk [7].

1.1. Aims and Objectives of This Project

The four aims of this project were as follows:

1. To establish, in selected soils, whether observable microbial resistance to Cd, Zn and Hg shows an association with agricultural land use history.
2. To establish, in the same soils, whether parallel associations exist for resistance to selected Abs.
3. To determine if HMR and co-selected AbR is associated with the presence and magnitude of contamination with Cd, Zn and Hg.
4. To quantify and provide information to distinguish bacterial community structure differentiations due to selection pressure caused by HMs (Cd, Zn, or Hg) presence in soil.

To explore Aims 1 and 2, soil samples were collected from farming areas in the Waikato and Wellington regions of New Zealand. Waikato samples were from nearby properties of the same soil type that had very different land use histories. One was a background ('native') soil, one was a long-term arable farm, and one was a long-term pastoral farm. Across these three land use types, additions of the metals and other trace elements from fertiliser, pesticide or animal remedy use increased in the order background (none) > arable > pastoral (most). Wellington region samples were from a farm airstrip site which has a long history of being used for fertiliser topdressing. This site (all of the same soil type) features a strong concentration gradient of the

contaminants found in superphosphate fertiliser. The most prominent anthropogenic enrichments in soils of the Waikato and Wellington farm sites are P, Ca, F, Cd, and U (from P fertiliser) and Zn (from three sources, depending on land-use).

Mercury is not significantly enriched on Waikato region farmed sites, and so provided a useful comparator element. For Hg in these samples, induction of resistance would be either natural or secondary to resistance induced by the other contaminants.

Further investigations using microcosms comprising background and pasture soils spiked with Cd, Zn or Hg were performed. Due to soil availability these soils used for the microcosm experiments from a separate Waikato region site.

Aims 3 and 4 were investigated using the bacterial isolates extracted from the soil samples from WR and BRP and also the soil samples from microcosms.

Across the work various workups, chemical and biochemical measures were undertaken on selected samples to progress all four of the aims.

1.2. Structure and Layout of This Thesis

Chapters 1 and 2 are the introductory parts of the thesis. **Chapter 1** provides the general background and research aims, and **Chapter 2** is a literature review providing a more detailed overview of the study context and current research gaps.

The research parts of this thesis, which took the form of four different studies, are presented as **Chapters 3 to 7**.

- **Chapter 3** provides details of all methodologies used.
- **Chapter 4** presents findings of investigations into bacterial HM and Ab resistance in WR and BRP soils. This part of project focuses on bacterial populations, and the resistance or tolerance of bacterial isolates to different concentration of HMs and Abs in the selected soil samples. The hypothesis was that there are higher number of bacterial population resistant

to HMs and Abs in soils with higher levels of HMs compared to those with lower concentrations of HMs. In addition, it was hypothesised that there are greater level of resistance to HMs and Abs in bacterial isolates from soils with higher levels of HMs.

- **Chapter 5** covers bacterial resistance to HMs and Abs in microcosms soils spiked with a range of Cd, Zn and Hg. This study focused on the bacterial community dynamics in microcosms, containing soil and various concentrations of the HMs, while incubating over a span of time. The hypothesis was that some specific concentration(s) of HMs could induce resistance in bacterial isolates while present in soil, and concentrations outside this range have different effects.
- In **Chapter 6** results are given for experiments examining bacterial community structures using DNA-based molecular methods, including TRFLP and Next Generation 16s rDNA Sequencing to investigate the probable differences into the bacterial genome and genes due to the presence of difference levels of HMs in their biosphere. This study focused on the genomic DNA extracted from the 6-week incubation microcosms' soil and BRP selected soils to explore the presence and frequency of resistance genes to HMs and Abs in varied conditions.
- In **Chapter 7** results are presented for work investigating horizontal transfer of Cd resistance genes. This was examined by conjugating recipients with suitable Cd resistant bacterial donor cells. The hypothesis tested in this study was that genes encoding resistance to Cd were able to be transferred horizontally to other bacterial isolates and resistance induced in the new recipient of these resistance genes.

Of these, **Chapters 4 and 5** relates particularly to Aims 1, 2 and 3, and **Chapters 6 and 7** relate to Aim 4. However, there is some overlap as all results are relevant.

Each of the results chapters is provided with its own discussion. These are brought together in **Chapter 8** as an overall discussion of key findings, conclusions, and recommendations.

Chapter 2, Literature Review

2.1. Heavy Metals and Their Biological Effects

Most HMs have a fundamental task in the life processes of microorganisms and higher organisms and act as essential elements in the environment. Heavy metals can be added to the environment by a variety of different human-related activities or natural processes [8, 9]. Heavy metal ions catalyse many complicated biochemical reactions in cells and form redox-active complex compounds, thus playing a substantial role as trace elements. Some HMs, such as Cd (with the exception of functioning as an essential element in the physiology of some bacterial species [10]), Hg, Ag, Al, Pb and Au, have no beneficial effect. Instead they bring about detrimental consequences in the cell.

Some other metals, for instance cobalt, calcium, magnesium, zn, iron, manganese, sodium, copper, nickel, potassium and chronium are necessary elements for cellular metabolism and other life processes [10-15]. For example, Zn and magnesium are involved in stabilizing many enzymes, RNA and DNA via electrostatic forces [11, 16]. Iron, nickel and copper are required for redox reactions [10, 11]; and magnesium, iron, cobalt, chronium, copper and nickel are components of vital molecules involved in diverse biological reactions e.g. photosynthesis, vitamin B12 metbolism, urease, cytochrome and cytochrome-c-oxidase [10, 17, 18]. However, in high concentrations these ions can have toxic effects, by producing complex compounds in the cell. For example, the presence of high concentrations of metals in cells results in metal ions with higher affinity binding to thiol groups or oxygen sites than metals required for cell function. Essential cations like Zn^{2+}, Cu^{2+} and Ni^{2+} can be toxic at higher concentrations [18-21].

The formation of deleterious toxic complexes by some cations like Cd^{2+}, Hg^{2+} and Ag^{+} makes them hazardous agents to all kinds of life [19, 22]. Fortunately, because of their unavailability to living cells in the environment, most HMs are not particularly harmful and do not have major toxic effects on biological processes. This is due either to the low concentration of these

elements in the environment or low solubility of the metals [10, 14, 16, 18, 21, 23]. For instance, tin, cerium, gallium, zirconium and thorium have low solubility and do not have any characterised influence on biological reactions [14]. Bio-availability and toxicity of metals in the environment is considerably influenced by environmental conditions such as pH, redox potential and organic matter content [10, 16, 18, 24, 25]. Briefly, oxygen levels and the concentration of organic matter act inversely; hence, higher concentration of oxygen and lower pH increase metal solubility, whilst higher concentrations of organic matter decrease metal solubility. Some metals are considered trace elements with low toxicity (e.g. iron, molybdenum and magnesium). Zn, nickel, vanadium, cobalt, copper, tungsten and chronium are also considered trace elements, but with more toxicity compared to the previous group [10, 14, 18, 26]. Cd, Hg, silver, arsenic, antimony, lead and uranium have few advantageous effects on organisms and are mostly considered as toxic metals [10, 12, 14, 17, 18, 24]. Since the focus of this thesis is on Zn, Cd and, Hg (members of Group 12 [IIb] of the periodic table), the role and importance of these metals on cells and biological processes are discussed.

Zinc, as one of the most important and necessary elements for the living cells, can be found in the environment particularly as the divalent cation Zn^{2+} and is involved in diverse cellular processes. For example, as well as being found in a huge number of enzymes, Zn is an important part of DNA-binding proteins [11, 14, 15]. Zn is not active as a biological redox agent; so does not have any action in respiration [21]. Especially at high concentrations of Zn bacterial cells can absorb Zn by a fast and universal mechanism. This kind of accumulation uses several mechanisms including the CorA (MIT) Mg^{2+} transport system or MgtE system [14, 21].

Cadmium is more toxic than Zn because of differences in their chemical compounds solubility; for example the solubility is 1.4×10^{-29} K_{sp} (at 25°C) for CdS and 2.91×10^{-25} K_{sp} (at 25°C) for ZnS [14]. Cd can enter cells as divalent ions using uptake systems for manganese and some other metals and can be accumulated 3-15 times more in Cd-susceptible bacterial cells compared to resistant ones [11-13, 15, 19, 20, 23, 24]. The assumed mechanisms of the toxicity of Cd for living

cells imply an interaction with Zn metabolism, thiol-binding and protein denaturation, single strand breakage of DNA and interaction with Ca metabolism and membrane damage. Cadmium is probably able to enter yeast, bacteria and plant cells by using Mg, Mn and Ca uptake systems, respectively [10, 14, 16, 20, 22, 27].

Hg^{2+} has the strongest toxicity on *Escherichia coli* of the 3 HMs studied in this project and the MIC is 0.01 mM; the rate of solubility of HgS is 6.38×10^{-53} K_{sp} (at 25°C) [12, 14, 27]. Mercury's unique features include its redox ability (electrochemical potential of Hg(II)/Hg(0) is 430 mV at pH 7), and its low melting and boiling points, -39°C and 357°C respectively. Because of these characteristics and high probability of exposure of bacterial cells to toxic concentrations of this metal, resistance to Hg^{2+} is prevalent due to *mer* resistance determinants among bacteria [14]. Detoxification strategies by bacteria consists of reduction of Hg cations, which leave the cell by passive diffusion. This HM attaches to protein and enzyme thiols and inhibits their cellular functions [10-12, 16, 18, 22].

2.2. Heavy metals in New Zealand

Long-term and widespread use of phosphate fertilisers on New Zealand farms during the last several decades has caused major accumulation of Cd in patoral and arable soils. It has happened in parallel to increase of the nutrient elements levels (e.g. sulphur, and calcium) in soil. The highest levels of Cd accumulation has happened in the dairying regions of NZ with a long history of intensive livestock production, where the current average Cd concentration is ~0.7 mg/kg compared with a background average of 0.16 mg/kg [5]. Early concerns about this issue were reviewed by Bramley in 1990 [28]. In the mid-late 2000s a Cadmium Working Group (CWG) was convened by the then Ministry of Agriculture and Forestry to consider the risks of Cd accumulation in NZ agricultural soil. A long-term National Cadmium Management Strategy was developed, which is overseen by a Cadmium Management Group (CMG) through the Ministry for Primary Industries. Warne (2011) and Cavanagh (2012) report that the recent average rate

of Cd accumulation in NZ soil is about 5 µg/kg soil/year, which is likely to be slightly lower than the historic accumulation rate for a number of reasons, e.g. the source of the phosphate rock which is used for manufacture of superphosphate and response of farmers to financial constraints [5, 29].

More recently, the substantial use of Zn (oxide and sulphate) in sheep and cattle as a preventative for facial eczema (pithomycotoxicosis disease) has resulted in a rapid accumulation of Zn in soils of many farms in the upper North Island of New Zealand [30]. Sowry (2011) showed that average Zn concentrations in Waikato farm soils have doubled (from 30 mg/kg to 60 mg/kg) in last decade, with about 10% of pastoral soils exceeding 100 mg Zn /kg. Sowry (2011) estimated 60-100 percent of pastoral farms in the Waikato region use Zn compounds against facial eczema disease annually, most of which is excreted and deposited in the paddock (about 8500 tons/year) [6]. Zinc accumulation happens more easily in allophanic and granular soils, because these soils are more adsorptive [31]. The most common type of Zn available in soil solutions and the main form responsible for Zn toxicity is Zn^{2+}. Zinc also accumulates in many horticultural soils through use of Zn-containing fungicides (zinc dithiocarbamates) on various plant crops [30].

Mercury is another naturally occurring metal with a high toxicity for biota. Common environmental forms of mercury are mercuric mercury (Hg^{2+}), methylmercury (CH_3Hg^+) and elemental mercury (Hg^0) [32]. The Ministry for the Environment commissioned reports on discharges of Hg to the New Zealand environment. Rumsby et. al. (2008, 2009) indicated the Hg cycle in New Zealand is influenced equally by both natural (e.g. volcanic activities and soil Hg volatilization) and human-related activities (e.g. cement and steel manufacturing, fuel consumption, waste deposition) and estimated its emission is about 3000 kg/year over the whole country. Of the various sources, the Hg naturally contained in superphosphate fertilizers is estimated to contribute about 40 kg of Hg to New Zealand soils each year [33]. This mass load is not enough to cause noticeable accumulation of Hg in New Zealand's productive soils (the

comparative figure for Cd is 30-40 tonnes/annum). Overseas however, contamination of productive soils with Hg can become a problem. One common example is where Hg is discharged to local environments through its use at illegal ('wildcat') gold-mining sites [34].

2.3. Metal Uptake Systems

Metals enter eukaryotic and prokaryotic living cells by two different methods, either ATP-dependent or ATP-independent. The first type needs the energy provided by ATP hydrolysis and is established by the cells in necessary and special situations. The second and more common process does not require ATP hydrolysis and is faster and less substrate-specific compared to the ATP-dependent process. ATP-independent metal transport works based on chemiosmotic gradients through the cytoplasmic membranes of bacterial cells [8, 10-15, 17, 19, 24, 35]. The first mechanism plays an important role in uptake and accumulation of HMs in the cell. However the ATP-independent uptake processes are more efficient either in terms of energy use and results in the influx of a wider range of metals. This may result in higher concentration of some metals in the cell, than result from energy-dependent mechanisms [14, 23]. The reason for the greater prevalence of ATP-independent metal uptake systems among microorganisms is probably due the lower biological cost that resistant cells invest in efflux pump encoding genes compared to specific uptake systems for each metal [20].

The biochemical cycling of the metal ions is strongly influenced by bacterial metal resistance and the relative solubility and toxicity of the metals as well. In Gram-negative bacteria, Archaea and some yeasts, like *Saccharomyces cerevisiae,* Zn^{2+}, Cd^{2+} and Hg^{2+}can enter cells by a membrane-integrated, nonspecific magnesium uptake system protein (CorA), a member of membrane inorganic transporters (MIT) family. In this process the metal cations are accumulated in the cells by the CorA Mg^{2+} uptake system [13, 14, 16, 23].

In addition, there are energy-dependent metal channels, such as ATP-binding cassette (ABC) transporters, for the uptake of Zn^{2+}, Ni^{2+}, Fe^{2+} and Mn^{2+} [10, 12-15, 17, 23, 36]. Membrane-bound

P-type ATP-dependent transporter protein is active in carrying many metal cations into cells, for example, Zn^{2+}, Cd^{2+}, Ag^{+}, Pb^{2+}, Ca^{2+}, Mn^{2+}, Mg^{2+}, K^{+} and Cu^{2+} [8, 10-15, 19-21, 23, 24]. Although the concentration of metals in the cells increases markedly, these non-specific transport proteins can lead to the uptake of high amounts of toxic HMs indiscriminately in contaminated environments [11, 20, 23]. This can facilitate a common reason for metal toxicity. The activity of enzymes can be blocked by binding to cations, such as Cd^{2+} and Hg^{2+}, due to their binding to sulphydryl groups and producing sulphide substituents immediately after entering to the cells [10, 14-16, 25]. The toxic activities of some HMs can be observed by their interference with the physiological action of other metals, e.g. Cd^{2+} with Zn^{2+}, Cd^{2+} with Ni^{2+}, Cd^{2+} with Ca^{2+} and Zn^{2+} with Mg^{2+}. These toxic features of HMs along with interference of metal oxyanions in action of fundamental metabolites and producing harmful products, such as chromate and arsenate, are strong selective pressures on microbes to develop HM resistance mechanisms [10, 12, 16, 17, 23, 37].

2.4. Wide Range of Metal Resistant Organisms in the Nature

There is mounting evidence that microbial biofilms show more tolerance to HMs and antibiotics than single microbial cells. This appears to be due to different structural and physiological transformations, which are in many aspects similar to the cellular processes of multicellular organisms [8, 16, 18, 36]. Biofilm production, as the most prominent lifestyle of microorganisms accumulating in a self-produced extracellular matrix in nature, is a tolerance strategy against HMs and antimicrobial substances. This kind of resistance can be varied in different biofilms based on the nature of the microorganisms and antimicrobial agents, and finally the environment conditions; as some examples, (i) metabolic differences among microbe populations is due to the different biofilm structures; (ii) the extracellular materials surrounding the biofilm influence on cells physiology; (iii) the biosorption of metal ions affects the metals movement; (iv) metal ions undergoing chemical reactions with extracellular matrix decreases their toxic properties [8, 10, 16, 37].

A wide range of organisms can tolerate an array of different HMs concentrations. For example, Gram-negative bacteria are more tolerant to metal ions and can protect the normal cellular functional pathways more effectively in comparison with Gram-positive bacteria. For example *E. coli* and *Pseudomonas aeruginosa* are able to tolerate Cd(II) several times more than Gram positive isolates like *Staphylococcus aureus* and *Bacillus subtilis*. Conversely, some Gram positive isolates are able to bind to Cu(II) significantly more compared to *E. coli* [11, 36, 38, 39]. Transportation of Cd ions attached to glutathione molecules with YCF1p protein is one example of metal resistance in *Saccharomyces cerevisiae* and multidrug resistance in human and many other eukaryotic cells may be a related product of *YCF1p* gene [14]. *Candida tropicalis* and *C. albicans* are widespread human pathogens which show dramatic resistance to some of HMs like Cd, Hg and Pb and are able to accumulate and remove Cd cations especially from aquatic environments [16, 25, 27]. The human pathogen *Rhodotorula* sp. are able to tolerate significant concentrations of metals in the range of under 100 mg 1^{-1}[40]. The soil-borne human pathogenic fungus *Trichosporon asahii* is resistant to Cd^{2+} and is able to tolerate significant concentrations of this metal (10 mM) [25]. Different species of *Aspergillus* sp., such as *A. aculeatus* and *A. foetidus,* show high resistance to Cd and are able to improve the capability of their herbal host to bio-accumulate metal from contaminated soils [41, 42]. The species of *Thiobacillus* sp. resistant to Cd, Hg, Zn and Cu not only show significant resistance to these metals, but indicate potential to bio-remediate contaminated soils [12, 13, 17, 26].

2.5. Metal Resistance Mechanisms

Development of metal resistance in cells can be achieved by reducing metal availability and modifying cells constituents to protect vital cell components. This issue can be influenced by various factors such as specific characteristics of metal uptake mechanisms, the function that each metal performs in the cell during physiological reactions, and the structure and location of acquired genetic determinants of resistance on plasmids or chromosomes [10, 11, 16, 41]. The importance of the last factor is due to the differences between resistance systems induced by

plasmid, chromosomes or transposons genes. Plasmid-located resistance genes encoding metals' toxic-ions efflux mechanisms can be transferred to new recepients by horizontal gene transfer mechanisms, e.g. conjugation, more easily than chromosome-located genes. The more convenient transfer of toxic-ion efflux mechanisms between bacterial cells, suggests that these mechanisms are plasmid-borne [8, 10, 22, 23].

Although, some scientists recognise three different HM resistance mechanisms in microorganisms, others categorize these mechanisms into 5-6 different sorts including: (i) active efflux of toxic metal ions from cells; (ii) the intracellular or extracellular segregation of cations, especially those with affinity for sulphur groups of cellular components and accumulation as intracellular complexes by thiol-containing molecules; (iii) reduction of toxic metal ions to a less toxic oxidation state; (iv) HM exclusion using permeability barriers and (v) diminishing the sensitivity of cellular targets to toxicity of HMs [10-12, 14, 18, 19, 21].

2.5.1. Active Efflux of Metals from Bacterial Cells

Chromosomal or plasmid-encoded efflux mechanisms to transfer metal ions out of the cells can be considered as the most common metal resistance mechanisms among microorganisms [11, 14]. These mechanisms are either energy-dependent or independent which can be observed in ionophores and uncoupling proteins which block efflux of ions by transforming the energy to heat instead of producing of ATP [11, 15]. There are various examples of the plasmid-borne systems in bacteria, such as Pb and Zn resistance caused by ZntA, ZiaA and CadA proteins in *E. coli*, *Synechosystis* sp. and *S. aureus* respectively [12, 13, 19, 21, 23]. Other examples of plasmid-borne systems are ATP-independent Cd, Zn and Co resistance system of *Alcaligenes sp.* encoded by the *czc* operon [11, 14, 17, 18, 20, 23, 24, 36], *ars* operon-encoded resistance of *S. aureus* and *E. coli* to arsenic and arsenite as an ATP-dependent system [11, 15, 17, 20, 21, 24], and P-type *cad* operon-mediated resistance of *S. aureus, E. coli* and *Bacillus sp.* to cadmium and zinc [12, 13, 19]. There is a group of metal efflux mechanisms designated as the RND family, powered by

the proton gradient involved in expelling the Cd, Zn, potassium, copper and magnesium ions outside the cell. Unlike the P-type transport system, which is able to expel metal ions from the cytoplasm, the RND is a special efflux system that transfers metal across the outer membrane and cell wall in Gram-negative bacteria; so designated as a trans-envelope transport system [14, 15, 21, 23, 36].

The plasmid-mediated *cad* and *czc* systems are the most important Cd resistance mechanisms [14, 20]. The investigations proved the existence of two main different patterns, including a single *cadA* locus and a complex of the *cadA*, *cadB* and *cadC* loci involved in Cd resistance [11, 12, 17, 19, 20]. The cadA protein which has many similarities with P-type and E_1E_2 ATP-dependent metal transporting systems, acts as the main efflux agent [11]. It has been hypothesized that, on the other hand, CadC protein produces two cysteine domains that bind Cd^{2+} and makes these ions accessible to CadA in the cytoplasmic membrane [13, 15]. When Cd ions are present, CadA becomes phosphorylated and the high energy of this protein is stable until metal ion efflux. One of the novel examples of CadC function can be seen in *E. coli*; owing to lack of ion antiporters in this bacterium, Na^{+2} is delivered to CadA to be expelled out of the cell. In addition, it has been suggested that CadC binding to the *cad* promoter leads to downregulating transcription. A Cd resistance transposon Tn5422-encoding CadA and CadB has been found in *Listeria* species, which exist in a variety of environments such as food [11, 13, 15, 17, 20].

The *czc* system is a well-known example of an RND system, like *cad*, that can combine with the Mn^{+2} transport system and trigger the efflux of Cd. This system, which acts as a zinc-proton antiporter, is encoded either by plasmids such as pMOL28 and pMOL30 or chromosomal loci including *czcA*, *czcB*, *czcC*, *czcD*, *czcI* and *czcN* [11, 17]. However, the function of CzcI and CzcN proteins has not been demonstrated, the proteins encoded by the four first genes construct the efflux pump, constituting a membrane channel and transferring cations out of the cytoplasm via CzcA protein. This protein, as the biggest *czc*-encoded protein, acts like a proton tunnel and

provides metal transmission by a charged-relay mechanism. CzcA can induce resistance to Cd, Zn and cobalt. Presumably, binding ions from czcA and transferring them to the outer membrane is the function of czcB. This protein is probably responsible for metal specificity and has 8 histidine residues for metal binding and removal of these residues reduces, but not eliminates, Zn resistance. CzcC, like CzcB, resides in the periplasm and acts as a sub-factor for the *czc* system to prepare the membrane channel and exports metal ions outside the cell. Lacking CzcC leads to sensitivity to Cd and to some extent to cobalt, but does not alter Zn resistance. CzcD as the product of a fragment located downstream of *czcA*, *B* and *C* genes, activates the efflux pump. The *czcD* gene is related to a large family of transporter proteins named CDF, which are mostly involved in Zn and cadmium transport [11-15, 17, 19, 20, 23, 24, 36]. Two members of this family act in *S. cerevisiae* to induce Zn and Cd resistance [14, 15, 23]. Detoxifying Zn from the cytoplasm by ZnT1-encoded efflux proteins is one example of a functioning CDF system in mammalian cells [14, 23].

2.5.2. Intracellular and Extracellular Sequestration of Metals Using Protein Binding

The most common metals undergoing intracellular sequestration are Cd, Zn and copper. They are accumulated in the cytoplasm of the cell to prevent their exposure to cellular constituents [11, 36]. Two good examples of this mechanism have been described in *Synechococcus* spp., and sewage isolates of *Pseudomonas* spp., which produce metallothionein and Cys-included proteins respectively [11-13, 19, 20, 24]. *Synechococcus* spp. carry the *smtA* and *smtB* genes. The metallothionein encoded by *smtA* gene binds to the metals. The product of the *smtB* gene has a regulatory function and impedes the transcriptional activities of *smtA* and prohibits of metallothionein production. The Cys residues of metallothionein absorb and accumulate metal cations. The Cys-enriched metallothioneins encoded by *Pseudomonas* spp. are active in binding and accumulating Cd ions in the cell [10, 11, 13, 20].

The extracellular segregation of HMs is achieved by attaching the metals to different complexes and inhibiting their ability to enter cells. This phenomenon has been found in a wide range of organisms such as bacteria, yeasts and fungi [11]. Constructing Cd complexes outside the cell leads to the resistance of some yeast and bacterial spp. like *Citrobacter* to this metal [11, 17]. Producing oxalate extracellularly and binding metal ions helps some fungal species to show resistance to copper and Cd [11, 41]. Strong attachment of glutathione to HMs results in diminished absorption of the nickel in resistant yeasts carrying the methylglyoxal resistance gene [10, 14, 23, 25, 37].

2.5.3. Enzymatic Detoxification of Metals

The efficiency of all metal resistance mechanisms against Cd has been demonstrated, except minimizing the toxic state of ions by enzymatic detoxification [11]. Metals, such as Cd, Zn, nickel and cobalt do not involve in enzymatic NADPH-related resistance. In addition, because of high toxicity and instability of these metals, the covalent states of these metals are not physiologically compatible with the living cells processes. Therefore, they undergo oxidation for being transformed to the neutral state. The best example of resistance by enzymatic detoxification and reduction to metal is with mercury. The resistance to this metal has been shown in several bacterial species such as *S. aureus*, *E. coli*, *P. aeruginosa* and some species of *Bacillus* spp. [11, 12, 14, 19, 20, 25].

Investigation has demonstrated the involvement of 5-6 genes of the *mer* operon in mercury resistance and there are two groups of organisms resistant to this metal, designated as narrow and broad spectrum resistance [10, 12, 14, 15, 17, 18, 20, 36]. The *mer* operon is found on plasmids or transposons in different bacteria, for instance transposons Tn501 in *P. aeruginosa* and Tn21 in *Shigella flexneri*, pTM314 plasmid in *Thiobacillus ferrooxidans* and pDU1358 plasmid in *Serratia marcescens*. [11, 12, 26, 36, 43]. The similarity of genes located on these plasmids and transposons is high. For example, *merP* and *merT* genes exist in all HgR resistant organisms

and are required for complete mercury resistance, whereas the presence of *merC* and *merB* varies in different oranisms [11, 15]. Detoxification of mercury is triggered by a group of proteins encoded by the *mer* operon as well as its transmission out of the cell. MerC and T, and P are considered transportation proteins, functioning in the cytoplasm and periplasm respectively. The results of mutagenesis of *merT* and *merP* revealed that these genes can function independently and *merT* products show more importance in inducing metal resistance. When there are no Hg ions in the cells, some regulatory genes are expressed and produce periplasmic binding proteins which participate in membrane transportation. These regulatory proteins then transfer the mercury ions out of the cell and transfer them to inner periplasmic space for subsequent detoxification. The periplasmic action of MerP supports survival of periplasm and inner membrane proteins containing thiol residues. The function of MerC is not clearly understood [11, 12, 14, 15, 17, 18, 20, 26, 36].

Acquiring resistance to mercury, in addition to these transfer proteins, is related to two essential enzymes encoded by the *mer* operon, including organomercurial lyase and mercuric reductase. The mercuric reductase is encoded by the *merA* gene, located downstream of the transport genes of the *mer* operon. The organomercurial lyase hydrolyses the bond between mercury and carbon to produce mercury thiolate and substituting glutathione with cysteine ligands. The product of this reaction is then used by mercuric reductase. Hg ions undergo reduction by mercuric reductase in a NADPH-dependent reaction [12, 14]. Unlike the organomercurial lyase which is a monomer and acts slowly, mercuric reductase is a homodimer and functions rapidly and completely specifically to mercury. At the end of reaction, the reduced mercury is expelled from the cell [11, 12, 14, 17, 19, 20]. Lacking the organomercurial lyase encoding genes in organisms makes them to suffer from sensitivity to mercury, and the presence of these genes induce production of mercury resistance-related enzymes and also high resistant to Hg [11, 14]. The regulation of the mer proteins' function is the responsibility of merR protein in the presence or absence of Hg ions. In the presence of Hg in the cell, an ion binds the Cys residues of merR,

which is attached to RNA polymerase binding sites of the promoter and induces an allosteric structural change in the merR binding sites and results in the attachment of RNA polymerase to the promoter and initiation of transcription. Expressing *merA* in yeast and different kinds of plants has been a great step toward the detoxification of HMs in environment especially agricultural soils [11, 12, 17, 19, 20].

2.5.4. Metal Exclusion by Permeability Barrier

Exclusion of metals by permeability barriers as a novel approach to preserve sensitive cellular components from harmful effects of HM ions, is achieved by conversion of the cytoplasmic membrane, cell wall and envelope of bacteria. As an example, a single mutation in the *E. coli* genome leads to a change in the cellular membrane channel protein porin, which results in exclusion of Cu (II). The ability of many bacteria to produce excess exopolysaccharides results in biosorption of several different cations. This function which is pH-dependent, helps bacteria such as *Pseudomonas putida* and *Arthrobacter viscosus* to prevent entry of toxic ions into the cell and interfering in important physiological processes [11, 22, 26, 36]. Membrane permeability results from conformational changes in membrane lipids and due to genes located on penicillinase plasmids in several *S. aureus* strains, prohibits entry of Cd cations and various other HMs [11, 20, 22]. A four gene operon in several *Pseudomonas* spp. encodes some membrane-integrated proteins. These proteins' function results in general absorption of metal ions, saturation of periplasmic binding sites and inhibition of metal ions inflow to the cytoplasm [10, 12].

2.5.5. Reduced Metal Sensitivity of Cellular Components

This mechanism, a non-biochemical natural resistance mechanism, induces resistance by decreasing the sensitivity of substantial cellular components to HMs or amplifying production of these components due to mutation in plasmid or chromosomal genes. These mutations reduces metal sensitivity and do not leave any effects on the respective components functionally [11,

20, 27, 36]. Increasing the production of cellular materials can be accomplished by changing regular cellular pathways to overexpress their componenets. Glutathione induces superficial resistance to HMs, such as Cd, Hg, copper and silver, presumably by inhibiting metal free radical production. An example of this mechanism has been described in *E. coli* with Cd ions. The bacterial resistant cells show a longer lag phase in sensitive cultures compared to subcultures in which the lag phase due to the establishing of resistance is shorter. This behaviour probably is because of DNA repair functions which have been damaged at the earlier exposure to Cd [11].

2.6. Heavy Metals and Agriculture and Health Issues

Environmental contamination, especially of soil, by HMs is one of the most important issues which endangers the environment and ecosystems, especially agriculture lands and aquaculture environments due to entering a huge amount of animal manure and water waste [18]. The most important HM contaminations in environment which endanger human ands animal health and trigger the resistance are copper, Hg, lead, Zn and Cd. Accumulation of HMs in biosphere, e.g. soil, is considered a leading issue endangering human and animal health [9, 12, 44]. Fertilizing agricultural land with organic or inorganic fertilizers, such as animal manures, sewage sludge and pesticides containing HMs and irrigation of farms with wastewater leads to the discharge of potentially large concentrations of metals into the soil. This is common, in developing countries, but also in developed countries. For example, arable soils are the most important HM contaminated environments in Britain [9, 18, 44]. Permeation of HMs into the environment due to agricultural activities and from fish farms, animal production and husbandry practices causes selection for bacterial HM resistance. The occurrence of metal resistance parallel with co-selection toward antibiotic resistance in microorganisms underlines the importance of preventing the accumulation of metals and subsequent resistance in the environment. The co-selection mechanisms of HMs and antibiotic resistances result in higher health concerns in human as well as imposing burdensome detriments to livestock industry and imposes immense losses socially and financially [21]. In spite of the potentially harmful effects of microorganisms

resistant to HMs in the environment, HM resistant bacterial species have potential for use in bioremediation of agricultural soils as do yeasts, fungi and plants species with metal resistance and bioaccumulation capabilities [14, 16, 17, 21, 26, 41, 42].

2.7. Antibiotics and Environmental Quandaries

The earliest antibiotics used in modern medicine were discovered from synthetic compounds from dyes [45]. The earliest records of the phenomenon of growth inhibation of some microorganisms by other microorganisms have been reported from 19th century. These observations led to the discovery of the first natural antibiotic in 20th century by Alexander Fleming [46]. Antibiotics are one of the most important and successful drugs historically, and have been faced with a great amount of resistance reactions among bacteria [47]. The first observation of resistance to an antibiotic was reported in 1950s [48]. The resistance to these drugs influences animal and human health in different aspects, e.g. getting difficult dealing with infectious diseases and other medical procedures resulting in immunosuppression. For example, tuberculosis caused by *Mycobacterium tuberculosis* is one of the diseases posing lots of treatment challenges due to multi-drug resistance [49]. Bacterial reaction to different antiobiocs can be categorised into susceptible, resistance and intermediate groups based on Minimal Inhibitory Concentrations (MICs) [47, 50]. The use of antibiotics can have a significant effect on natural environments because of the specific effects they might have on evolutionary resistance mechanisms. These effects can result in “biological pollution” due to the dissemination of resistance genes, located on transferable elements, among other bacterial species [47, 51-56]. Due to the transfer of antibiotic resistance genes among microorganisms, the diversity of these resistance elements might not be decreased by increasing the time and distance from the site of their direct selection. The abundance of bacterial isolates in environments, like farms and waste water treatment facilities makes them important sites for the selection of resistance mutations and propagation of microbial antibiotic resistance genes among strains and this process can be amplified in the presence of HMs [47, 51, 57-64].

2.8. Antibiotics in New Zealand

The annual antibiotic usage in New Zealand is ~75 tons and about half of this is used in farms and livestock industries. The use of streptomycin on NZ farms was more than 1.2 tonnes per year in 2000s [65]. The lack of adequate information about antimicrobial resistance among bacteria in New Zealand farms and livestock husbandries, prompted the establishment of a working group under supervision of the New Zealand Food and Safety Authority (NZFSA) in 2005 to monitor the issue in NZ agriculture [66]. Following this a comprehensive investigation was completed in 2009 to identify and measure the susceptibility or resistance of environmental bacteria to antimicrobial agents. The results showed some *Salmonella* isolates were resistant to streptomycin and sulphonamide and some *E. coli* isolates showed high resistance to ampicillin, chloramphenicol, tetracycline, streptomycin and sulphonamide [66].

In New Zealand, antibiotics are not used as general growth promotants, and a strong emphasis is placed on ensuring that antibiotic residues do not enter food products, such as milk. Sick animals requiring treatment are typically isolated to prevent the infection from spreading, and their effluent is not delivered directly to the farm soil. For these reasons, at a broad-scale level the direct inputs of antibiotics to New Zealand productive soils are generally negligible and (where they do occur) only temporary. As organic compounds, antibiotics which do reach soil will also be subject to degradation [7]. By contrast, at a broad-scale many New Zealand soils have been receiving significant inputs of both cadmium and zinc, that are not degraded and largely remain in topsoil [Kim, N.D. Personal communication 16 April 2016; based on information provided by Dr Grant Northcott (Northcott Research Consultants, Hamilton)].

2.9. Antibiotics and Gene Function

Prior to the introduction of the sulphonamides as the first generation of antibiotics in 1930s, the influence of human beings on the environment by introducing antibiotics was negligible. This period is named the pre-antibiotic or antibiotic naive era. However, antibiotic resistance had

been being induced in bacterial cells by exposure to naturally occurring antibiotics in the environment and the use of HMs to treat some diseases for a long period before the introduction of antibiotics (e.g. Hg was a common, long-standing treatment for syphilis) [67]. Resistance genes had been transferred horizontally and existed in non-antibiotic producer bacteria e.g. *E. coli* and some of *Enterobacter sp.* [47, 55, 64]. The presence of AbR genes in the genomes of all of the studied organisms shows that these genes have existed for a very long time before the human use of antibiotics [47, 56, 60, 68-70]. The role of the resistance genes in antibiotic producer microorganisms is preservation of the cell against the destructive effects of produced antibiotic. Conversely, there may be other roles for these genes in microorganisms with no ability to produce antibiotics, e.g. peptidoglycan recovery by chromosome-encoded β-lactamases; preventing the destructive effects of antimicrobial compounds on bacteria and signal transduction in bacterial cells. Genes encoding various functions in bacterial cells would be active as resistance genes in the presence of antibiotics. For example, although the *mexF* gene, which encodes a multidrug efflux pump, increases the adaptation of *Shewanella oneidensis* cells to anoxic aquifer sediments, can induce resistance to chloramphenicol (Cm) and tetracycline (Tc) [47, 69, 71-73]. The parvome produced by some of bacterial isolates contains different bioactive molecules which act as antibiotics. Parvomes are multi-functional bioactive cellular compounds with low molecular weight in bacteria, plants and yeast cells. For example most Actinobacters are able to produce these molecules [64, 74]. Most of the resistance genes found to date are located on plasmids, integrons and transposons. Transferring these genes to new recipients results in no physiological function, other than to act as resistance determinants. It means these genes have acquired new function due to mutation without any changes in them structurally; this phenomenon is named exaptation. The family of TEM and CTX-M β-lactamases are well-known examples of this and it has been shown that these genes will experience antibiotic selection pressure in presence of antibiotics [47, 52, 56, 60, 64, 74-78].

2.10. Antibiotics and Resistance and Susceptibility Issues

Susceptibility or resistance to a specific antibiotic is a complicated issue influenced by various factors. The resistance of parental (wildtype) generations are considered as intrinsic or natural resistance and acquired resistance. Generally speaking, if a strain shows less susceptibility to a given antibiotic compared to its parents, it would be considered as resistant regardless of the specific antibiotic MIC [47, 50, 51, 55, 56, 68]. These resistance mutations imply acquired resistance. On the other hand, if a strain represents higher susceptibility to an antibiotic in comparison with its prior generations, because of structural or functional features, it would be regarded as a susceptible mutant. There are several reports that this natural resistance is due to absence of cellular target, susceptible to antibiotics, or the presence of cellular barriers for antibiotics' cellular permeability. These cellular barriers are regulated by the products of various genes involving in cellular physiological processes [60, 64, 68, 69, 79, 80]. As some examples: resistance to triclosan in *Pseudomonas spp.* carrying an insensitive allele of *fabI* encoding enoyl-ACP reductase enzyme as a target of triclosan; resistance of Gram negative bacteria to the lipopeptide daptomycin because of reduced phospholipids with negative charge in the cytoplasmic membrane. This results in less antimicrobial activity due to less aptitude of Ca^{2+}-mediated binding of daptomycin to cytoplasmic membrane; resistance to the glycopeptide antibiotic vancomycin due to inability of transmission of these glycopeptides through the outer membrane in Gram negative bacteria [47, 68, 74, 80-82].

High density genome mutant libraries are considered as suitable tools to investigate susceptibility or resistance of bacterial isolates to different antibiotics or drugs. These libraries have been produced by targeted insertion or random transposon mutagenesis in *P. aeruginosa*, *E. coli* and *S. aureus*. For instance, silencing all of the non-essential genes of *E. coli* has led to potential new targets e.g. thioredoxin (TrxA), thioredoxin reductase (TrxB), SapC, FabI, RecQ and DacA [47, 59, 60, 64, 68, 74].

Apart from the abovementioned heritable antibiotic resistance mechanisms, there are some non-heritable resistance mechanisms due to the physiological characteristics of bacteria including biofilm formation and bacterial persistence. Bacterial persistence is a phenotypic epigenetic trait of slow growth and antibiotic resistance. Some bacterial persisters challenged with antibiotics may survive and produce new generations due to their physiological processes after removal of antibiotics [47, 53, 57, 60, 62, 74, 83]. It has been shown that inducing some metabolic changes result in the susceptibility of bacterial persisters to aminoglycosides. For example, in the presence of high concentration of oxygen, persistence of *M. tuberculosis* against antibiotics is decreased due to producing reactive oxygen species (ROS). Clofazidime induced ROS can increase the susceptibility of bacterial isolates to this antibiotic even in low concentration of oxygen [47, 80].

2.11. Antibiotics in Soil

Using molecular methods e.g. PCR and metagenomics to detect antibiotic resistance genes is one of the best ways to investigate the genome of the bacterial population genome in soil. PCR-based methods can detect known resistance genes, while metagenomics means the study of the collective genome using DNA analysis straight from the environment. It should be noted that there are difficulties in investigating soil, because of various heterogeneities in soil microhabitats due to chemical and physical factors even in a few square meters [47, 53, 55, 56, 59, 64, 71, 74, 84].

Metagenomic studies have revealed the presence of resistance genes in every environment investigated [47, 64]. These genes are mostly located on mobile genetic elements which can spread the resistance features among other bacterial strains, or are chromosomal which can be spread by transduction. Detecting the *ampC* gene, a chromosomal β-lactam resistance gene of *E. coli* and *P. aeruginosa*, can prove the presence of *E. coli,* but is not a threat to the environment as a mobile Ab resistance gene [47, 50, 52-54, 56, 70, 82, 83]. The transfer of chromosomal genes

to mobile elements is considered as an important issue, because this may introduce resistance genes to new hosts. Chromosome-located MDR genes in Gram negative bacteria exhibit this phenomenon. RND (tripartite resistance nodulation division) chromosomal genes are capable of transfer to IncH1 plasmid in *Citrobacter freundii* along with genes encoding New Delhi metallo-β-lactamase 1 (NDM1, a carbapenemase) [50, 54, 68-70, 80, 83, 85].

Because identification and inactivation of enzymatic reactions of well-known resistance genes will not provide a comprehensive understanding of resistance mechanisms, investigations of new resistance mechanisms play a more important role in improving our understanding of antibiotic resistance [47, 68].

2.12. Antibiotics and Heavy Metals

The prevalence of human and animal infectious diseases due to antibiotic resistant microbes is increasing worldwide. Using antibiotics as growth promoting agents has been proscribed in intensive animal production systems and it seems we are facing a post-antibiotic era [18, 50, 61, 64]. The problem is not confined to using antibiotics and cannot be rectified by preventing their use. Selection for AbR can be triggered, or aggravated, by dissemination of HMs in the environment. The prevalence of HMs, which are used in organic and inorganic fertilizers, feed additives and pesticides, can increase AbR among microorganisms by co-selection [18, 36, 61, 64, 70].

The dissemination of antibiotic resistance genes can be influenced by physical forces e.g. wind and rainfal, animal behaviour, immigration and human proximity, and human activities, in conjunction with typical molecular gene transfer mechanisms like HGT [53, 64, 74]. Using metal contaminated fertilizers including sewage sludge and manure raises the concentration of HMs in soil and run-off of contaminated drainage increases the concentration of HMs in aquatic environments [53, 55, 57, 61, 62].

Genes encoding metal resistance and antibiotic resistance are often located on the same genetic elements. Metal resistance genes can act as indirect selective factors for antibiotic resistance. Some of these resistance genes act as biosynthesis genes in antibiotic producers and their products function as antibiotics; so, some of the metal resistance genes located next to these genes can regulate the expression of neighboring genes. [36, 53, 55, 57, 61, 64, 70, 76, 82, 86].

Co-selection is coupled with two resistance mechanisms including cross-resistance and co-resistance. Cross-resistance is related to physiological resistance mechanisms and provides tolerance to more than one antimicrobial agent e.g. antibiotics and HMs. The best examples of cross-resistance mechanisms are multi-drug efflux pumps which, rapidly extrude the toxic material out of the cells. Co-resistance describes two or more genetically linked genes encoding resistance and shows that these genes are located close to each other on a genetic transmissible element [18, 36, 55, 56, 58, 70, 76, 82, 83]. For example, the presence of genetic linkage of the *tcrB* gene encoding copper resistance, macrolide [*erm*(B)] and *vanA* gene encoding glycopeptide resistance in *Enterococcus faecium.* It means, copper in the environment can induce the resistance to antibiotics e.g. vanamycin as well as copper [18, 61, 68, 83, 87]. As another example, pUUH239.2 plasmid isolated from a clinical outbreak in Sweden carries genes encoding resistance to a range of antibiotics e.g. tetracycline, β-lactams and various metals e.g. silver and copper [61]. It is worthy of note that class 1 integrons are considered to accelerate co-selection of resistance to HMs and Abs because of the presence of gene cassettes causing resistance to Abs and HMs [50, 52, 53, 57, 60, 74].

Genetic elements capable of acquisition and exchange of DNA fragments are called integrons. Bacterial isolates containing integrons show more tolerance in stressful conditions e.g. HM presence. For example, the multi-drug efflux pump CzcA which is active in expelling Zn^{2+} and Cd^{2+} out of the cell [18, 50, 52, 55, 64, 74, 82, 87]. Even though co-selection and less Ab susceptibility might happen in the presence of HMs , there are some reports indicating more susceptibility to antibiotics in the presence of HM ions [18]. Hölzel et al. (2012) showed decrease

of MIC for various Abs in presence of $HgCl_2$. The influence of mercury on enzymatic degradation of Abs and also on the enzymes involved in DNA and RNA expression and protein synthesis is supposed to be the reason of this observation [88].

Principally, MIC is the least Ab concentration needed to inhibit bacterial growth; so, an increase in MIC indicates higher tolerance to an Ab in bacterial isolates. On the other hand, minimum co-selective concentration (MCC) and minimal selective concentration (MSC) defines the required HM concentration which causes co-selection; thereby, the concentrations of HM greater than MCC are considered as probable co-selection agents [47, 58, 60, 61, 80]. Cd, Zn, Hg and copper have been found to be the most important co-selection factors in water and soil environments [18, 61]. Interestingly, in the presence of various antibiotics and metals, the minimal selective concentration (MSC) needed to trigger resistance in bacterial isolates is much lower than the required concentration in the presence of a single antimicrobial agent. Metals and antibiotics show synergetic influence on resistance selection. Additionally, the MSCs in the both states (single or combination) of antibiotics and HMs is significantly lower than MICs needed to inhibit bacterial growth [60, 61].

In addition to co-selection for antibiotic resistance caused by HMs in environment, co-regulation of resistance genes might enhance tolerance of bacterial isolates to antibiotics. In this phenomenon antibiotic resistance genes are regulated by metal ions, leads to less susceptibility of bacterial cells to antibiotics. For instance, the *soxS* gene encoding the AcrAB efflux pump regulator in *E. coli*, is regulated by different ions e.g. $Cr_2O_7^-$ and Cu^{2+} under oxidative stress, which results in more expression of *soxS* gene and increasing tolerance to different antibiotics, e.g. tetracycline, chloramphenicol [18, 36, 50, 60, 68, 76, 80, 82].

2.13. Effects of Antibiotics on Microorganisms

Antibiotics resistance can result from two different processes including Horizontal Gene Transfer (HGT) and mutation. In HGT, the genes coding for antibiotic resistance in environmental bacteria

are transferred to other bacterial strains. Since a wide range of bacterial isolates produce different antibiotic metabolites, they possess specific genes to protect their cell against their self-produced antibiotics [47, 52]. Two human pathogens, *Kluyvera* sp. and *Shewanella algae* carrying CTX-M β-lactamase resistance genes and quinolone resistance *QnrA* gene respectively, are examples of this phenomenon [47, 55, 70, 79, 82, 83, 89-91].

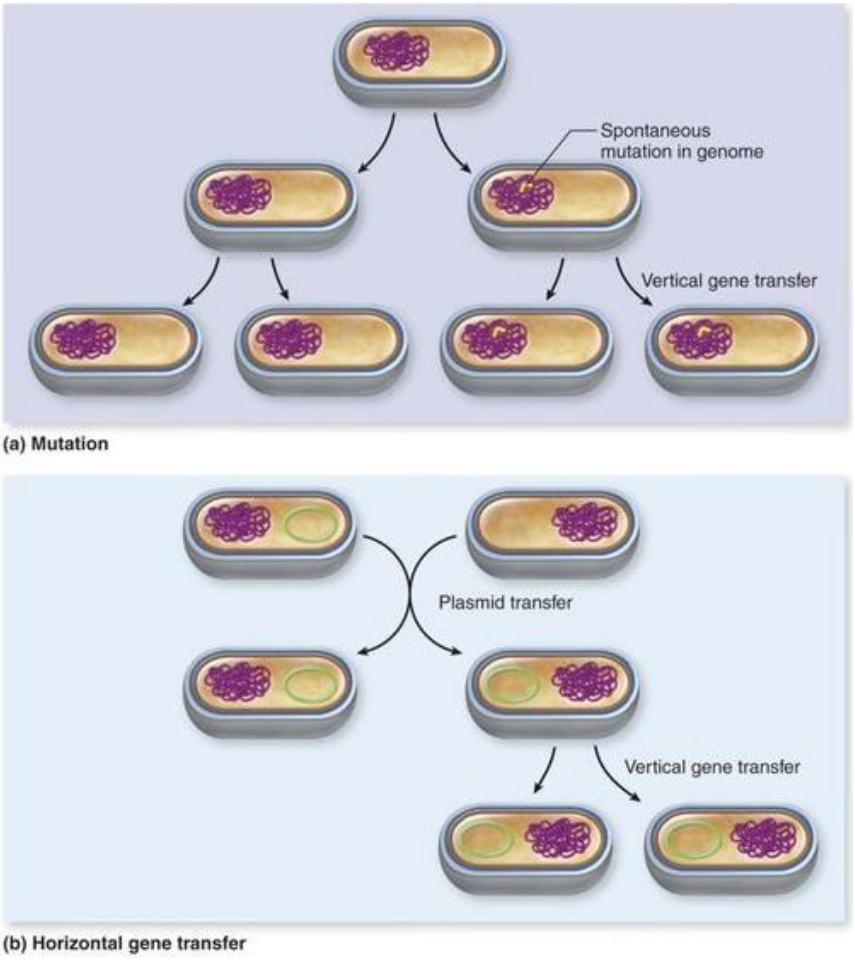


Fig. 2. 1. Horizontal Gene Transfer versus Mutation. These are two methods of AbR genes transfer among bacterial cells (Image: Nester's Microbiology: A Human Perspective [92]).

Transfer of resistance genes to other bacterial isolates is influenced by different factors, e.g. connection of the gene donor and recipient, which is not a real head-on contact necessarily and needs positive selection for resistance-gene transfer. The original location of quinolone resistance *qnrA* gene in aquatic environments and its subsequent location on plasmids of recipient cells revealed that this kind of gene transfer can be accomplished in antibiotic contaminated aquatic environments [47, 52, 54-56, 83]. As another factor, influence of an

acquired resistance gene on acquiring another dispersed resistance gene is considered as founder effect factor and presumably is one of the most important factors of less variability of resistance genes in pathogens compared to natural environments [47, 56].

Two different factors are involved in the stabilization of resistance genes in new hosts. The first one is being collaborated with the gene-exchange community, and the second one is fitness cost imposed on the recipient cell. the first one deals with occurring the mutations and their divergence and the second one is involved in extension and stabilization of resistance genes. Indeed, fitness costs means the pressures of the acquired resistance genes imposed on the host cell to make the cell physiologically compatible with the newcomer gene; hence, bacteria are able to acquire those mutations and resistance genes with the ability of compensation for this fitness cost [47, 52-56, 60, 69, 82, 83].

2.14. Antibiotic Resistance Mechanisms

Antibiotic resistance mechanisms can be grouped into three different categories:

- Those that affect the target of the antibiotics using genetic mutation or post-translational modification.
- Those that alter the effective concentration of antibiotics.
- Those that detoxify antibiotics by hydrolysis and modification [47, 50, 64, 68, 69, 79, 80, 83].

Modifications to antibiotics' targets can be caused by mutation or protection of the target, substitution and enzyme alteration. Note that because of pleiotropy of mutated genes, resistance to antibiotics is not the only result of the mutation in genes [64, 74, 82, 83]. Examples of these respective mechanisms are that mutated bacterial topoisomerases produce QnrA protein and cause quinolone resistance, chimeric PBP proteins lead to beta-lactam resistance, and cell wall transformation leads into vancomycin resistance [72, 89, 91, 93-99].

Changes leading to reduction of antibiotics concentrations in target cells can be achieved by mechanisms which either modify or do not modify the structure of antibiotics. Obstructing antibiotics cell entry (e.g. *P. aeruginosa* resistance to imipenem due to absence of the OprD2 transporter) and removing antibiotics from cells using efflux pumps are examples of mechanisms in which antibiotics remain structurally unchanged. Enzymatic inactivation of antibiotics and alterations in enzymes which activate antibiotics are the examples of mechanisms which change the structure of antibiotics e.g. isoniazid in *M. tuberculosis* can be used as a source of carbon and nitrogen. Also, *Pseudomonas fluorescens* grows on streptomycin (Sm), and *Streptomyces venezuelae* grows on Cm [68, 90, 96, 98, 100-102]. A wide range of cellular physiology can lead Abs resistance [47].

2.14.1. Inhibition of Target Accessibility

Due to outer membrane barriers, Gram negative bacteria are intrinsically more resistant to the entrance of antibiotics into the cell compared to Gram positive bacteria [64, 68, 79, 82]. Although, hydrophilic antibiotics are able to distribute through outer membrane porin proteins, reduction in expression of genes encoding porin proteins (e.g. OmpF and OmpC) and substitution of them with channels with more selective features happens in a wide range of Gram negative bacteria (e.g. Enterobacters). This is considered as the reason for innate Gram negative bacteria resistance and their ability to be impermeable to antibiotics. However, it has been shown that the prior hypothesis involving drug binding sites in outer membrane drug channels was wrong [68, 69, 76]. For instance, it has been shown Entrobacteriaceae members like *E.coli* and *Enterobacter spp*, and *Pseudomonas spp*. and *Acinetobacter spp*. are resistant to recently introduced antibiotics e.g. carbapenems and cephalosporins, and that this is mediated by enzyme degradation of these antibiotics. Carbapenemase is the necessary enzyme for inactivation of carbapenem; so, if mutation happens in genes encoding porins which decrease porin production, parallel to lack of carbapenemase results in resistance of enteric bacteria to carbapenems [50, 68, 76].

Gram negative bacteria compared to Gram positive isolates have more variety of efflux pumps to excrete antibiotics and other drugs out of the cell. Over-expression of efflux pumps in Gram negative bacteria is an important resistance mechanism and results in resistance to a wide range of antibiotics [62, 68]. Some of the efflux pumps in bacteria, for example, Lmr, MdeA, FuaABC and KexD in *Streptomyces aureus*, *Streptococcus mutants*, *Stenotrophomonas maltophilia* and *Klebsiella pneumoniae* respectively can confer resistance to a range of drugs [68, 80]. Homotrimeric inner membrane pumps, *E. coli* AcrB pump and *P. aeruginosa* MexB pump, are examples of RND family MDR efflux pumps, which make a trilateral binding to AcrA and MexA periplasmic proteins and outer membrane transferal proteins like TolC and OprM, and act as multidrug efflux pumps [58, 60, 68, 69, 76, 80].

Local and global regulator genes located beside the efflux pumps-encoding genes can control production of pumps. AraC-XylS gene regulator family members e.g. *MtrA* and *RamA* genes, are considered global regulators with a wide range of functions. MtrA and RamA are involved in increased transcription of *Neisseria gonorrhoeae mtrCDE* and *Salmonella spp*. *acrAB-tolC* [68, 82]. Along with the efflux pump gene expression regulators, there are lots of MarR (multiple antibiotic resistance proteins) family repressor-encoding genes located next to transcription-regulator genes which inhibit regulator genes functions, e.g. *marR* and *ramR* from *E. coli* and *Salmonella typhimurium* respectively. If MarR repressor proteins functions decrease, the overproduction of AraC-XylS transcription regulators, e.g. MarA, SoxS, RamA and Rob transcription regulator proteins from Entrobacteriaceae result in more MDR efflux pump production and suppression of porin protein function [47, 50, 68, 76, 82, 83].

Gene expression of RND pumps is influenced and suppressed by a local repressor, TetR. AraC transcription factors act to regulate and suppress the TetR repressors. AraC genes, in turn, are suppressed by MarR global repressors. So, overexpression of AraC encoding MDR pumps, can occur in MarR repressors. Mutation can occur in local repressors and intergenic sites too (e.g. mutation can happen in the TetR local repressor genes located upstream of *acrA* (encoding

adaptor protein) and *acrB* (encoding efflux pump) resulting in their overexpression [64, 68, 82]. Mutation can occur in promoters too and change the promoter activity (e.g. a single bp mutation in the upstream region of *mtrC* of *N. gonorrhoeae* established a more active promoter and increased efflux pump expression [47, 68].

Overexpression of efflux pumps production might happen in necessary conditions. In this mechanism, binding a molecule, like an antibiotic, to a transcriptional repressor protein is one of the most important mechanisms of resistance (e.g. *acrAB* in *E. coli* and *Salmonella* sp., and MtrCDE in *N. gonorrhoeae* and NorA in *S. aureus*). Structural analysis of the Rv1219 transcriptional repressor protein from the TetR group which controls expression of the RV1219c-Rv1218c transporter of ABC family revealed there are several multidrug binding pockets in this protein [68, 76, 80].

2.14.2. Mutation in Antibiotic Targets

Preventing an Ab binding to its target is another Ab resistance mechanism. A wide range of antibiotics bind to their targets to restrict the target actions. Mutation in one copy of several target gene copies in bacterial cells results in multiplication of the mutant allele. For example, there are several copies of the 23S rRNA ribosomal subunit, targeted by linezolid; so selection for mutation with linezolid will lead to more proliferation of bacteria containing the mutated allele in Gram positive bacteria like *S. aureus* and *S. pneumoniae* [55, 68, 69, 103].

Mosaic genes formed by recombination of DNA from the environment with resident genes and target protein modification are resistance mechanisms occuring via natural transformation. For example, penicillin resistance in *S. pneumoniae* is due to mosaic penicillin binding protein genes (*pbp*) can occur by recombination with *Streptococcus mitis* DNA [68, 97, 104].

Attaining a gene homologous to the target is another antibiotic resistance mechanism, for example acquisition of the staphylococcal cassette chromosome *mec* element (SCC*mec*) in methicillin resistant *S. aureus* (MRSA). This element carries *mecA* gene encoding β-lactam

insensitive protein PBP2a allowing protein cell wall biosynthesis to occur although the native PBP action is inactive in the presence of antibiotic [64, 68, 75, 80, 105].

2.14.3. Target Protection by Modification

This mechanism does not change the Ab target by mutation, with addition of chemical residues compound to the target to prevent Ab binding to the target. There are no mutational changes in the target and means the target is protected from any changes induced by mutation [68, 76, 105]. There are several examples of clinically important antibiotic resistance involving this mechanism, e.g. alteration of drug binding sites of macrolides, lincosamines and streptogramins by methylation of 16S rRNA by erythromycin (Ery) ribosome methylase (*erm*) family genes. Also, the recently discovered methylation of A2503 of 23S rRNA by Cm-florfenicol resistance (*cfr*) methyltransferase in different Gram positive and negative bacteria (e.g. *S. aureus* and *E. coli*) leads to resistance to various compounds including phenicols, pleuromutilins, streptogramins, lincosamides and oxazolidonones like linezolid [58, 68, 89, 104, 106].

The plasmid-borne *qnr* genes encoding resistance to quinolone produce pentapeptide repeat proteins (PRPs) that bind topoisomerase IV and DNA to protect against quinolone. The interaction of PR proteins with complexes of quinolone and topoisomerase results in augmentation of quinolone efflux and inhibition of double-stranded DNA breaks [50, 68, 83].

Targeting lipopolysaccharides (LPS) of the cell of Gram negative bacteria by polymyxin antibiotics (e.g. polymyxin B and E) is another example of a mechanism affecting targets. The long hydrophobic chain of these antibiotics binds to LPS and interacts in both inner and outer membranes. This group of antibiotics, also called colistin, has been used against infections caused by *P. aeruginosa* and *Acinetobacter spp.* and a range of Enterobacteriaceae [47, 68, 79]. Resistance to these Abs results in reduction of the LPS production-regulator gene expression and therefore less drug binding to the target [68, 104, 106].

Daptomycin resistance in *S. aureus* is an example of effect of point mutations to change the target. This antibiotic which is used against Gram positive infections, targets anionic phospholipids in the cytoplasmic membrane and enters the membrane and causes depolarization of intracellular contents when calcium ions are present [68, 107]. Resistance to this Ab can occur by point mutation to *mprF* (multiple peptide resistance factor) and modification of membrane phospholipids which results in membrane charge alteration and less daptomycin binding [68, 80, 105, 106].

2.14.4. Antibiotics Modification

These Ab resistance mechanisms are as important as abovementioned mechanisms in bacterial populations and are categorized into two different groups [68, 104]:

- The hydrolysis and inactivation of Abs.
- Alteration of bacterial activity by adding chemical changes.

In the first group, the process is production or acquisition of an enzyme with activity to cleave the Ab. The destroyed antibiotic will not be able to bind its target and, resistance to the Ab results. This resistance mechanism is very common against β-lactams, aminoglycosides, phenicols and macrolide antibiotics [68, 69, 104].

Chemical changes can confer resistance to some antibiotics. Addition of chemical residues leads in structural modification of Abs and prevents the Ab bindings to its target. This phenomenon results from steric hindrance and is facilitated by bacterial enzymes (e.g. aminoglycoside-modifying enzymes) [68, 104, 106]. There are a broad range of chemicals residues that can be added to the antibiotic structure by a wide range of antibiotic-resistance enzymes to inhibit their antimicrobial activities (e.g. nucleotidyl, ribitoyl, phosphate, and acyl groups). There are three different groups of aminoglycoside-modifying enzymes including, acetyltransferases, phosphotransferases and nucleotydiltransferases [68]. Aminoglycosides are not very tolerant to enzymatic changes, because of their size and the presence of several hydroxyl and amide groups

in their structures. All these three modifying enzymes can bind to aminoglycosides, although they have different affinities for specific aminoglycoside groups [68, 104]. The recently discovered genomic island encoding six different aminoglycoside-modifying enzymes from *Campylobacter coli* showed the resistance of this bacterial isolate to various aminoglycosides [68]. Another example of this mechanism has been found in some Actinomycetes species with rifamycin-resistance genes and involves a group of phosphotransferases [68, 106].

2.15. Antibiotics Used in the Project

Tetracycline (Tc) was discovered as a natural product in 1945. Tc is bacteriostatic and binds to the 30S subunit of bacterial ribosomes and prevents introduction of new amino acids to the developing peptide chain [108]. Resistance mechanisms to Tc are efflux pumps, ribosomal protection and enzymatic inactivation [108]. The Tc resistance genes are *tet* family genes which associate with conjugative and transferable elements (e.g. *tetA* and *tetE* genes encode an outer membrane-associated efflux pump protein in Gram-negative bacteria) [109].

Chloramphenicol (Cm) was discovered and isolated from *Sterptomyces venezualae* in 1947 and in 1949 was synthesised as the first laboratory-synthesised Ab. Cm is bacteriostatic and inhibits protein chain elongation by suppressing the peptidyl transferase activity of the 50S subunit of the bacterial ribosome [110]. The most frequent resistance mechanism to Cm is inactivation of the Ab by attaching acetyl residues to hydroxyl groups of Cm, which inhibits binding of Cm to the 50S ribosomal subunit. This resistance mechanism is facilitated by Cm acetyltransferase encoded by *cat* genes.[111].

Ampicillin (Amp) was introduced by a British company, Beecham, in 1961. Amp is bactericidal and can enter Gram positive and some of enteric bacteria. Amp inhibits transpeptidase enzyme activity and bacterial cell wall production required for binary fusion [112]. Resistance mechanisms against Amp include modifications of penicillin binding proteins (PBPs) in the cell,

reducing the affinity for Amp binding, and inactivation of extracellular Abs by β-lactamases (e.g. *E. coli*, has plasmid-borne *amp* genes, encoding β-lactamase enzymes) [113].

Carbenicillin (Cb), like Amp, is a bactericidal β-lactam and its mode of action, in several aspects, is the same as ampicillin [114]. This Ab is more efficient at killing Gram-negative bacteria and is less susceptible to degradation by beta-lactamases compared to Amp. As with Amp, the most frequent resistance mechanism against carbenicillin is β-lactamase production (e.g. genes encoding resistance to carbenicillin by beta-lactamases production, are *bl2c bro*, *bl2c pse1* and *bl2c pse3* in *P. aeruginosa*, *Acinetobacter* sp., *Salmonella enterica* and *Oligella urethralis*) [115].

Erythromycin (Ery) was discovered from a soil bacterium, *Streptomyces erythraea* and was introduced commercially with the name of Ilosone in 1952. This is a bacteriostatic Ab of the macrolide class and interacts with ribosomal 50S subunit, resulting in protein synthesis inhibition [116] [117]. The most frequent resistance mechanism to Ery is modification of its target site by a rRNA-methylating enzyme with elimination or reduction of Ery's binding to its target. Resistance genes for Ery include the *ermAM* genes found on plasmid pAM77 of *Streptococcus* sp. and confer resistance to macrolides, lincosamide and streptogramin-B [118].

In the current project, I tried to include one representative of some of Ab classes. Carbenicillin and ampicillin which are from the same antibiotic class, penicillins, were chosen, because carbenicillin is more effective against a wide range of bacteria like Pseudomonads which show high resistance to ampicillin. By choosing these two Abs from the same class I tried to have a better contrast of penicillin class of Abs.

2.16. Mechanisms of Transferring Resistance Genes by Horizontal Gene Transfer (HGT)

Horizontal Gene Transfer (HGT) a major gene transfer mechanism plays an important role in the evolutionary changes and different kinds of resistance to antimicrobial compounds and HMs [119]. HGT was discovered in microorganisms in 1940s and since then its effect in variation of other organisms, including eukaryotes was revealed [120].

Some of the transmitted genes show a neutral effect in the recipient cell, especially in closely related cells [121]. The most important feature of a gene to be accepted in a new host is to be harmless to the recipient. Integrated genes are able to be expressed in the new host cell. Complete integration of the transmitted gene in the host cell leads in development of new phenotypes with new behaviours in the long term behaviors. If transferred genes have no specific function or are detrimental to the host cell, the horizontally transferred genes might be removed from the cell [119, 122, 123].

2.16.1. HGT Mechanisms

The most common mechanisms of HGT in prokaryotes are conjugation, transformation, transduction, and the recently discovered ones e.g. GTA (gene transfer elements) and cell fusion [119].

Fig. 2. 2. Mechanisms of gene transfer among bacterial cells. Conjugation (**a**) happens between donor and recipient cells, transferring single-stranded DNA. Cell fusion (**b**) transfers DNA bi-directionally after bridge formation. Transduction (**c**) gene transfer by phage. Gene transfer agents (GTA) (**d**) are phages that only carry random fragments of host DNA. Transformation (**e**) DNA uptake from the environment. Endosymbiotic gene transfer (**f**) means incorporation of genetic material from an endosymbiotic or organelle into the host cell genome which are mostly eukaryotes. (Image: Soucy et. al., 2015 [119]).

Physical contact of donor and recipient cells is important for HGT by conjugation. This contact is facilitated by a bacterial pilus [121]. With the exception of *Agrobacterium* conjugation to plant cells, HGT by conjugation happens among bacterial cells [124]. When conjugation occurs, a single strand of DNA is moved from donor to recipient cells [119].

Phage can facilitate HGT by transduction. Integration of donor genes into the phage genome leads to transfer of these genes to the recipient. General transduction and specialised transduction are two types of transduction which describe incorporation of a random fragment

of host DNA in the phage genome, or a specific section of host chromosome by an activated prophage [119, 125].

GTA, found in both prokaryotes and archaea, has some similarities to the transduction system. GTAs evolve from prophages with inability to recognise and transfer their genes to encode their protein packaging [125]. These mobile agents transfer random fragments of host DNA to the new recipients. As a host chromosome-integrated system, GTA is involved in transferring genes mostly under the regulatory system of host. The small random fragments of host genome are transferred in capsids by GTAs to neighbouring hosts. Lang et. al. showed that transferring genes by GTAs has no advantage for the host, when its genes are transferred by GTA to other hosts, nor for GTAs encoding genes; this is suggested because there is no preference for GTAs to transfer their encoding genes to the new hosts, although how the GTAs gene remain under selection has not been explored yet. [125, 126]. An example of GTA-dependent gene transfer is the antibiotic resistance genes transfer from *Rhodobacter capsulatus* to other bacterial species [126].

Cell fusion is considered as bi-directional gene transfer which occurs on the joining of two adjacent microorganisms' cell membrane by several small bridges [127]. There are some similarities between conjugation and cell fusion systems, but the action of both cells as either donor or recipient of genes in cell fusion makes this system similar to sexual reproduction system in eukaryotes. The most common examples of cell fusion have been observed in archaea e.g. *Haloferax volcanii* and *Sulfolobus* spp. isolates or even between *Haloferax* isolates from distinct species [128-130].

Fig. 2. 3. Schmatic representation of cell fusion mating mechanism. (**A**) Two cells with close proximity. (**B**) Cytoplasmatic bridges between two cells. (**C, D**) Cell fusion is established (**E**) creation of a hetero-diploid cell, leading to (**F**) segregation of chromosomes and plasmids resulting in reversion to the original state, and (**G**) recombination between the chromosomes to create a hybrid cell. (Image: Noar and Gophna, 2013 [127]).

The Lateral Gene Transfer (LGT) system is the incorporation of whole or some parts of cellular organelles genetic component (e.g. chloroplasts and mitochondria) to their host cells genome [131]. Similar to LGT, endosymbiont bacteria in the eukaryotic cells are able to incorporate some part of their genome to the host genome and this is described as Endosymbiotic Gene Transfer (EGT). The presence of endosymbiont in the eukaryotic cells does not guarantee the occurrence of EGT, because EGT has not been demonstrated in some host-endosymbiont integrations [132]. As one of the common animals' endosymbiont choanoflagellates, *Monosiga brevicollis* is an example of unicellular eukaryotes which obtain a broad range of genes encoding different treatments to carbohydrates and amino acids metabolism, HMs and antibiotics tensions and etc. by EGT [133].

Hybridization and consecutive backcrossing with parent in, for example two different species of eukaryotes leads in incorporation some part of genes in the recipient cell and occurrence of introgression system [134]. This HGT system not only is one of the most common exchanging genes in plants, also its effects on human ancestors brain evolution has been proven [135].

2.17. DNA-related Molecular Techniques

DNA-related molecular methods, e.g. Polymerase Chain Reaction (PCR), Terminal Restriction Fragment Length Polymorphism, Sanger Sequencing and Next Generation Sequencing, are the in-situ methods providing the capability to detect, differentiate, categorise bacteria [136]. The following are the review of the molecular methods used in the current project. The molecular methods were used in this project to investigate bacterial community structures and groups in soil samples and probable differences among bacterial HM and Ab resistance genes in bacteria from soils with different history of usage and different levels of HMs. These methods were used along with the culture-based methods as only less than 1% of soil bacteria are culturable on common media and these number of bacteria are not the proper representative of the total phylogenetic diversity. The unculturable bacteria they play an important role to shape the structure of bacterial communities in soil, therefore, identification and abundance of these bacteria in soil is important [137].

2.17.1. Terminal Restriction Fragment Length Polymorphism (TRFLP)

TRFLP analysis is applied to investigate the taxonomic composition of complex bacterial communities in soil according to variation in the 16S rDNA gene [138]. TRFLP analysis is used to interrogate the soil bacterial community structures and dynamics in response to variations induced due to various environmental factors, e.g. levels of HMs in soil. There are several reports of using TRFLP method to investigate complex bacterial communities in soil [139], sludge systems [140] as well as in human health and pathology research projects [141]. This method does not need cell culture-based methods, and provides comprehensive, fast and sensitive analysis to explore the structure and diversity of bacterial communities in various environments. TRFLP is cheaper than common nucleotide sequencing methods and does not need require genomic sequence information [142]. TRFLP amplifies 16S rDNA genes from extracted total genomic DNA using fluorescent labelled primers. The PCR product is subjected to digestion to obtain terminal restriction fragments with fluorescent labels at the 5’ ends [143].

2.17.2. The History of DNA Sequencing

DNA sequencing technology has developed in a variety of ways since the invention of the early sequencing technologies of two-dimensional chromatography and chain-termination sequencing by Frederick Sanger et al. in 1977 [144]. The DNA sequencing method based on chemical modification developed by Maxam and Gilbert in 1977 [145]. In addition, capillary electrophoresis (CE)-based sequencing adopted in the first automated sequencing machine, AB370 by Applied Biosystems in 1987; this system employed a fluorescent labelling technique. These methods are recognised as the first generation of DNA sequencing. The Maxam-Gilbert method could not compete with the Sanger method because of its lower performance. These early sequencing technologies were precursors of more complicated technologies, used in the Human Genome project, which was provisionally completed in 2001 [146]. The massively parallel signature sequencing technique (MPSS) was a major breakthrough in sequencing science, reading a large number of overlapping short fragments of the genome simultaneously and represented the beginning of Next Generation Sequencing (NGS) of genomes [147]. Consequently, the price of fragment sequencing fell significantly and sequence data output increased. Sequencing technology in 2005 was able to sequence 1.3 human genomes per year, but it increased to 18000 human genomes in 2014.

A wide range of NextGeneration Sequencing (NGS) methods have been developed, which differ based on the nucleic acids (DNA or RNA) and the organisms, tissue or environment they are obtained from. The common applications of NGS methods are in Genomics, Transcriptomics and Epigenomics.

2.17.2.1. Genomics

The most important methods related to genomics are whole-genome sequencing, genome resequencing, Exome Sequencing, *de Novo* Sequencing and Targeted Sequencing [146]. Resequencing methods are used to detect all the variations that exist in the genome across

individuals of a species, which were not demonstrated in a single index individual genome sequence [148].

Sequencing of an organism's genome without any reference sequence available is accomplished by *de novo* sequencing methods using contig (a conting is a group of overlapping DNA fragments, which represent the consensus residues of the sequence alignment, either nucleotide or amino acid) assembly. In this strategy, the sequenced fragments are aligned as contigs. The quality of *de novo* sequence data can be determined by the cohesion and length of these fragment sequences as well as the diversity of insert sizes of fragments used in library preparation [149, 150]. Using a combination of fragments sizes helps to cover a wider range of structural variants and results in a more accurate sequencing data, especially for complex genomes.

Targeted Sequencing refers to experiments which are aimed at sequencing specific genes or regions of genomes with higher coverage levels compared to whole genome sequencing, for instance 500x-1000x vs 30x-50x. This capability results in higher accuracy of rare variant detection and money saving compared to CE-based sequencing. Flexible and scalable kits for Targeted Sequencing help scientists to adjust the sequencing panels according to their experiments' requirements [151].

The Amplicon Generation method workflow leads to multiplexing the desired regions by PCR, followed by amplification and purification of the fragments. This is a flexible method to researchers' demands including metagenomic studies of phylogeny of bacterial species using 16S rRNA [152-154].

Sequencing of total RNA (RNA-Seq) or other RNA fragments including mRNA, tRNA, rRNA, small RNA, noncoding RNA (ncRNA), targeted RNA and micro-RNA, can be done by transcriptomics methods [146]. In this method, the process is started by total RNA sample preparation and then rRNA deletion. This stage is followed by RNA reverse transcription to cDNA in order to prepare an NGS library. [155].

Epigenetics is study of heritable changes of phenotypic features due to changes in genes activity which is caused by external factors. These factors that make the genes to switch on or off are including DNA methylation, small RNA-mediated regulation, DNA-protein interaction, histone modification and etc. [146, 156].

2.18. NGS systems

There are several NGS systems, e.g. SOLiD and Ion Torrent PGM (Personal Genome Machine), GS FLX Titanium and GS Junior of Roche Company, and Genome Analyzer, HiSeq and MiSeq series of Illumina Company. The novel NGS machines with Massively Parallel Sequencing technology were introduced following the human genome project (e.g. the Genome Analyser machine, the 454 machine and SOLiD [Sequencing by Oligo Ligation Detection]) [146].

2.18.1. Illumina NGS Systems

Massively parallel signature sequencing (MPSS) was one of the earliest NGS methods developed in 1990s. This method used adapter ligation and adapter decoding to sequence DNA and was susceptible to sequence specific bias [147]. The high complexity of this method resulted in no commercial representation of this method in a specific sequencing machine. Lynx Therapeutic Company representing this technology along with Solexa Company were amalgamated to form Illumina and led to the development of SBS technology [146].

The Genome Analyser (GA) was introduced in 2006 with output of about 1 Gb per run. Its efficiency enhanced dramatically in a few years and reached 85 Gb per run.

The Illumina HiSeq and MiSeq machines, introduced in 2010s, and progressively improved in 2014 and the lowest price per base ever [146, 157]. The Illumina HiSeq and MiSeq are based on the base-by-base sequencing method and DNA colony sequencing method. Using P5/P7 primers and adapters which have multiplexing incorporation, the HiSeq and MiSeq machines are able to sequence thousands of samples concurrently [158]. The only difference between these machines is the magnitudes of the reads of HiSeq (300 million) compared to MiSeq (25 million).

There are two laser apparatuses and four filters to detect the four different nucleotide types in these machines and sequencing quality is influenced by the distribution of bases. This happens because of cross-talk among of the 4-types nucleotides' emission spectra [157].

The MiSeq machines (used for the current project) not only have lots of HiSeq systems' traits allow it to sequence various kinds of samples, they are also specially designed to handle bacterial genome and amplicon sequencing as well as ChIP-Seq and clone checking. It is able to complete the sequencing process in addition to library preparation and quality measurement in less than 10 hours [159, 160]. Nextra and Illumina 16S V3-V4 library preparation methods are used in this instrument by a combination of this instrument with other automated systems e.g. Agilent Bravo, Tecan, Hamilton Banadu, etc. The more effective performance of this system in contig assembly compared to HiSeq systems has been achieved by improvement of reads lengths in this system. Higher output and on-instrument data analysis feature of MiSeq are the advantages of this system versus the Ion Torrent PGM system [146].

Illumina sequencing by synthesis (SBS) technology consists of 4 stages including library preparation, cluster generation, sequencing and finally data analysis. In the first step, random fragmentation of DNA or cDNA, followed by 5' to 3' adapter ligation produces the sequencing libraries. These two tasks can merge into one step by tagmentation which enhance the sequencing output significantly. Tagmentation is the name of a library preparation method which uses Tn5 transposase to fragment *in vitro* DNA instead of using chemical ligation [161]. The adapters are oligos which are bound to the 5' and 3' ends of fragments while preparing the sequencing library. In the second step, the flow cells containing the surface-bound oligos, which are complements of the adapters, are loaded with the library fragments. A bridge amplification process will lead to fragment amplification and insertion into different clonal clusters. The process starts by leaning over the strand and binding other end of the ligated strand with another promoter to another complementary oligonucleotide on the flow cell surface which results in making a bridge. Same as PCR, replication of this process which consists of

denaturation and extension cycles, leads to generation of millions of similar fragments which produce specific fragment clusters [162, 163]. Illumina uses SBS technology which is an exclusive terminator-based method. In this method the single reversible terminator bases (RT-bases) are detected as their incorporation into the DNA strands. All of 4 cleavable fluorescent-dye labelled dNTPs and removable blocking groups are available in each cycle of process and one only base act as complementary to the template strand [164]. The labelled dNTPs are detected by laser excitation and imaging procedures using charge-coupled device (CCD) [146, 165]. The last step is analysing the obtained data of sequencing. In this stage, the obtained sequence will be aligned to a reference genome. By aligning the data, various analyses can be done such as single nucleotide polymorphisms (SNPs) and insertion deletion (indel) identification, and read counting for RNA-related experiments, phylogenetic and metagenomic analyses and etc. [146].

2.18.1.1. Illumina NGS Technology Features

One of the most important features of Illumina NGS is Paired-End (PE) sequencing method. In this method, both ends of DNA fragments are sequenced and aligned as forward and reverse reads. The importance of this method is in high accuracy of sequencing because of duplicate reads, enabling researchers to find indels, removal of PCR duplicates and other errors which are difficult or impossible to detect in single strand reads [163]. Producing more SNV calls is another advantage of Illumina NGS technology [146, 166].

There are lots of similarities between NGS and Capillary Electrophoresis (CE) sequencing; in both, incorporation of fluorescently labelled deoxyribonucleotide triphosphates (dNTPs) in a DNA strand within DNA sequencing cycles are catalysed by DNA polymerase, and fluorophore excitation results in recognition of each labelled nucleotide. In addition, there is a fundamental difference between the two techniques which makes their outputs quite distinct. The difference in the number of DNA fragments which are sequenced simultaneously, which is one fragment in CE sequencing and millions in NGS methods [146].

Compared to CE Sanger Sequencing methods, NGS uses a simpler and more rapid library preparation protocol. While the early library preparation methods took 1-2 days and were involved in making DNA or RNA random fragmentation, selecting fragments based on their size on gels, ligation of oligonucleotides to their specific platforms, PCR amplification etc., the new methods need only ~2 hours to prepare the library which are mostly PCR and gel free. To prevent the common errors in previous methods while working on CG/AT rich fragments, promoters and homopolymeric regions, NGS does not use PCR [146, 167].

In addition to the high output of NGS methods which is obtained at the end of experiment, the capability of this method to run multiplex libraries makes this a suitable method to increase the throughput of the experiments. This feature helps different index DNA fragments to get sequenced simultaneously in the span of one sequencing run. During the data analysis the sequencing data are separated and sorted out in distinct datasets based on the related index fragments and aligned to the related references [163].

2.18.2. NGS Applications in Microbiology

The applications of NGS to microbiology, especially environmental microbiology, include the detection and identification of bacterial isolates, and measurement of gene expression. A broad range of microbial genomes have been sequenced so far, but these data reveal just a small amount of information about microbial populations. There are many non-essential genes which differ among various strains, including pathovars of a species. To explore the genetic variation among bacterial communities more sequencing could reveal the key genetic factors involved in pathogenicity, drug resistance and susceptibility, ecological behaviours, phylogenetic relations, etc. [168].

One of the earliest examples of using NGS to investigate the genome of bacterial species was using the Roche 454 GS20 sequencer to explore the genome of *Myxococcus xanthus,* a soil-borne proteobacterium with social motility and cooperative predation. The project aimed to find

mutations resulting from different starvation periods triggering production of fruiting bodies, as a starvation-resistance behaviour [169].

Using metagenomic studies of microbial populations in various environments helps the understanding of unculturable microbes. With metagenomics the genes of unculturable bacteria encoding specific traits like metabolic features and drug resistance can be sequenced and compared with reference genomes. [168].

The impact of NGS systems, as precise and speedy methods, on different fields of microbiology, from environmental to clinical, has been substantial. There are a broad range of investigations on identification, categorizing, management and control of bacterial, fungal and viral diseases of plant, animal and human which have been accomplished or are in progress around the world because of the novel properties of NGS systems in reduction of sequencing costs and high accuracy [170].

2.19. Knowledge gaps

There are several knowledge gaps about bacterial resistance to HMs and Abs in the presence of elevated levels of HMs in soil. Some of these gaps are:

- Explicit mechanisms involved in the resistance of bacteria to HMs and subsequent resistance to Abs.
- If mechanisms of resistance to a given HM will lead to different mechanisms of resistance to different Abs, and if these differ among various classes of bacteria.
- If resistance to Abs induces resistance to HMs too, or this is only the way coselection occurs, from HM resistance to Ab resistance.

Some of the knowledge gaps that were covered by the current thesis are:

- Comprehensive comparison of bacterial resistance to HMs and Abs in soils with different history of usage.

- Comprehensive investigation of bacterial resistance to HMs and Abs in microcosms and if examination of bacterial resistance to HMs and Abs can act as a simulation of bacterial HM and Ab resistance in a real soil environment.
- Bacterial community structure changes (due to presence of elevated levels of HMs in soil) investigation using both TRFLP and/or 16S rDNA sequencing and on soil samples with different history of usage.

The following flow chart (Figure 2.4) illustrates the key concepts discussed in the literature review chapter.

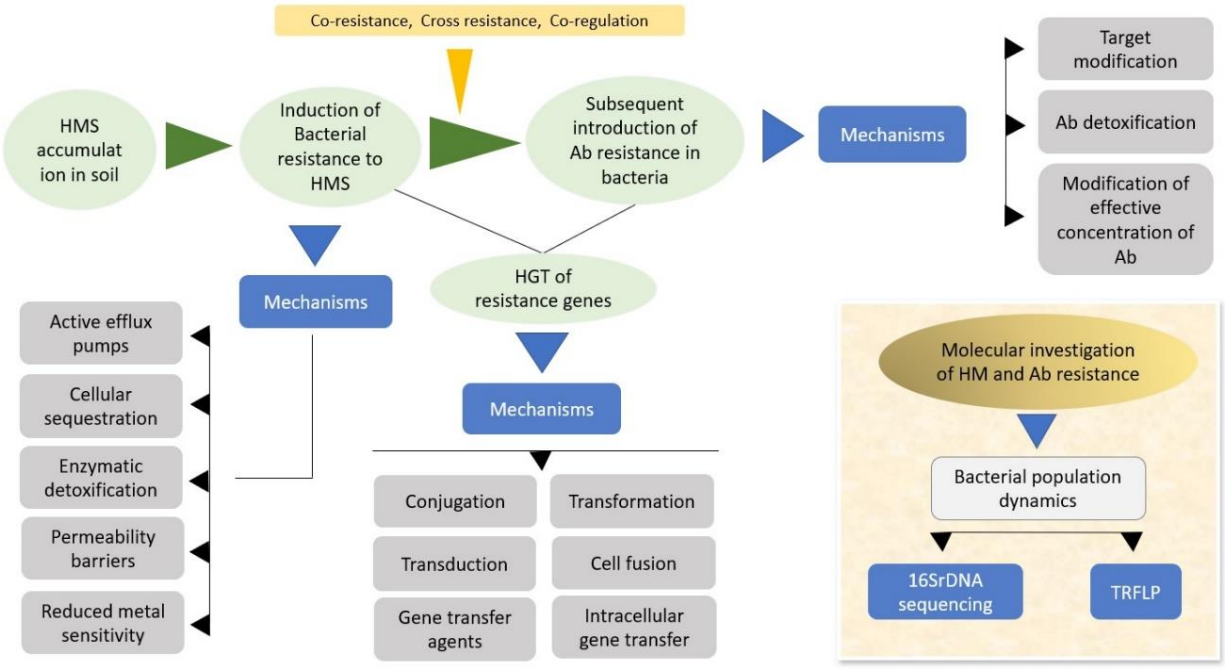


Fig. 2. 4. Flow chart summarising the key concepts discussed in the literature review chapter and their relevance.

Chapter 3, Methodology

Figure 3.1 illustrates different methods and steps used in the current project.

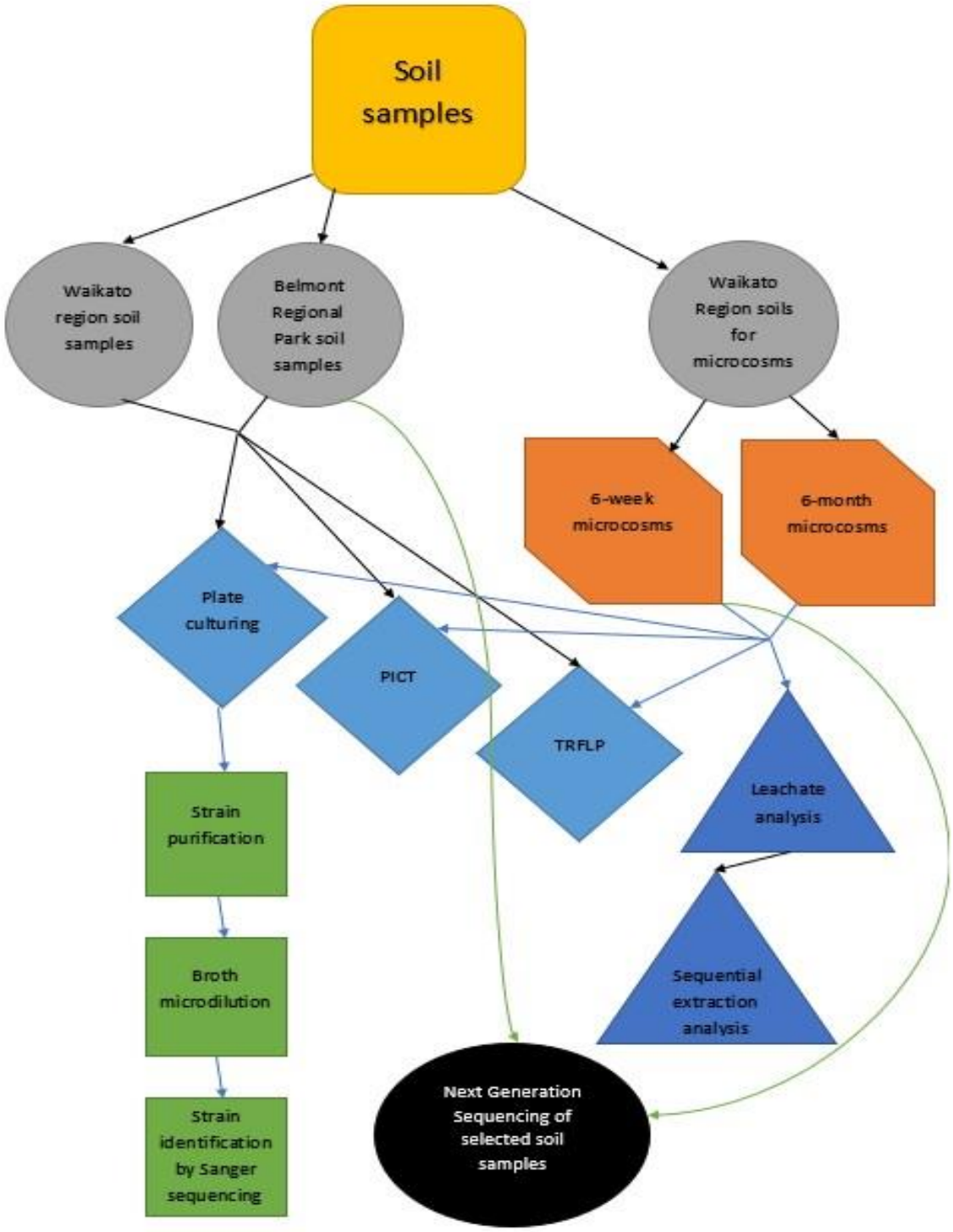


Fig. 3. 1. Flow chart summarising different steps and methods used in the current project. Various equipments and consumables used in this project are listed in Table 3.1.

Table 3. 1. Equipments and consumabeles used for the current project.

Product	**Brand**	**Location**
90 mm round petri-dishes	LabServ™	Lower Hutt, NZ
96-well polystyrene microtiter plates	CELLSTAR®	Dunedin, NZ
Bovine Serum Albumin (BSA))	Thermo Fisher Scientific	Lower Hutt, NZ
Brain Heart broth	Bacto™, Becton Dickinson	Auckland, NZ
Cycloheximide	Sigma-Aldrich®	Auckland, NZ
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich®	Auckland, NZ
DNA Isolation Kit	Mo Bio PowerSoil®	Auckland, NZ
Dream Taq PCR Buffer, dNTPs, Forward and Reverse Primers and DreamTaq DNA polymerase	Thermo Fisher Scientific	Lower Hutt, NZ
Electronic 8-channel pipette	Eppendorf	Auckland, NZ
Falcon tubes	LabServ™	Lower Hutt, NZ
High-resolution Continuum-Source Atomic Absorption Spectrometer	Analytik Jena ContrAA® 700	
ICP Multi-Element Standard Solution IV	Certipur®	Auckland, NZ
Labelled and unlabelled primers	Thermo Fisher Scientific	Lower Hutt, NZ
Lysozyme	Sigma-Aldrich®	Auckland, NZ
Manual colony counter	Chiltern®	Auckland, NZ
*Msp*I oligonucleotide restriction enzyme	Thermo Fisher Scientific	Lower Hutt, NZ
Nitric acid	Baker	Auckland, NZ
Nutrient agar	Difco™	Auckland, NZ
PCR grade nuclease-free water	Dnature	Gisborne, NZ
pH meter	ORION™ 420A	Lower Hutt, NZ
plate reader	FLUOstar® OPTIMA BMG LABTECH	Auckland, NZ
Polypropylene micro-centrifuge tubes	Eppendorf	Lower Hutt, NZ
R2A broth	LAB M™ Ltd.	Lancashire, UK
Reasoner's 2A (R2A) Agar	OXOID™	Lower Hutt, NZ
Refrigerated incubator shaker	Innova™ 4230	Auckland, NZ
Spectrophotometer	DeNOVIX® DS-11FX+	Auckland, NZ
sterile 0.45 µm syringe filters	MILLEX®-HV	Auckland, NZ
Tetracycline, Chloramphenicol, Carbenicillin, Erythromycin, Ampicillin and Streptomycin	Sigma-Aldrich®	Auckland, NZ

3.1. Soil Samples

3.1.1. Waikato Region (WR)

Three sets of soil samples, with each set including samples originating from pastoral, arable and native bush sites were collected from the Waikato Region (WR) in collaboration with the Waikato Regional Council (WRC) based on standard soil sampling protocols [171, 172]. Samples were collected from the upper soil horizon from 0-10 cm depth in a representative way and aggregated to provide sufficient mass (˜20 kg) for a range of experiments [173]. Sampling was done in different seasons from February 2014 to June 2015. A total of five different rural properties were sampled (Table 3.2) and these were drawn from the larger set that makes up WRC's 'Regional Soil Quality Monitoring Programme' [174]. All sites and regional boundaries are shown in Figure 3.1. These soil sampling sites were chosen as sites which receive high levels of HMs including Cd and Zn due to usage of high levels of HM-containing compounds on them annually.

Table 3. 2. WR soil sampling sites sampled for this study. EW stands for Environmental Waikato.

Samples	Acronym	Time	Native bush site	Arable site	Pasture site
1st sample lot	WRSS1	Feb. 2014	EW-73	EW-85	EW-69
2nd sample lot	WRSS2	Aug. 2014	EW-73	EW-86	EW-135
3rd sample lot	WRSS3	June 2015	EW-73	EW-85	EW-69

Under arrangements made between the WRC and property owners who participate in regional soil monitoring, confidentiality is maintained around the exact locations of privately-owned sampling sites, but more general areas can be identified.

Samples from sites EW-69, EW-73, EW-85, EW-86 and EW-135 (Table 3.1 and Figure 3.1) were used in the project. These samples were collected from Pukekawa and Tuakau, on properties that were nearby each other, to ensure a match of the soil type. The soil series for all samples was Patumahoe Clay Loam, in the 'granular' soil order (N. Kim, personal communication). Pastoral soil samples were collected from WRC sites 69 and 135, which are livestock production sites with a long drystock history. The arable cropping soil samples (WRC sites EW-85 and EW-

86) were from sites used in horticulture and have a long history of potato and onion cropping (Figure 3.1). Both arable cropping and pastoral sites have received regular inputs of products containing HMs, in particular Cd from superphosphate fertiliser and Zn from facial eczema remedies (in pastoral soils) and Zn-containing fungicides (in horticultural soils). Soil samples from a native bush site were collected from WRC site EW-73 at Alexandra Redoubt Rd Reserve, Tuakau, Waikato, which has been covered in bush for more than 100 years and not received any inputs of products containing HMs.

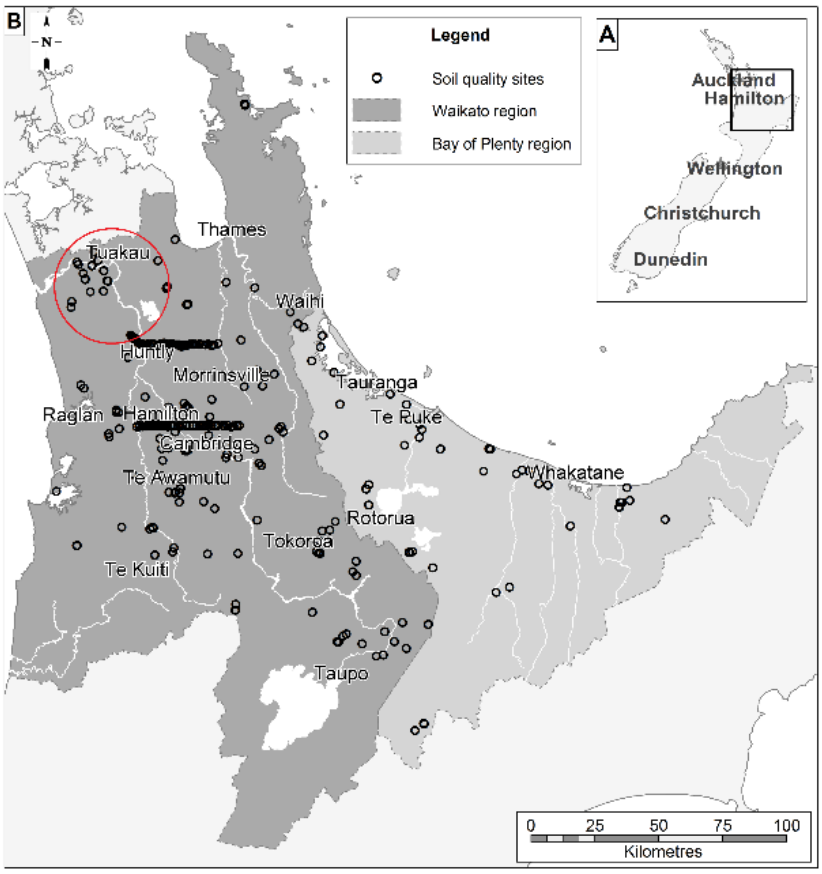


Fig. 3. 2. Locations of the soil sampling sites in WR (Image: courtesy of Kim N., et. al., 2015).

The sites' soil structure and chemical information was assessed in collaboration with WRC by Hill Laboratories Ltd., Hamilton, New Zealand. Briefly described methods for these assessments are shown in Table A1.1, Appendix 1.

3.1.2. Belmont Regional Park soils (BRP)

Soil sampling from the BRP (GPS Coordinates -41° 11' 24.00" S and 174° 52' 30.00" E), close to Wellington city, was performed at a pastoral livestock farm with granular soil and a fertiliser storage shed adjacent to an airstrip. Sampling was performed in December 2016 and April 2017 from eight sub-sites, 10 m apart, starting from the beginning of the airstrip (Figure 3.3) representing a gradation of fertilizer concentrations along the airstrip (Kim, N., pers. comm.). Sub-samples (n=10) of the upper soil horizon (0-10 cm) were collected in a straight line transect at each sub-site using a 10 cm-depth foot corer. Sampling was performed from least to most contaminated sub-sites. BRP's sampling location and sampling scheme are illustrated in Figures 3.2 and 3.3.

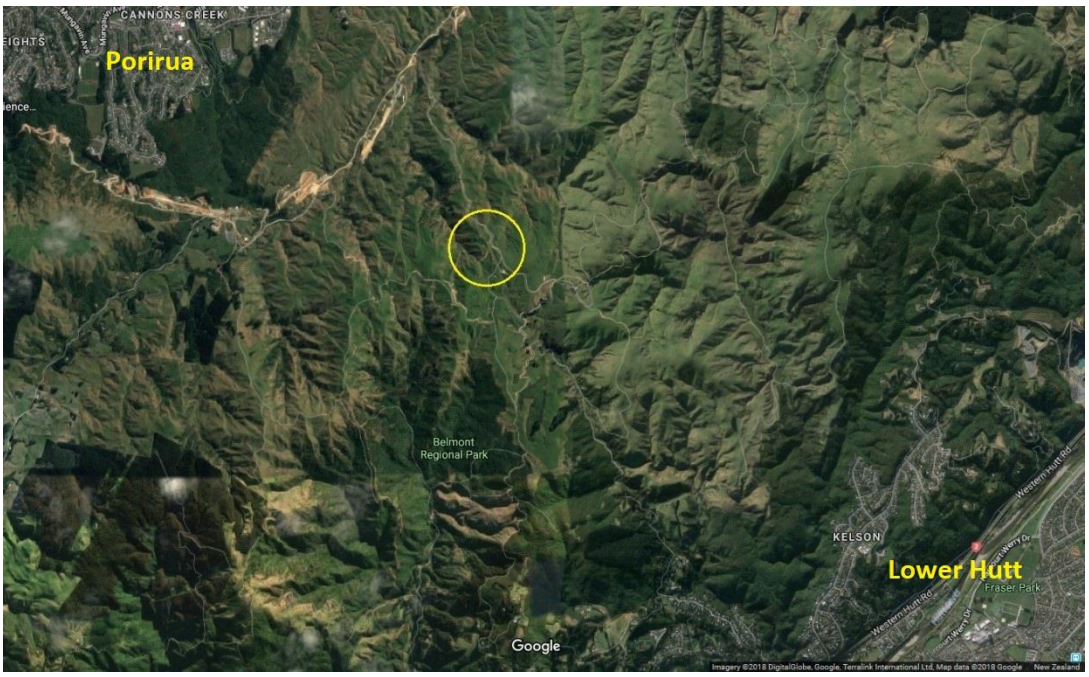


Fig. 3. 3. Belmont Regional Park map and airstrip location. Image: Imagery ©2018DigitalGlobe, Google Map

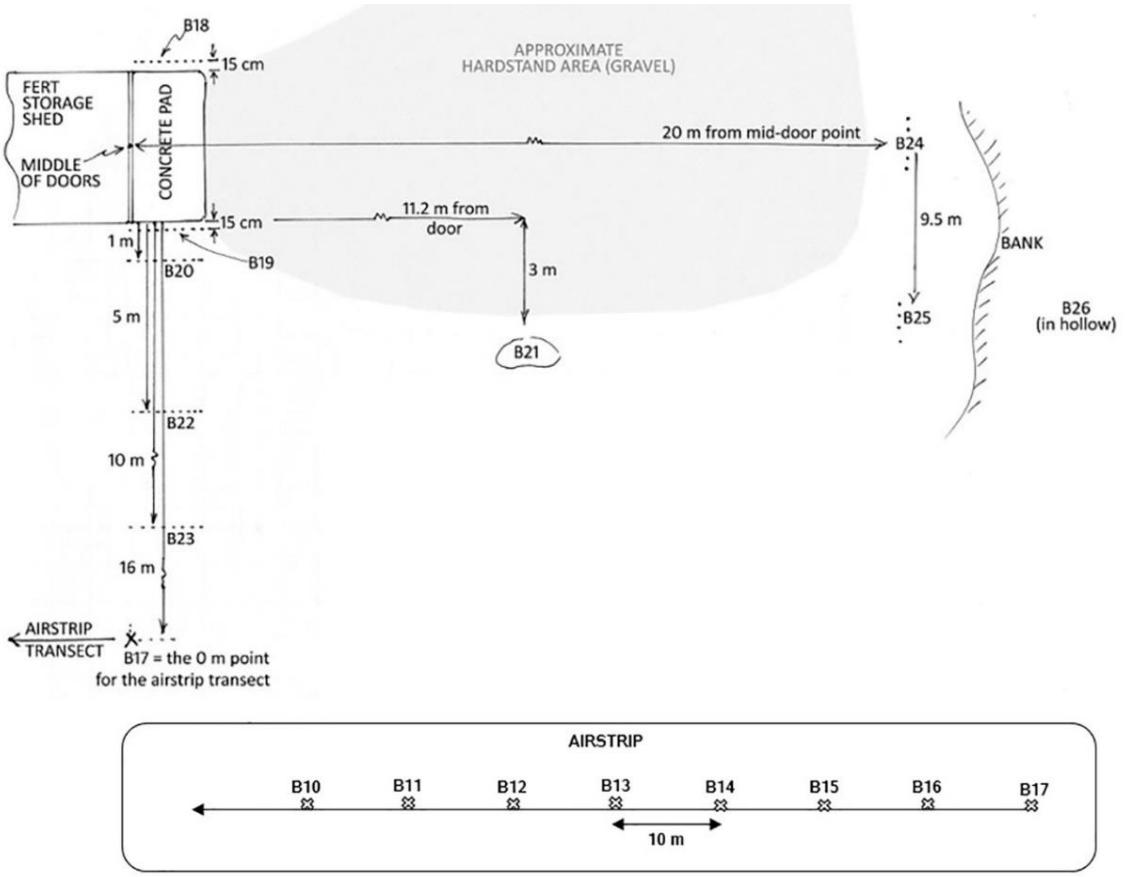


Fig. 3. 4. Sampling scheme at Belmont Regional Park airstrip. (image: Kim, N. Used by Permission).

The BRP sub-sites soil structure and chemical information was assessed by Hill Laboratories Ltd., Hamilton, New Zealand, using the methods described in Table A1.1, Appendix 1.

Soil samples from WR and BRP were kept at the most for 12 hours in the dark in air impermeable bags at 4 °C until commencement of laboratory studies. Water content and Ph of the soil samples were measured on arrival.

3.2. Extraction of Soil Bacteria and Preparation of Serial Dilutions

Soil samples were first sieved (aperture= 5 mm) to remove large soil particles, such as stones and plant debris [175]. The moisture content of the soil samples was measured by overnight drying 1 g of each sample and subtracting the dry weight from the 1 g initial wet weight [176, 177]. Soil samples (10 g, dry weight) were then added to a 200 mL Schott bottle containing 90 mL of sterile 1X Phosphate Buffered Saline (PBS) buffer (pH=7.0) and shaken at 200 rpm and 4

°C for 1 hour using a refrigerated shaker incubator. All of the pH adjustments for the PBS buffer and other reagents used in this study were performed using a pH meter and 0.1 M HCl and NaOH. Six universal bottles washed with 50% nitric acid, containing 9 mL of sterile 1X PBS buffer were allocated to each soil sample to make serial 10-fold dilutions [178].

3.3. Preparation of HMs Stocks and Abs

Initial stocks of metal solutions were made in distilled water to reach the concentrations of 1 M for Cd and Hg ions, and 5 M for Zn ions, using $CdCl_2$ (MW=183.31 g mol^{-1}), $ZnSO_4.7H_2O$ (MW=287.55 g mol^{-1}) and $HgCl_2$ (MW=271.52 g mol^{-1}). The pH of metal solutions was adjusted to neutral with 0.1 M NaOH and solutions were autoclaved (121 °C, 15 min, 1 bar). These were used as source solutions for HM additives to media to culture soil bacteria. The high water-solubility of these HMs' salts made them suitable options for bacterial culture-based experiments [179-183].

Five different Abs of various Abs groups, including Tetracycline (Tc), Chloramphenicol (Cm), Carbenicillin (Cb), Erythromycin (Ery) and Ampicillin (Amp) were used to prepare the Ab stocks. These Abs are members of the tetracyclines, chloramphenicol, antipseudomonal penicillins, macrolides and penicillins groups respectively. Initial stock concentrations of 0.2 g mL^{-1} of these Abs were prepared in dimethyl sulfoxide (DMSO). The Abs stocks were sterilised by passing them through sterile 0.45 µm syringe filters and stored at -20 °C [184, 185].

3.4. Plate Culturing

3.4.1. Media

Reasoner's 2A (R2A) Agar was used as a general solid culture media using 90 mm round petri-dishes. A high number of soil and water bacteria are slow growing and can be suppressed by faster growing isolates if they are cultured in more nutritious media e.g. Nutrient Agar [186]. After sterilisation (121 °C, 15 min, 1 bar), media (~37 °C) were poured into petri-dishes (20-25

mL) to make basal media and HMs and Abs supplemented media plates. HMs and Abs were added to the madia and when required supplemented with HMs or Abs.

3.4.2. HMs and Abs Additives Concentration for Plate Culturing Using R2A Agar

A range of HM and Ab concentrations were added to R2A Agar plates using the stock solutions of HMs and Abs (Abs addition at ~37 °C). Final HM ion concentrations were used 1, 0.1, 0.01 and 0.001 µg mL^{-1} for $CdCl_2$, 5, 1, 0.1, 0.01 and 0.001 µg mL^{-1} for $ZnSO_4$, and 0.1, 0.01 and 0.001 µg mL^{-1} for $HgCl_2$. The following equation was used to calculate the volume of 1 or 5 molar initial metal compound solution to be added per L kg^{-1} of media/soil:

Equation 3.1:

$$i = \frac{X \times 1000000}{1000(M/(C/100))}$$

Where *i* is the volume of metal compound solution (mL) to be added to one litre of media or one kg of soil, *X* is the preferred final concentration of metal in media/soil by m*M*, *M* is the molarity of metal's initial stock, *C* is the percentage of metal atoms in its 1 *M* compound solution.

A range of concentrations of Abs (dissolved in DMSO) were used as media additives at concentrations of 20, 100 and 200 µg mL^{-1}. Based on the literature these concentrations are the average concentrations to determine the bacterial resistance to Abs [187].

3.4.3. Cycloheximide Additive

A concentration of 100 µg mL^{-1} of cycloheximide was added to the basal, HM and Ab-supplemented media before pouring the plates to prevent the growth of fungi and yeasts [188, 189], which spread over the plate surface inhibiting the growth of bacteria and obscuring colonies to be counted. A 100 mg mL^{-1} stock of cycloheximide solution in DMSO was prepared. After sterilisation of the solution by filtration through sterile 0.45 µm syringe filters, 1 mL of that stock solution was added per litre (final concentration of 100 µg mL^{-1} of cycloheximide) of media.

3.4.4. Plate Spreading of Aliquets of Serial Dilutions

A 100 µL aliquot of each serial dilution, 10^{-2} to 10^{-8}, of extracted soil bacteria from 10 g (dry weight) in PBS buffer was spread on plates containing basal, HM- or Ab-supplemented media in triplicate. Plates were incubated at 25 °C for 14 days [188, 190].

3.4.5. Colony Counting and Colony Forming Units (CFU) Calculation

Colonies on plates were counted using a manual colony counter [191, 192].

The total Colony Forming Units (CFU) of plates containing 10-300 colonies were counted, because of lack of precision counting colonies >300 per plate. Counting colonies on plates with less than 10 colonies can lead to statistical errors [193]. The mean values from each set of three replicates were calculated and total CFU obtained per g of soil dry mass using the following equation: Equation 3.2:

$$CFU_{DM} = \frac{N \times D \times 100}{TW_w - SW_w}$$

Where CFU_{DM} is CFU per soil dry mass, N is number of colonies per plate, D is inverse dilution factor, TW_w is total weight of soil, which is for example 1 g, and SW_w is the weight of water in the soil. The total CFU (CFU_T) in 1 g of soil was determined using the following equation:

Equation 3.3:

$$CFU_T = \frac{CFU_{DM}}{TW_w}$$

3.5. Morphology Assessment and Gram Classification of Selected Bacterial Isolates

Approximately 100 independent colonies from plates, containing a range of HMs and Abs, were selected from each soil sample of WR and BRP soil, and categorised in various groups based on their morphological features (Table 3.3). The selected bacteria were selected from all of the morphological categories, not only the dominant morphologies [194].

Table 3. 3. Morphological descriptors used to describe bacterial colonies.

Whole colony	Edge	Elevation	Surface
Punctiform	Entire	Flat	Smooth, Glistening
Circular	Undulate	Raised	Rough
Rhizoid	Lobate	Convex	Wrinkled
Irregular	Filamentous	Pulvinate	Dry, Powdery
Filamentous	Curled	Umbonate	

Gram staining to differentiate the selected isolates into Gram negatives and Gram positives was carried out by both methods of Gram staining and use of 3% KOH solution [195, 196].

3.6. 6-Week and 6-Month Microcosms

3.6.1. Soil Samples

Microcosm experiments were performed in two separate stages and sets of experiments, the first involving 6-week incubations, and the second with 6-month incubations. The 6-month-incubation microcosms was performed to determine if any changes would occur in the profile of bacterial communities and diversity over a longer incubation period compared to the 6-week incubation. The pastoral soil-contained microcosms with 6-month incubation was used to investigate the bacterial community structure changes in presence of different levels of HMs in comparison with the bacterial community dynamics in native bush soil-contained microcosms incubated for 6 months [197-199].

The native bush soil sample from sites EW-13 was used for 6-weeks and 6-month microcosms' experiments, and EW-24 were used for 6-month microcosms' experiments. The sample from EW-13 was a sandy loam soil from Taupo, with a long history of plantation forestry. This soil was classified as an Immature Orthic Pumice soil and contained relatively low contamination levels, because little fertiliser is used on plantation forestry. This soil can be considered as 'close to natural.' Sample EW-24 was a clay loam soil from Coromandel (West of Whangapoua) from a property used for dry-stock farming, containing higher levels of Cd, Zn and other contaminants. This soil was classified as a Typic Orthic Brown Soil.

3.6.2. Preparing Microcosms

Polypropylene boxes with 500 g capacity (Dimensions L=20, W=15, H=5 cm) were used as containers for the microcosms with 10 x 1 mm diameter drainage holes in the bottom, and 5 x 10 mm ventilation holes in the lids to avoid excess soil moisture. Soil samples (300 gram) were added to each microcosm container after the removal of large stones, debris etc. by sieving (aperture= 5 mm) (Figure 3.5).

Fig. 3. 5. Microcosms stored in a 25 °C incubator room.

3.6.3. Microcosms- 6-Week Set

The first microcosm experiment involved a total incubation period of 6 weeks. Topsoil from WRC site EW-13, with no or limited HM pollution history was used for this part of experiments. Each prepared microcosm was spiked by one of the five concentrations of $CdCl_2$, $ZnSO_4.7H_2O$ or $HgCl_2$ in triplicate to reach the final concentrations of Cd ions of 5, 10, 50, 100 and 200 mM, Zn ions by 20, 50, 100, 200 and 300 mM, or Hg ions by 0.5, 1, 5, 10 and 50 mM. The selected concentrations cover the Landcare Research recommended limit concentrations for metals in New Zealand soil [197, 200, 201]. Three microcosms were assigned as controls and received no spiked HMs. HM stocks were prepared as described in Section 3.4.2. Each microcosm had a tray to collect the drainage fluid. Aliquots of 300 mL of sterile distilled water were spiked with the appropriate

amount of metal stock solution and this was sprayed onto the microcosms' soil. A negative microcosm control, containing distilled water with no metal additive was allocated to the experiment. Microcosms were left for 6 hours and then their drainage fluids were collected for metal concentration analysis by Atomic Absorption Spectrophotometry. The microcosms were incubated for six weeks at 25 °C.

A total amount of 10 g (dry weight) of each microcosm soil sample was mixed with 90 mL of sterile PBS buffer and was shaken in a shaker incubator with 200 rpm for 1 hour. Aliquots of 100 µL of 10-fold serial dilutions of 10 g (dry weight) samples of the microcosms' soil were cultured on plates containing one of two concentrations of HMs (0.1 and 1 µg mL^{-1} for Cd and Zn, and 0.01 and 0.1 µg mL^{-1} for Hg) or one concentration of Abs (20 µg mL^{-1} for Tc and Cm, 100 µg mL^{-1} for Cb and Ery, and 200 µg mL^{-1} for Amp) as well as control plates (no added metal or Ab) in triplicate at time zero and two-weekly intervals. Plates were incubated at 25 °C for 2 weeks and colony counting was performed [176, 177, 202]. Morphology assessment of bacterial isolates from the plates and pH measurement of soils was performed at time 0 and every other culture round and compared to colonies from control microcosms [202-204].

The soil of the microcosms was mixed every week using sterile spatulas [176]. Weekly moisture measurement of the microcosms' soils was performed and adequate water was added to maintain constant soil moisture of about 20±5% (the original soil sample's moisture content) [177].

Duplicate 10 g of soil samples were added to 50-mL Falcon tubes, one for Pollution Induced Community Tolerance (PICT) and another one for DNA extraction for Terminal Restriction Fragment Length Polymorphism (TRFLP) tests and stored at 4 °C and -20 °C respectively.

3.6.4. Microcosms- 6-Month Set

Two types of soils were used for 6-month microcosms. These soils were from site EW-13 which was considered 'close to natural' in its soil contaminant status, and site EW-24 with a long history

of dry stock grazing, which contained medium levels of specific contaminants, most notably Cd and Zn. The same processes and experiments were carried out for the 6-month microcosms as for the 6-week series, but with an incubation period of six months, and the plate culturing being performed at time 0 and every 2 months. The 6-month microcosm work was performed to determine if any changes would occur in bacterial communities and diversity over a longer incubation period compared to the 6-week incubation. The aim of using 6-month microcosms with pastoral soil was to investigate the bacterial community structure changes in presence of various levels of HMs in comparison with the bacterial community structure changes in native bush soil-contained microcosms incubated for 6 months.

One potential limitation to be noted, for this part of the project, when making comparisons is that two different soil types were involved in the 6-month microcosm work. The 'near natural' soil from site EW-13 was in the 'Pumice' soil order (with the texture classification of 'sandy loam'), whereas the sample from EW-24 was a 'Brown' soil (texture: clay loam). The second soil contained higher organic matter and clay content. Presence of these soil components would be expected to reduce the bioavailability of anthropogenic Cd and Zn in soil EW-24, compared with that expected if the same total concentrations were present in a pumice soil. This difference was eliminated in experiments involving other soils (Table 3.1) which were all the same soil order ('Granular') and type.

Texture and chemical information of EW-13 and EW-24 soil samples was assessed by Hill Laboratories Ltd., Hamilton, New Zealand, using the methods briefly described in Table 3.2.

Soil samples (10 g) from each 6-month microcosm was collected in 50 mL Falcon tubes at time 0, and every two months thereafter. Duplicate 10 g soil samples were added to 50-mL Falcon tubes, one for Pollution Induced Community Tolerance (PICT) and another one for DNA isolation for Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis and stored at 4°C and -20°C respectively.

3.7. Microcosms' Leachate Metal Concentration Analysis

Metal concentrations of drainage fluids collected from microcosm trays were analysed using Atomic Absorption Spectrophotometry (see below). Based on the initial metal concentrations added to the microcosms, two different methods of metal concentration analysis were performed. Flame AAS (air/acetylene) was used for higher-level determinations [205], and Graphite-Furnace AAS was used to quantify metals present at concentrations below detection limits of the flame technique [206]. Samples of the negative-control microcosms drainage were used as negative controls for this part of the analysis. Starting levels of HMs were measured and the result are shown in Table 5.1.

After collection and prior to analysis, samples were preserved by adding sufficient analytical reagent (AR) nitric acid to achieve a final acid concentration of 0.5%. The analysis was carried out against suitably prepared matrix-matched standards on a high-resolution Continuum-Source Atomic Absorption Spectrometer. Testing carried out in graphite-furnace mode made use of appropriate matrix modifiers. Determinations were carried out at wavelengths of 228.8018 nm for Cd, 213.8570 nm for Zn, and 253.6519 nm for Hg, which are primary absorption lines for the three elements [207]. Standard solutions were prepared by dilution from ICP Multi-Element Standard Solution IV for Cd and Zn, and 0.1 *M* $HgCl_2$ solution for Hg. Standards and blank solutions were prepared using distilled water and preserved with AR nitric acid (final strength 0.5%).

3.8. Sequential Extraction of HMs

This analytical procedure of HMs extractions from microcosms soil samples was performed to assess the absorption of HM cations to the soil particles [208]. The method had five different stages, during which more strongly absorbed ions could be extracted on each successive step. The first step is to extract the exchangeable ions in water, and here just this step was performed. Eight mL of 1*M* $MgCl_2$ (pH=7.0) was added to 1 g of soil air dried overnight in 15 mL Falcon tubes

with vigorous shaking for 60 minutes at room temperature. Tubes were spun at 1000 rpm to remove soil residues and an aliquot of 5 mL of the supernatant was transferred to a new 15 mL Falcon tube containing 10 mL of deionised distilled water. Suitable standards were prepared (using the mentioned method above) and each sample's HM concentrations were measured by HR-CS Atomic Absorption Spectrophotometer. The negative control microcosms leachate were used as negative control for these determinations.

3.9. Purification and Storage Selected Isolates

All the selected isolates (n=900 for each WR soils set, n=300 for each BRP soils, and n=600 for each microcosm set) grown on R2A agar plates, containing a range of HMs and Abs, were sub-cultured and purified by streaking them onto R2A Agar plates. These were a total number of 100 bacteria isolated from each WR soil sample, and 50 isolates were selected from plates with lower (Cd 0.1 mM, Zn 1 mM, and Hg 0.01 mM) and 50 isolates were selected from plates with higher (Cd 1 mM, Zn 5 mM, and Hg 0.1 mM) concentrations of HMs. These isolates were a total number of 100 bacteria selected from plates with each metal (50 isolates from plates with lower HM concentration and 50 isolates from higher concentration of HM). These isolates were a total number of 150 isolates per each microcosm (50 bacterial isolates selected from plates with each HMs including Cd 1 mM, Zn 5 mM and Hg 0.1 mM). A total number of 50 HM-sensitive bacterial isolates from each soil sample from WR, BRP and microcosms and were selected from plates with no added HM. These sensitive isolates were then cultured on plates with the mentioned concentrations of HMs to validate their sensitivity to the HMs. A few colonies of each morphotype were used to prepare a liquid culture to prevent selection of atypical variant clones. These purified single colonies were added to 5 mL R2A broth (pH=7.0), and re-cultured in a shaker incubator for 48 h at 25 °C. These liquid culture stocks were stored at -80 °C in 30% (v/v) glycerol [178].

3.10. Pollution Induced Community Tolerance (PICT) Analysis

The tolerance of a community of bacteria to a range of antimicrobial agents at various concentrations was determined by a microtitre plate culturing-dependent method called Pollution-Induced Community Tolerance (PICT) [209, 210]. The PICT test was carried out for soil samples taken from WR, BRP, and the 6-week and 6-month microcosms. R2A broth was used as a common substrate for soil bacterial communities.

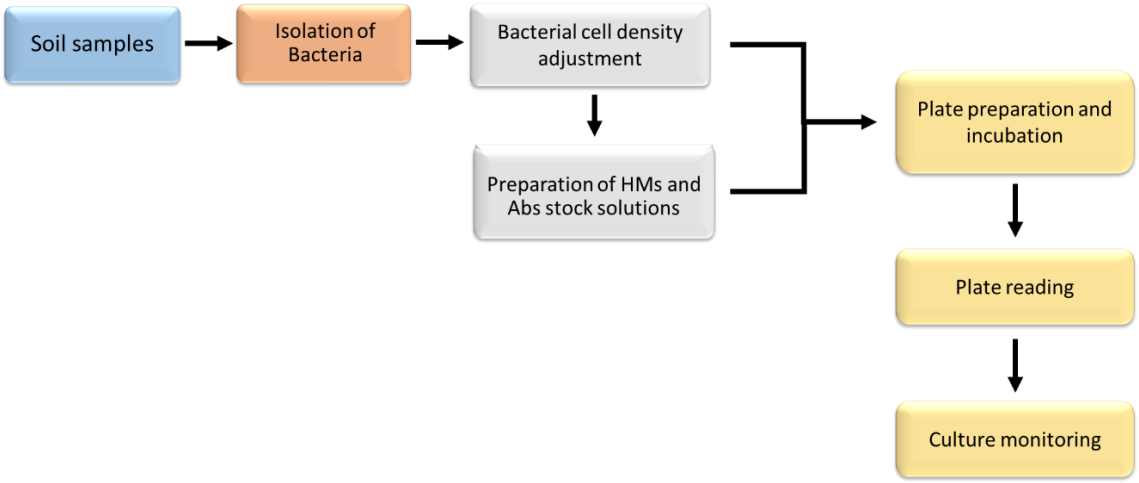


Fig. 3. 6. Flow chart illustratind different steps of PICT analysis

3.10.1. Measuring the Cation Content of Media

The concentrations of cations in media, may be higher than the cation content human or animal blood, and may induce higher MIC reactions to Abs. The cation content of culture media should be checked before plate culturing [193], and the results are more comparable with *in vivo* Abs activity results [193, 211]. Based on manufacturer's information, the content of $MgSO_4$ in R2A broth media was 50 mg L^{-1} corresponding to a ~10 mg L^{-1} Mg^{2+}, which is equivalent to the Mg^{2+} content of blood. The Ca^{2+} content of the R2A broth media was measured by Atomic Absorption Spectrophotometry and determined to be about 20 mg L^{-1}. Mg^{2+} and Ca^{2+} concentrations in R2A agar was taken from the manufaturer's information. The chemical contents of R2A agar and R2A broth media used in this project are listed in Table 3.4.

Table 3. 4. R2A agar and broth chemical contents

Typical formulation	Oxoid® R2A agar	LAB M™ R2A broth
	g L^{-1}	
Yeast extract	0.5	0.5
Meat peptone	0.5	0.5
Casein hydrolysate	0.5	0.5
Glucose	0.5	0.5
Starch	0.5	0.5
Dipotassium hydrogen phosphate	0.3	0.3
Magnesium sulphate	0.024	0.05
Sodium pyruvate	0.3	0.3
agar	15	-
pH at 25°C	7.2 ± 0.2	7.2 ± 0.2

3.10.2. Initial Bacterial Cell Stock Preparation

A 10 µL aliquot of an overnight culture of purified isolates (described in Section 3.4) was added to 25 mL of autoclaved R2A Broth media in 50mL screw-capped and 50% nitric acid-washed universal bottles. The bottles were incubated at 25 °C for 48 h in a shaking incubator (200 rpm) [212]. A 100 µL aliquot of the liquid culture was cultured on basal R2A agar and incubated at 25 °C for 48 h, to check culture purity.

3.10.3. Bacterial Cell Density Adjustment

The density of bacterial inoculum was measured and set spectrophotometrically —OD=0.1 at 600 nm wavelength— at 1×10^8 cells by adding adequate amount of fresh R2A broth. Spectrophotometer OD measurement validation was performed using a McFarland 0.5 turbidity standard. The turbidity standard solution was made by mixing 1 mL of 48mM barium chloride dihydrate ($BaSO_4$-$2H_2O$) solution with 199 mL of 1% sulphuric acid (H_2SO_4) and shaking vigorously. Its optical density (OD) was measured using both spectrophotometer and plate reader at 600 nm and showed 0.1 absorbance, proving both the prepared standard turbidity solution and spectrophotometer OD measurement accuracy [193].

To reach the desired number of bacterial cells in liquid culture of 5×10^5 mL^{-1}, 1:200 dilution of stationary phase of bacterial cultures were prepared —100 µL of liquid culture with OD=0.1 in

19.9 mL of fresh liquid media [213, 214]. Since the OD absorption can be different for various bacterial isolates with different cell size, shape and colour, the precise number of colonies mL^{-1} at a given OD for each bacterial isolate was determined; therefore, a 10 µL aliquot of each 1:200-diluted liquid culture was added to 990 µL fresh R2A broth and a 100 µL aliquot was spread on the surface of basal R2A agar. The number of colonies was counted (between 10-300 colonies) and CFU was determined for the 100 µL-volume positive control at the time zero point using the following equation:

Equation 3.4:

$$CFU = \frac{C \times 10}{10^{-D}}$$

Where CFU is the total number of individual colonies grown from 100 µL of liquid culture; C is number of colonies per plates; 10^{-D} represents the number of 10-fold dilution of liquid culture which is D=2 here; 10 (µL) represents the liquid culture aliquot volume diluted by 1:100. This scenario helped to monitor the precise number of individual cells/CFU of each bacterial isolate that were added to the microtiter wells [193].

3.10.4. Preparation of Heavy Metals (HMs) and Antibiotics (Abs) Stock Solutions

To measure the MIC (the lowest concentration of antimicrobial agents inhibiting visible planktonic cell growth), the desired range of HMs and Abs concentrations were selected based on literature reports and standard MICs for bacterial isolates reported by EUCAST respectively [215]. Stock solutions 1000X stronger than required final concentrations of HMs and Abs were prepared; these concentrations included 0.1, 0.5, 1, 5, 10, 20, 100, 200 and 500 µg mL^{-1} (dissolved in DMSO) for Abs. Final HM ion concentrations of 0.0001, 0.001, 0.01, 0.1, 1, 2, 5 and 10 mM of Cd using $CdCl_2$, 0.001, 0.01, 0.1, 1, 2, 5, 10 and 20 mM of Zn using $ZnSO_4.7H_2O$, and 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, 2 and 5 mM of Hg using $HgCl_2$, were prepared in sterile 1.5 mL polypropylene micro-centrifuge tubes [216].

3.10.5. Plate Preparation and Incubation

A 100 µL aliquot of the prepared extracted bacterial dilution with ~5×10^5 bacterial cells per mL was added to each well of microtiter plate containing 99 µL of 2× R2A broth [213]. To test if there are ~5×10^5 mL^{-1} bacterial cells, aliquots of 100 µL of extracted bacterial dilution was cultured on basal R2A agar and the number of CFUs was calculated using Equation 3.4 [193]. A 1 µL aliquot of HMs or Abs (prepared as described in Section 3.10.4) was added to the allocated wells in triplicate, as were negative and positive controls. OD adjustment was performed before inoculation of plates wells with HMs and Abs (as described in Section 3.10.3).

S. aureus NCTC 12973 was added to the experiment batches as a quality control, and resulting data compared with the EUCAST databases [17, 42]. Incubation was performed in a shaking incubator at 25 °C and 200 rpm for 72 h. OD reading from each well were recorded at time zero, before inoculation of wells with antimicrobials to monitor the possible OD changes due to antimicrobial agents addition, and at 6h intervals [212]. EC50 was calculated for each batch of results. Growth monitoring by plate culturing and measurements of metals' bioavailability was carried out after 72 h incubation by the methods described below (Sections 3.10.7 and 3.10.8). A half-filled 5 L beaker of water was put in the shaking incubator to provide enough humidity to prevent the liquid culture drying out.

3.10.6. Plate Reading

Plate reading was performed at time zero and 6-hour intervals using a plate reader at 600 nm. The data from the plate reader was normalised by establishment of linear relation of the plate reader with the spectrophotometer used for initial OD adjustment at 600nm wavelength [216]. Bacterial resistance was quantified as MIC based on the obtained MIC results at the exponential growth phase and data was analysed according to the EUCAST ECOFF (epidemiological cut-off) recommendations. To generate ECOFF values for Abs at least 250 isolates from each soil sample needs to be subjected to the MIC evaluation [217-219]. D'Costa et. al. (2007) and Bhullar et. al.

(2012) reported that Ab resistance in soil bacteria is defined as growth at 20 µg mL^{-1}, which is used for all bacteria and all classes of antibiotics [220, 221]. EC50 was calculated by GRAPHPAD Prism 6 software and Log (inhibitor) vs. response, Variable slope (four parameters) method.

3.10.7. Growth Monitoring

Agar plate culturing of the liquid growth in the microtiter plates was performed on R2A plates with the same method, in order to monitor the plate reader OD accuracy, and also the growth of the bacterial population in the presence of HMs and Abs after 72 h incubation [193]. Aliquots (100 µL) from each triplicate well were mixed and an aliquot of 100 µL of the cell mixture was cultured on basal R2A agar plates, after suitable dilution based on their OD. The equation 3.3 was used to calculate the number of bacterial colony forming units in each culture. The agar plate culturing helped to monitor and compare between the number of viable versus senescent bacterial cells in the microtiter plates liquid culture [193]. The positive and negative controls mentioned in Section 3.11.5, were used here too. Bacterial biofilm formation can be induced in the presence of low levels of Abs. However, this feature of some bacterial classes was considered as a natural characteristic which may or may not affect the accuracy of the bacterial growth in liquid media, since each assay was prepared consistently this phenomenon was not expected to significantly influence the data collected. On the other hand, this feature was outside the scope of this thesis.

3.10.8. Measuring Heavy Metals (HMs) Bioavailability in Plate Wells

Due to the supposition that absorption to the surface of microtitre plates wells by some HM ions might have occurred, HMs bioavailability in polystyrene microtiter plate wells was measured after the incubation time using Atomic Absorption Spectrophotometry (AAS). After mixing triplicate wells including those for the positive and negative controls, and inoculated wells with each concentration of HMs, bacterial cells were precipitated by spinning at 3000 rpm for 5

minutes. The supernatants after serial dilution detectable by the AAS, were applied to the machine.

3.11. Broth Microdilution (BM) Analysis

A BM experiment was performed for the selected isolates purified from WR, BRP, and microcosm (described in Section 3.9). MIC and EC50 values of HMs and Abs for each bacterial strain were determined from this data. Figure 3.7 illustrates different steps of BM analysis.

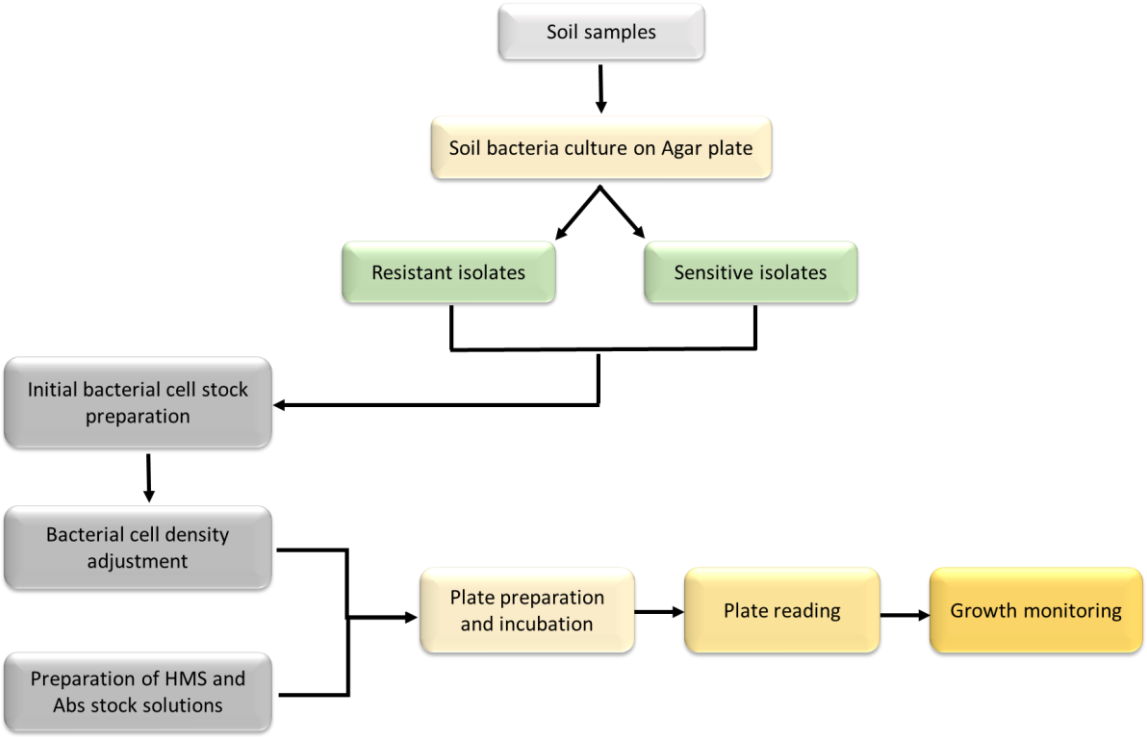


Fig. 3. 7. Flow chart illustrating different steps of BM analysis.

3.11.2. Plate Preparation

MIC was examined for a range of Abs and HMs concentrations. A 99 µL aliquot of liquid culture (cell density adjustment was explained in Section 3.11.3) was dispensed to each well of 96-well polystyrene microtiter plates using an electronic 8-channel pipette [193, 213]. A 1 µL aliquot of the Abs and HM stocks was added as antimicrobial additives to the wells of 96-well plates in triplicates. An aliquot of 1 µL DMSO/99 µL fresh broth media, positive (growth without any

antimicrobial additives) and negative (sterile; fresh media without bacterial cells) controls were included in triplicate. *S. aureus* NCTS 12973, a standard sensitive control strain was added to each batch as a quality control. The *S. aureus* control strain's MIC test values were compared with the EUCAST (European Committee on Antimicrobial Susceptibility Testing) epidemiological cut-off databases in order to quality control [193, 213, 217]. According to culture-based investigations of Ab resistance in soil bacteria, the defined level of antibiotic resistance in soil bacteria is growth at > 20 μg mL^{-1} [217, 220, 221]. The whole process for each bacterial isolate was completed in ≤1 h based on the EUCAST MIC test recommendations, to prevent experiment errors due to bacterial cell divisions [215, 222, 223].

The time-zero plate reading was performed at 600 nm before inoculation of the plates' wells with a range of HMs and Abs to monitor OD changes due to antimicrobial agents addition [193].

Plates were incubated in a shaking incubator at 25 °C and 200rpm for 72 h [212]. Other methods used for PICT analysis, e.g. measuring the Cation content of media, initial bacterial cell stock preparation, bacterial cell density adjustment, preparation HMs and Abs stocks, were performed as described in sections 3.10.1-3.10.5.

3.12. Molecular Experiments

3.12.2. Soil DNA Extraction

Total genomic DNA of soil samples, (from WR and BRP sites, and 6-week and 6-month microcosms) was extracted to allow genetic/genomic analysis, including 16s rDNA Sequencing, and Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis. Microbial DNA suitable for Polymerase Chain Reaction (PCR), genotyping and Next Generation Sequencing (NGS) was extracted from soil samples using Mo Bio PowerSoil® DNA Isolation Kits [224] following the manufacturer's protocol [225]. Briefly, 0.25 g of each soil sample was added to PowerBead tubes and gently shaken. A 60 μL aliquot of Solution C1 (contains SDS and other

compounds required for cell lysis) was added to each tube and mixed vigorously for 10 minutes. Tubes were spun at 10000 rpm for 30 seconds.

Quantitative and quality assays control of the extracted DNA samples were achieved by 1.5% agarose gel electrophoresis in 0.5% TBE (Tris-Borate-EDTA) buffer, and also dsDNA spectrophotometry for A260/280nm (preferably 1.7≤) and A260/230nm (preferably 2.0≤) ratios to detect impurities, primarily humic acids and proteins [226, 227].

3.12.3. 16s rDNA Sequencing

In order to investigate the diversity of the bacterial communities in soil samples, 16s rDNA sequencing was performed [228]. Total genomic DNA samples from 6-week microcosms spiked with Cd 100, Zn 200 and Hg 50 μg mL^{-1}, and the BRP B17, B14 and B10 sub-sites were sent to the Massey Genome Service hub of NZ Genomics Ltd., Palmerston North. Illumina 16S V3-V4 rRNA library preparation and Illumina MiSeq 16s rDNA sequencing test was performed on these samples. The quality control for 16s rDNA sequencing and the subsequent analysis was done with SolexaQA++ and QIIME software tools to generate a comprehensive taxonomic overview of the soil samples bacterial communities.

3.12.4. Terminal Restriction Fragment Length Polymorphism (TRFLP)

Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis is a technique used to study complex microbial communities based on variation in length of restriction fragments of the 16S rRNA gene. TRFLP analysis can be used to examine microbial community diversity, as well as structure and community dynamics, in response to changes in different environmental parameters or to study bacterial populations in natural habitats [229].

Genomic DNA of WR and BRP, and 6-week and 6-month microcosms soil samples was extracted for TRFLP analysis by the method described in Section 3.13.1 [225].

Quality control of DNA samples was performed by trial PCR reactions using unlabelled 16s rDNA gene-specific primers with the same sequence as the labelled ones used for sequencing, and 2% agarose gel electrophoresis (100 V, 40 min). A negative control containing no DNA template was included with these PCR reactions. PCR reaction contents are listed in Table 3.5.

Standard TRFLP primers have been designed to amplify a selected region of the 16S rRNA gene of the bacterial soil community DNA, the amplified product was approximately 1000 bp long. The 63F forward primer was labelled at its 5'-end with 6-FAM™ phosphoramidite dye-labelled (blue) —5'-CAG GCC TAA CAC ATG CAA GTC-3'— [230] and the 1087R reverse primer was VIC® phosphoramidite dye-labelled (green) —5'-CTC GTT GCG GGA CTT AAC CC-3'— [231]. The maximum absorption and maximum emission of the labels were respectively at 494 nm and 522 nm for 6-FAM™ (6-carboxyflourescein) and 538 nm and 554 nm for VIC® [232]. PCR product quality was assessed by ethidium bromide staining after running the PCR product on a 2% agarose gel (100 V, 40 min) [233].

Table 3. 5. PCR contents to prepare TRFLP test DNA fragments.

Component	**Reaction volume**	**Base concentration**	**Final concentration**
Dream Taq PCR Buffer	5 µL	10X	1X
dNTPs	1 µL	10 *mM*	200 *µM*
Forward primer	1 µL	0.05 *µM*	10 *pM*
Reverse primer	1 µL	0.05 *µM*	10 *pM*
Template DNA	Variable	Variable	<1000 ng
DreamTaq DNA polymerase	0.25 µL	5U μL^{-1}	1.25 U 50 μL^{-1}
PCR grade nuclease-free water	To 50 µL	-	-

Table 3. 6. PCR conditions to amplify desired DNA fragments for TRFLP analysis.

Step	Temperature °C	Time	Number of cycles
Initial denaturation	95	3 min	1
Denaturation	95	30 sec	
Annealing	55	30 sec	30
Extension	72	1 min	
Final extension	72	20 min	1

A 25 µL aliquot of PCR product (~200ng PCR product) was digested with 2 µL of 10U µL^{1-} *Msp*I restriction enzyme (Thermo Fisher Scientific). 0.3 µL acetylated Bovine Serum Albumin (BSA) (Thermo Fisher Scientific) at 37 °C for 3 h and subsequent 30min enzyme deactivation at 65 °C and a final fast cooling at 4 °C [233]. This step generated fluorescently-labelled terminal restriction fragments. Restriction fragment lengths were measured by detection of terminal fluorescent labelled fragments' analysed by ABI3730 Capillary Genetic Analyser at Massey Genome Service, Palmerston North. The output was a series of peaks of various sizes and heights that represents the profile of each sample [234]. The enzyme digestion products visualised for quality control purposes by analysing on a 2% agarose gel run at 100 V for 40 minutes. A negative control without DNA template was included for the PCR reactions, as negative control of enzyme digestion.

The Applied Biosystems 3730 series DNA Analyzer is used as strong electrophoresis system for TRFLP analysis. This machine (available at Massey Genome Service and used in the current project) is a multi-capillary system. TRFLP data analysis was performed with GeneMapper® software v.4.1 for peak analysis and PRIMER v.7 (Plymouth Marine Laboratory) for analysis of relative abundance of terminal restriction fragments as a proportion of a total peak height of all the Terminal Restriction Fragments (T-RFs) in that profile. GeneMapper software v.4.1 contains the required analysis parameters to recognise complex Terminal restriction Fragments (T-RF) patterns and provide a comprehensive fingerprint from metagenomics samples [142]. Three-Way ANOVA was used for statistical analysis to compare the abundance of the T-RFs between

samples. A total of 30 PCR reactions were executed using the conditions shown in Table 3.6 [233].

3.12.5. PCR Reaction Using Specific Primers

Two of the most common genes encoding Cd resistance in bacterial isolates were amplified using two specific primer pairs. A total number of 900 purified, Cd resistant bacterial isolates showed Cd resistant in HM's BM experiments (Sections 3.11) were tested in this way.

Total bacterial genomic DNA was extracted from these isolates by the boiling method [235]. Briefly, 2 mL of overnight cultures of bacterial isolates in R2A Broth was centrifuged in 10000 rpm to pellet the cells. The cells pellets were re-suspended in 500 µL of TE buffer (Tris-EDTA buffer, pH=8) and boiled and ice cooled three times for 5-minute intervals. A final concentration of 100 µg mL^{-1} of lysozyme was added to the Gram positive bacterial cell suspension and incubated in a 37 °C water-bath for 3 h with occasional shaking [236]. The same boiling method protocol was performed for Gram negative bacteria. The boiled and ice cooled tubes were spun in 13000 rpm for 10 minutes and 200 µL of supernatant with extracted DNA was transferred to a clean 500 µL polypropylene microfuge tubes and stored at -20 °C. The quality of the extracted DNAs was quantified using dsDNA spectrophotometry at A260/280 nm and A260/230 nm ratios to detect impurity, and 1.5% agarose gel electrophoresis in 0.5% TBE buffer [226, 227].

The targeted *czcA* [237] and *cadA* [238] genes were amplified using the PCR reaction components listed in Table 3.5, and primers detailed in Table 3.7 under the PCR conditions listed in Table 3.8. The *czcA* gene encodes an RND family efflux pumps involved in bacterial resistance to Cd^{2+}, Zn^{2+} and Co^{2+} [239]. The *cadA* gene encodes efflux pumps involve in expelling Cd^{2+} from bacterial cells [240, 241]. Negative controls with no DNA template was included in these PCR assays. The PCR products were visualised via 2% agarose gel electrophoresis run at 100 V for 40 minutes in 0.5% TBE buffer.

Table 3. 7. Sequence of the oligonucleotide primers used to amplify Cd resistance genes

Targeted gene	Primers	Sequence (5'-3')	Length of amplified fragment (bp)	Reference
czcA	Upstream (czcAF)	GGSGCGMTSGAYTTCGGC	252	Kaci et.al., 2014
	Downstream (czcAR)	GCCATYGGNYGGAACAT		
cadA	Upstream (cadA1)	CAAAYTGYGCRGGHAARTTYGA	1052	Oger et.al., 2001
	Downstream (cadA2)	AACTAATGCACAAGGACA		

Table 3. 8. PCR conditions to amplify Cd resistance encoding genes

Step	czcA			cadA		
	Temperature °C	Time	Number of cycles	Temperature °C	Time	Number of cycles
Initial denaturation	94	5 min	1	94	5 min	1
Denaturation	94	30 sec		94	1 min	
Annealing	58	1 min	35	52	1 min	30
Extension	72	1 min		72	2 min	
Final extension	72	7 min	1	72	5 min	1

3.12.6. Identification of Bacterial Isolates using 16S rDNA Sequencing

The bacterial isolates carrying either *czcA* or *cadA* Cd resistance genes were identified by 16S rDNA sequencing. DNA extraction with the boiling and ice cooling method, and DNA quality control by spectrophotometry and agarose gel electrophoresis was achieved as described in Section 3.13.5. The specific primers, unlabelled 63F and 1087R [230, 231] (Section 3.13.4), were used in PCR reactions to amplify the 16S rDNA regions of the bacterial isolates' genomes. PCR reactions were performed using the PCR reaction components shown in Table 3.5 and under the PCR conditions described in Table 3.8. DNA ethanol precipitation method was performed following the PCR reactions to concentrate and purify of the PCR products.

The quality and quantity of PCR reaction products were performed spectrophotometry and agarose gel electrophoresis. PCR products were sent to Massey Genome Service for 16S rDNA

sequencing by ABI3730 DNA Analyzer. The 16s rDNA sequence data from the raw reads for each bacterial isolate were compared to the data bases at NCBI to find the most similar species.

3.13. Investigation of Genetic Mobility of CdR from Isolates by Horizontal Gene Transfer

3.13.2. Replica Plate Mating

A streptomycin resistant (SmR) and Cd sensitive *Pseudomonas aeruginosa* ICMP 6286 (International Collection of Microorganisms from Plants (ICMP), Landcare Research, New Zealand) as a recipient strain was streaked on a Nutrient agar plate and incubated at 37 °C overnight. A single colony of *P. aeruginosa* was used to prepare an overnight broth culture in Brain Heart broth with shaking at 200 rpm and 37 °C to reach the log phase growth [242-244]. The incubation duration was 18 h and following by a 6 h of high temperature incubation at 48 °C. The high temperature heat shock was performed to enhance the chance of horizontally transfer of gene during conjugation with the donor bacterial isolates by inactivating the restriction system of the recipient strain [245-247]. An aliquot of 100 µL of the fresh overnight culture was used to seed a lawn cultured on well-dried BH agar (containing 2% agar) and left to dry [248, 249].

Freshly overnight culture of bacterial isolates as donor strains, were patched using sterile toothpicks onto 2% Nutrient agar plates concurrent with preparation of recipient cells cultures. Plates were incubated at 37 °C for 24 h to reach log phase growth. The donor strains test for ability to transfer CdR were those bacterial isolates carrying *czcA* or *cadA* genes as determined by PCR reactions described in Section 3.13.5. Squares of sterile velvet cloth were used to print the donor cells onto the recipient inoculated on BH agar plates containing 100 µg mL^{-1} of Streptomycin (Sm) (sulphate salt) and 1 mM $CdCl_2$ (prepared as described in Section 3.3). The sensitivity of the donor bacterial strains to the concentration of 100 µg mL^{-1} of Sm was determined prior to cell mating. An Sm sensitive *P. aeruginosa* strain and a bacterial isolate without either *czcA* or *cadA* Cd resistance genes were used as negative controls. Plates

containing mated bacterial cells were incubated at 37 °C for 24 h and the growth of transconjugant patches was scored quantitatively [187].

The presence of the horizontally transferred *czcA* and *czdA* genes in transconjugant strains was investigated with PCR reactions using the specific primers to amplify these resistance genes as described in Section 3.12.5. Negative controls containing no DNA template was included for the PCR reactions. The PCR products were visualised on a 2% agarose gel run at 100 V for 40 minutes in 0.5 x TBE buffer.

3.13.3. Heavy Metal and Antibiotic Resistance Examination of the Recipient Strains

To ascertain the resistance characteristics of transconjugant strains, fresh overnight patches were replica plating onto plates with HMs or Abs. Resistance of strains to some selected concentrations of Cd (1 and 0.1 mM), Zn (5 and 1 mM), Hg (0.1 and 0.1 mM) and a range of Abs (20 and 100 μg mL^{-1} of Tc, Cm, Ery, Cb and Amp) was assessed according to the method from Section 3.4 [193, 248, 250].

3.14. Statistical analysis

Statistical analysis to find differences between different variables was conducted using ANOVA (ANalysis Of VAriance). ANOVA analysis was used to test the differences among bacterial CFUs, MIC and EC50 values of Pollution Induced Community Tolerance (PICT) and Broth Microdilution (BM) analysis, the number of TR-F reads in TRFLP analysis, and number of 16S rDNA gene reads related to the relative abundance of each bacterial phylum in 16S rDNA sequencing. This statistical analysis method compared the means of samples or groups in order to make inferences about, for example, bacterial CFUs means. Dependent and independent variables and co-variates were introduced to the ANOVA analysis. Depending on the number of independent variables in each analysis, two-way or more way analysis of variances was conducted. The dependent and independent variables and co-variates introduced to ANOVA analyses are listed below for each experiment:

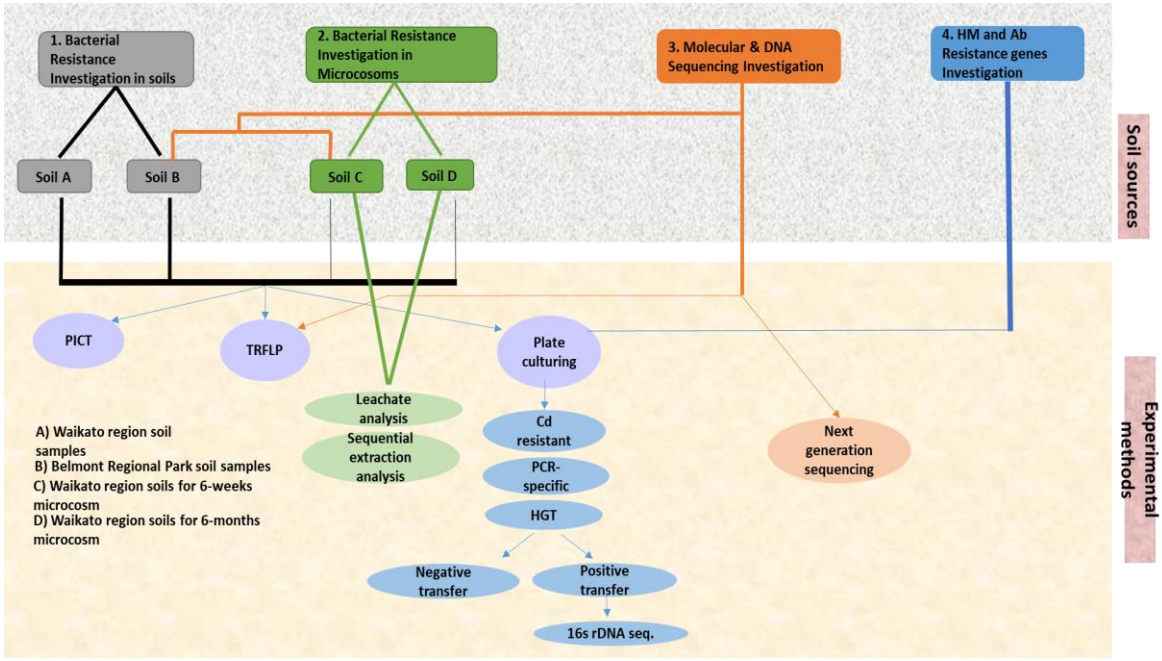
- Three-way ANOVA analysis was conducted for total bacterial count of Waikato Region soil samples. Soils sets, soils history of usage, and soils' initial HM concentration were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Monthly mean temperature and rainfall during the sampling months, soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates.
- Four-way ANOVA analysis was conducted for resistant bacterial counts of Waikato Region's soil samples on plates with HM and Ab additives. Soil sets, soils history of usage, initial HM concentrations of soils, and HM or Ab concentrations in media were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Monthly mean temperature and rainfall during the sampling months, soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates.
- Two-way ANOVA analysis was carried out for total bacterial count of Belmont Regional Park sub-sites soil samples. Soil samples with their distance from the storage fertiliser and initial HM concentrations in soil samples were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates.
- Three-way ANOVA analysis was conducted for count of resistant bacteria from Belmont Regional Park sub-sites' soil samples on plates with HM and Ab additives. Soil samples with their distance from the storage fertiliser, initial HM concentrations in soil samples and HM or Ab concentrations in plates were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates.

- Three-way ANOVA analysis was conducted for Pollution Induced Community Tolerance (PICT) test of bacteria extracted from Waikato Region and Belmont Regional Park soil samples. Soil samples, history of soils usage (for samples from Waikato Region) or sub-sets (for Belmont Park soil samples), concentrations of HM and Abs in microtitre plates were introduced to the analysis as independent variables. The dependent variable was MIC or EC50 value.
- Three-way ANOVA analysis was conducted for Broth Microdilution (BM) test of bacteria selected from Waikato Region and Belmont Regional Park soil samples. Soil samples, history of soils usage (for samples from Waikato Region) and distance of sub-sets from the fertiliser storage (for Belmont Park soil samples), concentrations of HM and Abs in microtitre plates were introduced to the analysis as independent variables. The dependent variable was MIC or EC50 value.
- Three-way ANOVA analysis was conducted for quantification of bacterial resistance in microcosm trials. Soil sampling from microcosms at appropriate intervals (2 weeks or 2 months), concentration of HM additives to the microcosms and HM or Ab concentrations in media were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils samples at time 0 and every 2-week or 2-month interval, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates.
- Three-way ANOVA analysis was conducted for Pollution Induced Community Tolerance (PICT) test of bacteria extracted from soil samples of 6-week and 6-month incubated microcosms. Soil sampling from microcosms at appropriate intervals (2 weeks or 2 months), concentration of HM additives to the microcosms and HM or Ab concentrations in microtitre plates were introduced to the analysis as independent variables. The dependent variable was MIC or EC50 value.

- Three-way ANOVA analysis was conducted for Broth Microdilution (BM) test of bacteria selected from soil samples of 6-week and 6-month incubated microcosms. Soil sampling from microcosms at appropriate intervals (2 weeks or 2 months), concentration of HM additives to the microcosms and HM or Ab concentrations in microtitre plates were introduced to the analysis as independent variables. The dependent variable was MIC or EC50 value.
- Three-way ANOVA was conducted for Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of Waikato Region and Belmont Park samples. Soil samples, history of soils usage (for samples from Waikato Region) and distance of sub-sets from the fertiliser storage (for Belmont Park soil samples), and soils HM concentrations were introduced to the analysis as independent variables. The dependent variable was the number of T-RFs reads. Three-way ANOVA was conducted for Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of soil samples from microcosms. Soil samplings at appropriate intervals (2 weeks or 2 months), initial soils HM concentrations and concentration of HM additives to the microcosms were introduced to the analysis as independent variables. The dependent variable was the number of T-RFs reads.
- Two-way ANOVA was conducted for the 16S rDNA analysis of Belmont Park soil samples. Sub-sets soil samples with their distance from the fertiliser storage and soils HM concentrations were introduced to the analysis as independent variables. The dependent variable was the number of 16S rDNA gene reads related to the relative abundance of each bacterial phylum. Two-way ANOVA was conducted for the 16S rDNA analysis of 6-week incubated microcosms at 6-week interval. Soil sample's initial HM concentrations and HM additive concentrations to the microcosms were introduced to the analysis as independent variables. The dependent variable was the number of 16S rDNA gene reads related to the relative abundance of each bacterial phylum.

Results

The results of the experiments investigating the hypotheses and aims of this project are presented in Chapters 4-7. The following flow chart summarises the different studies and experiments of the current project.



Chapter 4, Results Part 1

4.1. Study 1: Investigation of Bacterial Resistance in Waikato Region and Belmont Regional Park Soils

4.1.1. Introduction

New Zealand agricultural soils are subject to a range of contaminant inputs, of which inorganic contaminants in phosphate fertilisers and animal remedies are of special interest due to their capacity to accumulate over time [251, 252]. Accumulation of HMs in soil can introduce bacterial resistance to these HMs, as well as increasing the risk of occurrence of Ab resistance via cross or co-resistance [253-255].

As examples, Cd concentrations in soils of Waikato dairy farms are on average 5 times higher than their natural background levels after 7 decades of accumulation from phosphate fertilisers [5, 256]. The national average concentration of Cd in soil in New Zealand is about 0.35 mg kg^{-1}. This average concentration of Cd consists of 0.43 mg kg^{-1} for pastoral soils, 0.24 mg kg^{-1} for arable soils, and 0.16 mg kg^{-1} for native bush soils [257]. Average Zn concentrations have doubled during the last 2-3 decades through the widespread use of zinc as a preventative for facial eczema [30, 258]. Mean figures also hide the fact that some farms and areas have accumulated more Cd or Zn than others. For example, although the average concentration of Zn in Waikato soils is now 60 mg/kg, 11% of farms show levels exceeding 100 mg kg^{-1}, which has been suggested as a guideline for protection of soil microbial processes [259, 260]. These high levels of Cd and Zn in Waikato soils (Figure 4.1) can lead to resistance to these HMs and subsequent co-selection for Ab resistance in these soils' bacteria.

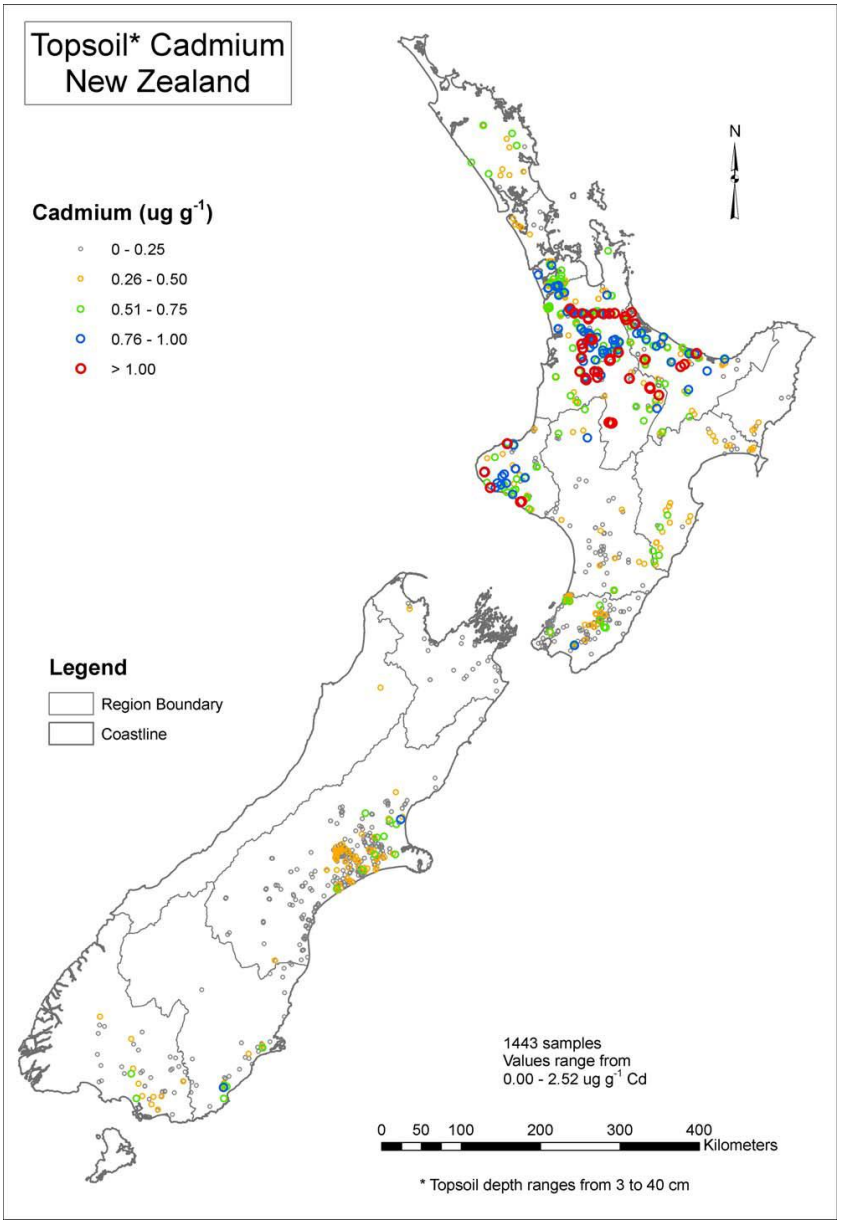


Fig. 4. 1. Regional map of topsoil cadmium levels (M. Taylor et. al., 2007).

The aim of this Chapter was to:

1. To establish, in selected soils, whether observable microbial resistance to Cd and/or Zn shows an association with agricultural land use history, and (if so) in which microbial classes (morphological).
2. If such resistance has led to increased resistance to antibiotics.

The approach was to examine resistance to a range of Cd, Zn and Hg concentrations, and to representative Abs, including Tc, Cm, Ery, Cb and Amp. This involved exploring if there are any

differences between the levels of soil bacteria resistant to the HMs and Abs, which were isolated from soils with higher levels of HMs compared to those from soils with lower amount of HMs.

The methodology used for this chapter is described in Sections 3.1-3.5 and 3.9-3.12, Methodology Chapter. In short, plate culturing, BM and PICT tests were applied to investigate the effect of soil HM concentrations on soil microbial resistance in soil samples from selected regions in New Zealand. Figure 4.2 illustrates different steps of methods used to investigate the aims of Chapter 4.

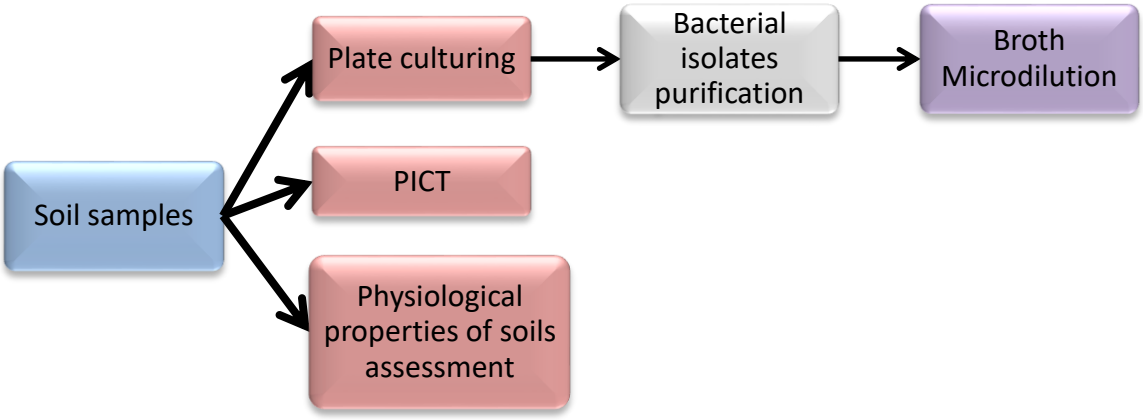


Fig. 4. 2. Flow chart illustrating different steps of methods used for investigations of Chapter 4.

4.1.2. Physicochemical Properties of the Soil Sampling Sites.

The three sets of soil samples collected from the Waikato Region (WR) and used for this project were categorised as Clay Loam soils with granular structure (M. Taylor, Personal Communication). Chemical analysis of representative samples revealed the concentration of the three HMs of interest. Cd concentration was higher in pastoral soils (sites EW-69 and EW-135) compared to arable (sites EW-85 and EW-86) and native bush (control) soil (site EW-73). Other physicochemical features of WR soil sampling sites from which these three soil sample sets were collected are listed in Table 4.1 and Table A2.1 of Appendix 1.

Table 4. 1. WR soil sampling sites physicochemical information.

Site No.	EW-73 (Native bush)	EW-85 (Arable)	EW-86 (Arable)	EW-69 (Pasture)	EW-135 (Pasture)
Annual rainfall (mm)	1400	1400	1400	1400	1400
pH	5.60	6.07	5.74	5.01	5.76
Total C (%)	8.00	3.79	3.40	10.10	8.63
Total N (%)	0.48	0.30	0.30	0.94	0.84
C:N	16.7	12.5	11.4	10.8	10.3
Olsen P *	6.00	110	89.0	54.0	73.0
Cd *	0.09	0.54	0.49	0.82	1.11
Hg *	0.19	0.31	0.42	0.20	0.21
Zn *	27.00	39.00	40.00	65.00	62.00
Fe *	28000	42000	53000	59000	39000
P *	290	1850	1540	2300	2500

*mg kg^{-1} of dry soil

At Belmont Regional Park (BRP) 8 sites (labelled B10-B17) were sampled at 10 m intervals along a transect running the length of a farm airstrip. These were categorised as Clay Loam soils with granular structure [261]. The Cd concentration showed a sharp increase from down-hill sub-site, B10 (assumed to be closest in soil nutrient/mineral composition to the BRP farm paddocks "background" state), to the top-hill sub-site, B17 which was closest to the fertiliser storage bunker (Figure 3.4). HM concentrations and other physicochemical properties of these sub-sites soil samples are detailed in Table 4.2 and Table A2.2 of Appendix 1**.**

Table 4. 2. BRP sub-sites soil samples physicochemical information.

Site No.	B10	B11	B12	B13	B14	B15	B16	B17
pH	5.90	6.80	5.90	6.70	6.70	6.40	5.50	6.10
Olsen P *	90.0	96.0	135	180	217	228	265	569
Total C (%)	6.00	5.20	6.40	5.70	6.00	5.90	7.00	8.60
Cd *	1.14	1.15	1.83	2.70	3.40	3.80	4.10	7.20
Hg *	0.109	0.076	0.091	0.079	0.103	0.081	0.129	0.125
Zn *	49.0	48.0	60.0	59.0	68.0	73.0	77.0	95.0
Fe *	19700	13400	21000	18000	19200	18300	20000	17100
P *	3300	2100	5000	6300	7700	8500	12300	22000

*mg kg^{-1} of dry soil

4.1.3. Total Bacterial Counts of Waikato Region's Soil Samples

Following isolation of the soil bacteria (see Table 3.2), bacterial colonies were counted and CFU were calculated for each soil sample set. Three-Way ANOVA analysis of the bacterial counts from each soil and comparison of mean values using BONFERRONI correction for multiple

comparisons was carried out. Soils sets, soils history of usage, and soils' initial HM concentration were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Monthly mean temperature and rainfall during the sampling months, soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. ANOVA showed the number of viable bacterial cells in pastoral and arable cropping soils was higher compared to the soil from the control (native bush) site ($P < 0.05$) for each WR region soil sample sets (WRSS1, the first sampling date in February 2014; WRSS2, the second sampling date of August 2014; WRSS3, the third sampling date of June 2015) (Figures 4.3 and A2.1, Appendix 2). There were higher numbers of bacteria in pasture soils compared to arable soils for each of these soil sample sets. There were no significant differences in total numbers of bacteria in pasture, arable and background soils between sampling dates, although the purpose of using soil samples from the different dates was to examine if there is any different among the number of bacteria in soil in different seasons. WR's monthly mean temperature and rainfall during the sampling months, including February, June and August, was introduced to the ANOVA as covariates, although including these values in the analysis made no differences to the number of significant p-values.

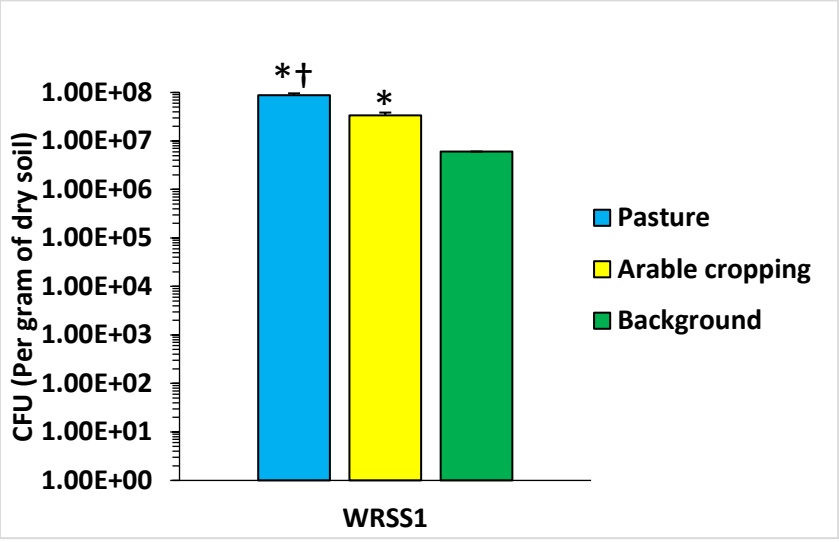


Fig. 4. 3. Total number of CFU (per gram of dry soil) from WRSS1 pastoral, arable and native bush soil samples, and selected on R2A agar. *$p < 0.05$ compared to background soil bacteria total CFU; †$p < 0.05$ compared to arable soil bacteria total CFU.

4.1.3.1. Resistant Bacterial Counts of Waikato Region's Soil Samples on Plates with HM additives

Plate culturing of WRSS1, WRSS2 and WRSS3 on R2A agar containing a range of concentrations of Cd, Zn and Hg was performed. Four-way ANOVA analysis was conducted and soil sets, soils history of usage, initial HM concentrations of soils, and HM concentrations in media were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Monthly mean temperature and rainfall during the sampling months, soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. The ANOVA analysis for these bacterial CFU ratios for WRSS1 (Figures 4.4-4.6) showed there were higher HMR CFUs/total bacterial CFU ratios for pastoral soil bacteria compared to those from background soil ($p < 0.05$). Similar results were determined for those bacteria from WRSS2 and WRSS3 (Figures A2.2-A2.4, Appendix 2).

4.1.3.1.1. Plates with Cadmium Additive

Four-way ANOVA analysis was conducted and soil sets, soils history of usage, initial HM concentrations of soils, and Ab concentrations in media were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Monthly mean temperature and rainfall during the sampling months, soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. Significant ($p<0.05$) differences were observed between CdR/total bacterial CFU ratios of WRSS1 pastoral and arable soils, pastoral and background soils, and also arable and background soils on plates with concentration of Cd 0.001 mM (Figure 4.4). In all cases the former were higher than the latter. Ratios of CdR bacteria increased with decreasing Cd concentration, and differences between soils were statistically significant at all Cd concentrations below 0.1 mM. In terms of land use types, the highest ratios (most relative resistance) were found for pastoral soils, followed by arable soils, with background soils showing the lowest proportion of CdR to total

bacteria. This outcome is consistent with the fertilisation history and Cd content of the three soil types, which also follow the order pastoral > arable > background. However, when plated onto increasing concentration of Cd in the agar plates, on the plates with higher Cd, the number of bacterial isolates resistant to these concentrations of HMs was less compared to when the levels of these HMs are lower; thereby, the ratio of HMR/total bacterial CFUs on higher concentrations of HMs is less than the ratio on lower HMs concentrations. Both this pattern, and the pattern of increasing ratios with decreasing HM concentrations, were also seen for Zn and Hg (see below). Similar results were determined for WRSS2 and WRSS3 (Figures A2.2, Appendix 2). There were no significant differences between the ratios of CdR/total bacterial CFU between WRSS1, WRSS2 and WRSS3 collected on different dates. Overall, the land-use types with the higher Cd level present in the soil have higher ratios, so, have more resistant bacteria as a percentage of the total culturable bacteria present compared to arable and background on each sampling date. However, when the concentration of Cd in plates is increased, the ratios of CdR/total decreases with increasing Cd concentrations in the plates.

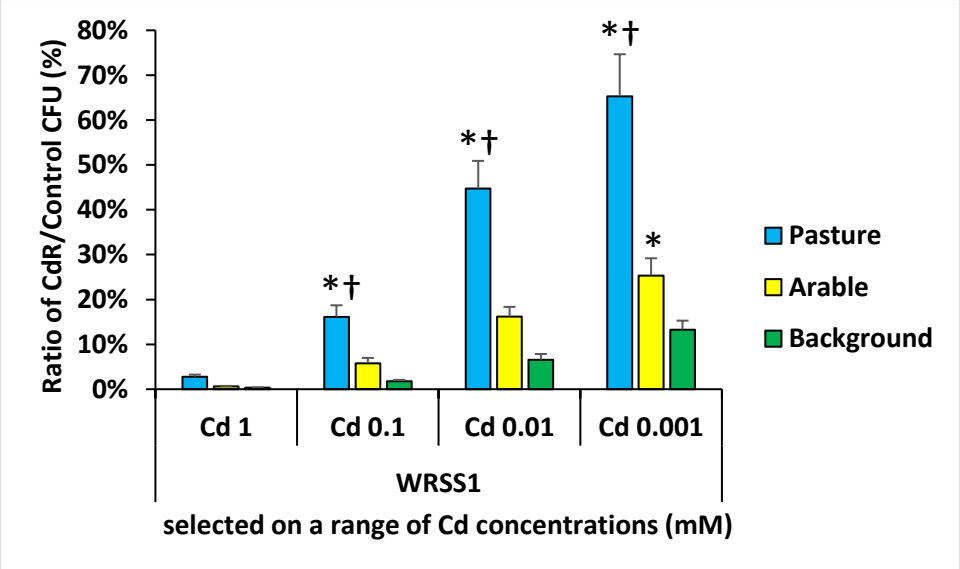


Fig. 4. 4. Ratios of CdR/total bacterial CFU (per gram of dry soil), selected on a range of Cd concentrations, for the three soil samples collected in the WR. (*$p < 0.05$ compared to background soil CdR CFU/total bacterial CFU ratio selected on the same Cd concentration; †$p < 0.05$ compared to arable CdR/total bacterial CFU ratio selected on the same Cd concentration).

4.1.3.1.2. Plates with Zinc Additive

In this case, investigations of resistance levels were carried out at five Zn concentrations to examine a wider range of Zn concentrations. The result of four-Way ANOVA analysis for ZnR/total CFU ratios of WRSS1, WRSS2 and WRSS3 bacteria showed the same overall patterns as for Cd. Ratios in pastoral and arable soils were significantly ($p < 0.05$) higher than those in background soils; and ratios in pastoral soils were significantly higher than those of arable soils. There was also one significant difference between Zn 1 mM-resistant/total bacterial CFU ratios of WRSS1 and WRSS2 pastoral soils (Figure 4.5). This result could be viewed as an outlier.

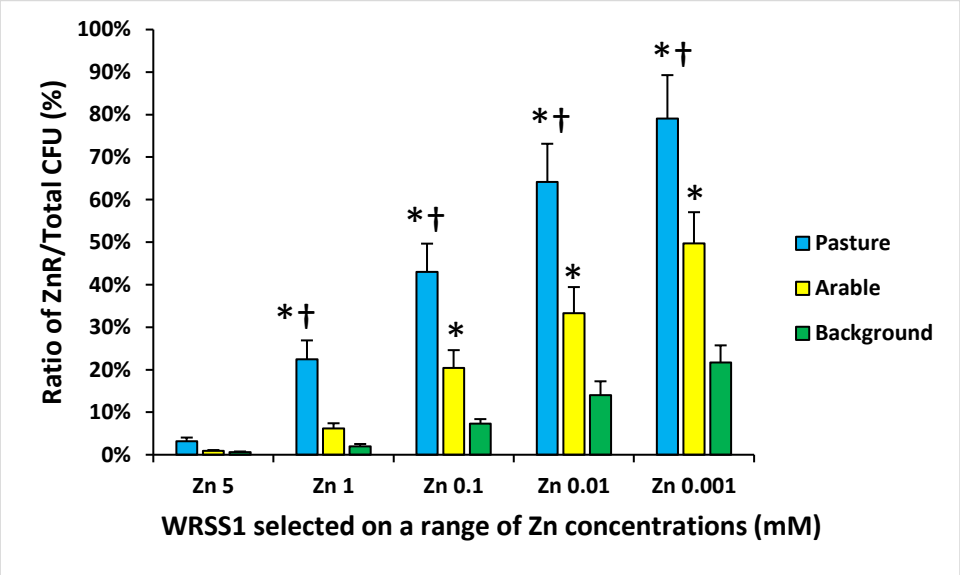


Fig. 4. 5. Mean ratios of ZnR/total bacterial CFUs, selected on a range of Zn concentrations, for WRSS1. *$p < 0.05$ compared to background ZnR/total bacterial CFU ratio selected on the same HM concentration; †$p < 0.05$ compared to arable ZnR/total bacteria CFU ratio selected on the same HM concentration; $^{\Psi}p < 0.05$ compared to the second pastoral soil HgR/total bacterial CFU ratio selected on the same HM concentration.

As for Cd, and for Zn the highest ratios are seen at the lowest HM concentrations on the agar plates; and the pattern of most to least resistance follows the order pastoral > arable > background soils. As with Cd, the latter pattern is consistent with Zn concentrations in the three soil types, with pastoral soils having the highest levels (with most anthropogenic Zn presumably from facial eczema treatments), arable next (with the anthropogenic component from Zn-containing fungicides), and background soils the lowest.

4.1.3.1.3. Plates with Mercury Additive

Tests relating to Hg were carried out at three concentrations. Four-Way ANOVA analysis showed that at one or more of these, there were significantly ($p < 0.05$ or lower) higher ratios of HgR/total bacterial CFUs from pastoral soils compared to background soils. Furthermore, there were significant differences between pastoral and arable soils, and arable and background soils (Figure 4.6). The highest relative resistance was again at the lowest exposure concentrations. Results for Hg are therefore consistent with those of Cd and Zn.

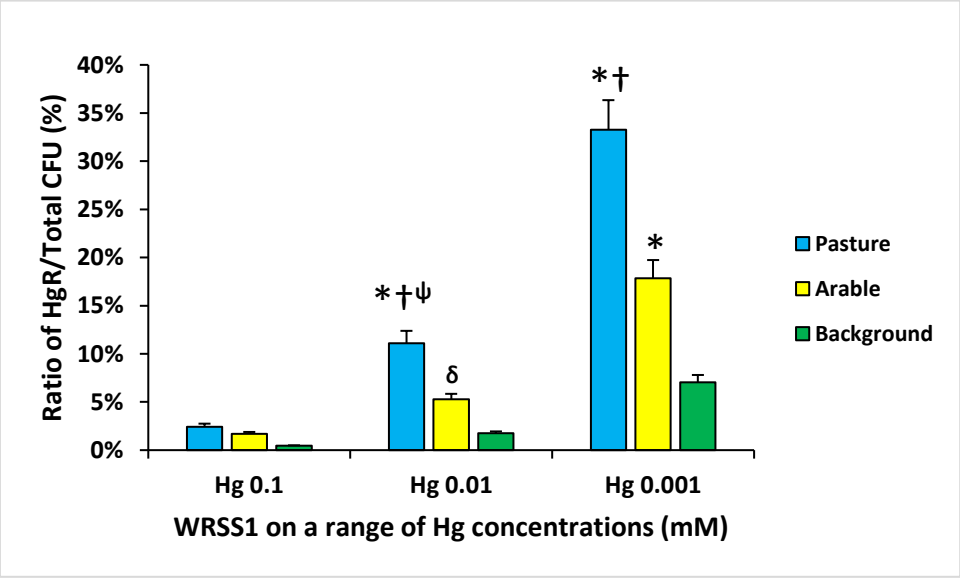


Fig. 4. 6. Mean ratios of HgR/total bacterial CFUs, selected on a range of Hg concentrations, for WRSS1. *$p < 0.05$ compared to background soil HgR/total bacterial CFU ratio selected on the same HM concentration; †$p < 0.05$ compared to arable soil HgR/total bacterial CFU ratio selected on the same HM concentration; $^{\delta}p < 0.05$ compared to the second arable soil HgR/total bacterial CFU ratio selected on the same concentration of HMs; $^{\psi}p < 0.05$ compared to the second pastoral soil HgR/total bacterial CFU ratio selected on the same HM concentration.

4.1.3.2. Resistant Bacterial Counts of Waikato Region's Soil Samples on Plates with Ab Additives

Figure 4.7 shows the ratios of AbR/total bacterial CFU of the WRSS1 pasture, arable and background soils when exposed to different Abs (Tc, Cm, Ery, Cb and Amp). Similar to the HMs, the pastoral and arable soils had significantly higher AbR/total bacterial CFUs compared to background, and the pastoral soil had higher AbR/total bacterial CFUs compared to arable soil.

In addition, similar to the metal plates, the lower concentrations of Abs have significantly higher ratios of AbR/total bacterial CFUs compared to the higher concentrations. Similar results were determined for WRSS2 and WRSS3 (Figure A2.5).

Similar to the HMs results, four-Way ANOVA showed there were significantly higher TcR/total bacterial CFU ratios of WRSS1 pastoral soils compared to those from arable soils ($p < 0.05$) (20 μg mL^{-1}). In addition, results showed higher Tc 20 and 100 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1 farmed (pastoral and arable) soils compared to those from background soil ($p < 0.05$). The similar results were determined for WRSS2 and WRSS3 (Figure A2.5). In addition, in one case, significantly greater Tc 100 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1 pastoral soil compared to those from WRSS2 pastoral soil were determined ($p < 0.05$).

The ratios of CmR/total bacterial CFUs are shown in Figure 4.6. As for Tc, there were higher Cm 20 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1 pastoral soil compared to those from the arable soils ($p < 0.05$). In addition, the result showed greater Cm 20 and 100 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1 pastoral and arable soils compared to those from background soil. Similar results were determined for WRSS2 and WRSS3 (Figure A2.5).

Likewise, analysis of the proportions of Ery, Cb and Amp were performed, and the results are presented in Figures 4.6. There were significantly higher Ery 20 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1, WRSS2 and WRSS3 pastoral soils compared to those from arable soils (the same overall patterns as for Tc and Cm on all three sampling occasions. Differences between resistant/total bacterial CFU ratios consistently reached $p < 0.05$ or less statistical significance. Differences in AbR/total CFU ratios between farmed and background soils were significant at the concentration levels (20 and 100 μg mL^{-1}). The results revealed significant differences between CbR/total bacterial CFU ratios of WRSS1, WRSS2 and WRSS3. The result showed there were higher Cb 20 μg mL^{-1}-resistant/total bacterial CFU ratios from WRSS1, WRSS2 and WRSS3 pastoral soils compared to those arable soils ($p < 0.05$). Moreover, there were significantly

greater Cb 20 and 100 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1, WRSS2 and WRSS3 pastoral and arable soils compared to those from Background soil. Significantly higher Amp 20 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1, WRSS2 and WRSS3 pastoral soils compared to those from arable soils were determined ($p < 0.05$). Higher Amp 20 and 100 μg mL^{-1}-resistant/total bacterial CFU ratios of WRSS1, WRSS2 and WRSS3 pastoral and arable soils compared to those from Background soil were revealed (Figure 4.7).

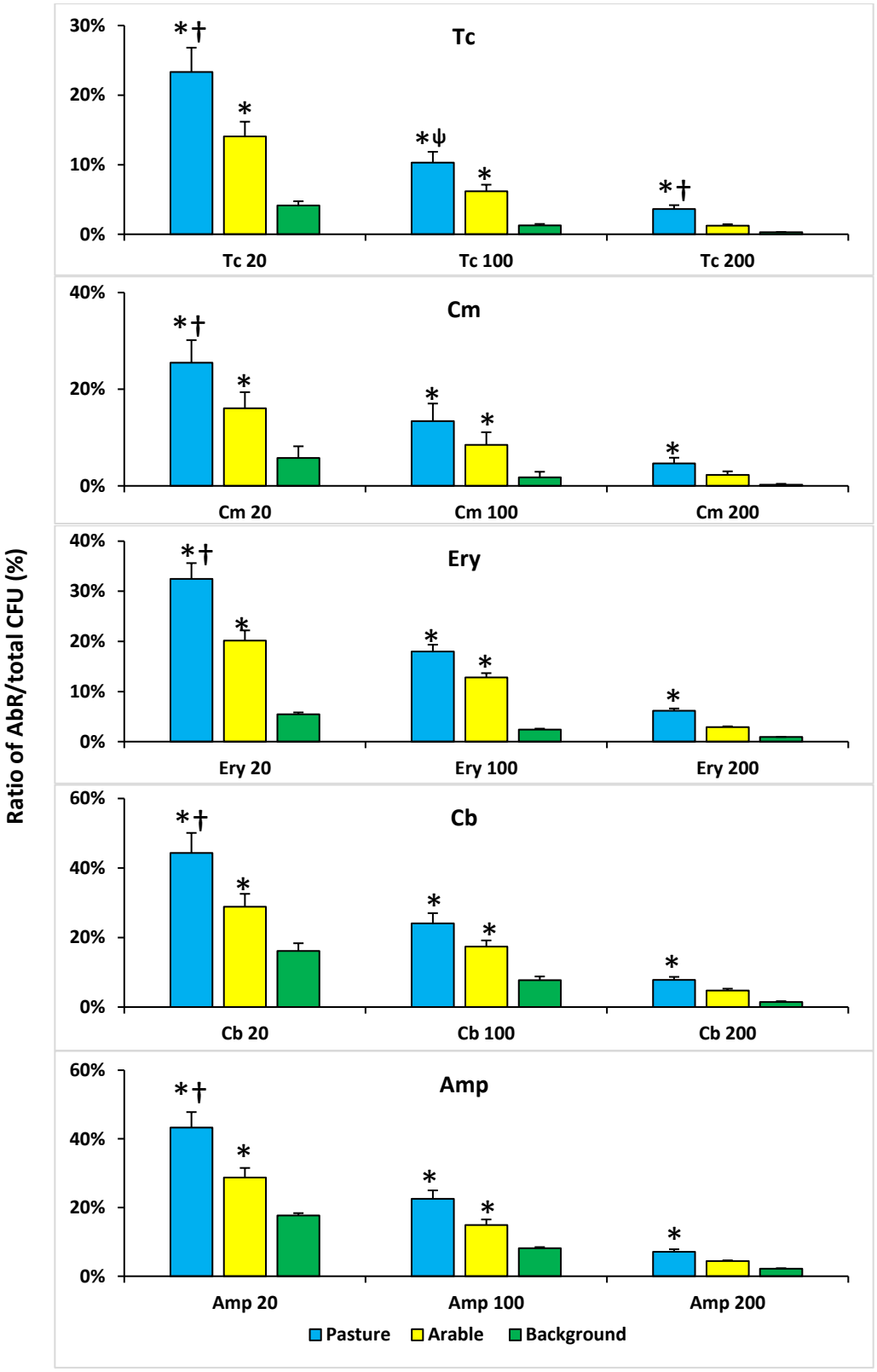


Fig. 4. 7. Mean ratios of AbR/total bacterial CFUs, selected on a range of Abs concentrations, for the WRSS1. The Ab concentrations are per µg mL^{-1}. *$p < 0.05$ compared to background soil AbR/total bacterial CFU ratio selected on the same Ab concentration; †$p < 0.05$ compared to arable soil AbR/total bacterial CFU ratio selected on the same Ab concentration; $^{\Psi}p < 0.05$ compared to the second pastoral soil AbR/total bacterial CFU ratio selected on the same concentration of Ab.

4.1.4. Total Bacterial Count for Belmont Regional Park Sub-sites' Soil Samples

Bacterial plate culturing on basal R2A Agar was carried out for soil samples from the Belmont Regional Park (BRP) sub-sites as detailed in Section 3.4. Following the colony counting, the number of bacterial CFUs was calculated for each soil sample using Equation 3.2. The exact distance of the sub-sites from the start of the airstrip is illustrated in Figure 3.3 of the Methodology chapter.

Two-way ANOVA was used to compare the differences between the total bacterial CFUs from basal R2A agar plates. Soil samples with their distance from the storage fertiliser and initial HM concentrations in soil samples were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. There were significant differences between total bacterial CFUs of B10 sub-site (70 m from the start of the airstrip) compared to those from B16-B17 sub-sites. In addition, significant differences between total CFUs of B11 sub-site compared to those from B17 sub-site were observed ($p < 0.05$) (Figure 4.8).

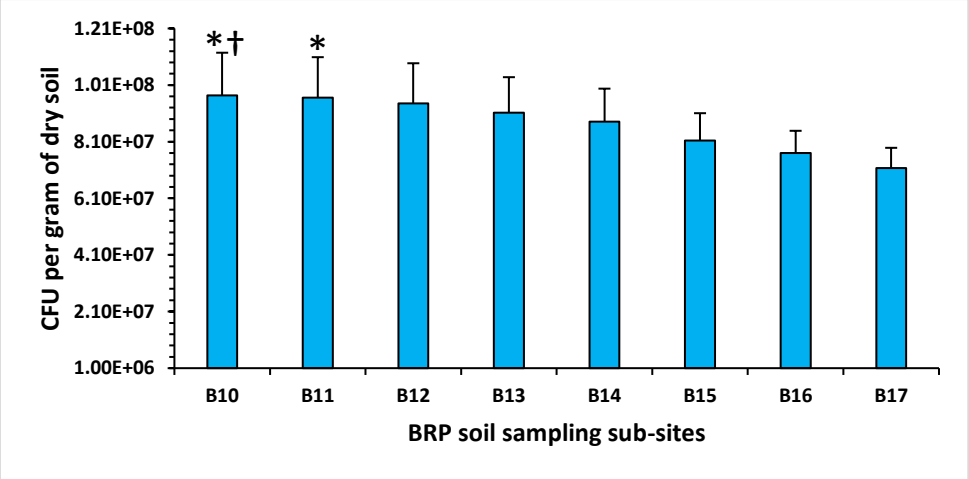


Fig. 4. 8. Total Plate Counts of bacteria (per gram of dry soil) from Belmont Regional Park sub-sites soil samples (*$p < 0.05$ compared to B17 soil bacteria total CFU; †$p < 0.05$ compared to B16 soil bacteria total CFU).

4.1.4.1. Counts of Resistant Bacteria from Belmont Regional Park Sub-sites' Soil Samples on Plates with HM additives

Plate culturing of BRP on R2A agar containing a range of concentrations of Cd, Zn and Hg was performed. Three-Way ANOVA analysis was carried out and soil samples with their distance from the storage fertiliser, initial HM concentrations in soil samples and HM concentrations in plates were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. ANOVA analysis for these bacterial CFU ratios (Figures 4.8-4.10) showed very similar trends to the BRP soils, in that soils with higher HMs concentrations (B17 and B16 sub-sites) had significantly higher ratios of HMR/total CFU compared to the sub-sites with lower HM concentrations (B11 and B10, i.e. further away from the start of the airstrip). In addition, there were significantly higher ratios of HMR/total bacterial CFU when the soils were plated onto agar plates containing lower concentrations of HMs ($p < 0.05$) (Figures 4.9-4.11).

4.1.4.1.1. Plates with Cadmium Additive

Three-Way ANOVA analysis was carried out and soil samples with their distance from the storage fertiliser, initial HM concentrations in soil samples and Ab concentrations in plates were introduced to the analysis as independent variables. The dependent variable was bacterial CFU. Soils moisture content, pH of soils, total C and N content of soils, and Olsen P values were introduced to the analysis as co-variates. Similar to the results from the WR soils, the soils with higher concentrations of HM had higher ratios of CdR/total bacterial CFUs compared to the soils with the lower concentrations of HMs. Furthermore, the result revealed that there were higher Cd 0.1mM-resistant/total bacterial CFU ratios of B17-B16 sub-sites compared to those from B11-B10 sub-sites. Lower Cd 0.1 mM-resistant/total bacterial CFU ratios of B17 sub-site compared to those from B12 sub-site were determined. Lower Cd 0.01 mM-resistant/total bacterial CFU

ratios of B10-B14 sub-sites compared to those from B16-B17 sub-sites were ascertained. Cd 0.001 mM-resistant/total bacterial CFU ratios were significantly lower for the B10-B14 sub-sites compared to those from B17 sub-site. In addition, there were lower Cd 0.001 mM-resistant/total bacterial CFU ratios of B10-B13 sub-sites compared to those from B16 sub-site. Lower Cd 0.001 mM-resistant/total bacterial CFU ratios of B10-B12 sub-sites compared to those from B15 sub-site were also found ($p < 0.05$) (Figure 4.9).

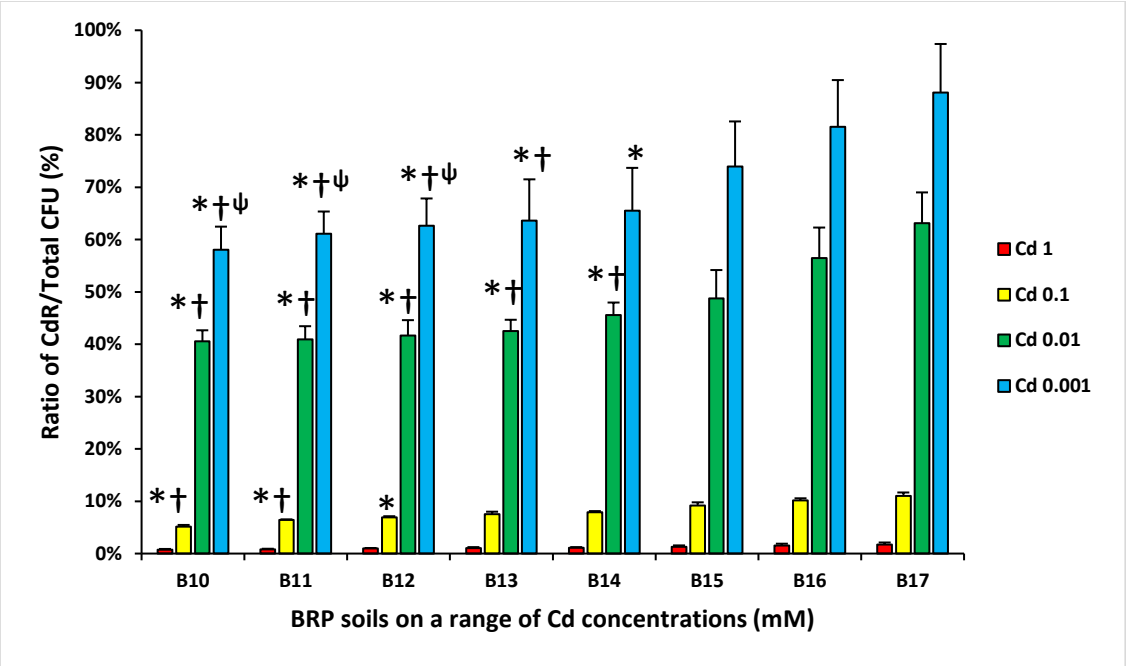


Fig. 4. 9. Mean ratios of CdR/total bacterial CFUs, selected on a range of Cd concentrations, for BRP sub-sites soil samples. *$p < 0.05$ compared to B17 soil bacteria CFU ratio selected on the same Cd concentration; †$p < 0.05$ compared to B16 soil bacteria CFU ratio selected on the same Cd concentration; $^{\psi}p < 0.05$ compared to B15 soil bacteria CFU ratio selected on the same Cd concentration.

4.1.4.1.2. Plates with Zinc Additive

The result revealed lower Zn 1mM-resistant/total bacterial CFUs ratios of B10-B11 sub-sites compared to those from B17 sub-site, from plates with Zn. Lower ZnR/total soil bacterial CFUs ratios of B10 sub-site compared to those from B16 sub-site were determined. Lower Zn 0.1mM-resistant/total bacterial CFU ratios of B10-B14 sub-sites compared to those from B17 sub-site were determined. Also, lower Zn 0.1mM-resistant/total bacterial CFU ratios of these sub-sites,

except for B14, compared to those from B16 sub-site were revealed. The result showed lower Zn 0.1mM-resistant/total bacterial CFU ratios of B10-B11 sub-sites compared to those from B15 sub-site. Additionally, there were lower Zn 0.01mM-resistant/total bacterial CFU ratios of B10-B14 sub-sites compared to those from B16-B17 sub-sites. The result showed lower Zn 0.01mM-resistant/total bacterial CFU ratios of B10-B12 sub-sites compared to those from B15 sub-site. Statistical analysis revealed that there were lower Zn 0.001mM-resistant/total bacterial CFU ratios of B10-B15 sub-sites compared to those from B17 sub-site. Lower Zn 0.001mM-resistant/total bacterial CFU ratios of B10-B13 compared to those from B16 sub-site were also determined. Lower Zn 0.001mM-resistant/total bacterial CFU ratios of B10-B12 sub-sites compared to those from B15 sub-site were revealed ($p < 0.05$) (Figure 4.10).

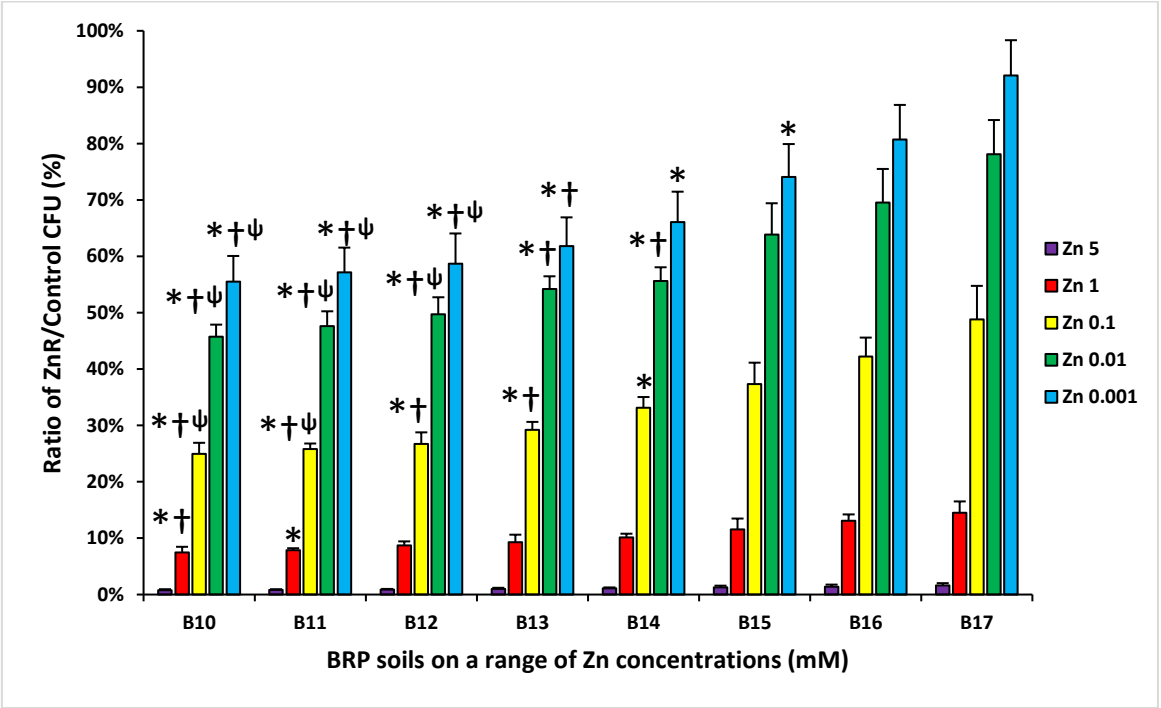


Fig. 4. 10. Mean ratios of ZnR/total bacterial CFUs, selected on a range of Zn concentrations, for BRP sub-sites soil samples. *$p < 0.05$ compared to B17 soil bacteria CFU ratio selected on the same Zn concentration; †$p < 0.05$ compared to B16 soil bacteria CFU ratio selected on the same Zn concentration; $^{\psi}p < 0.05$ compared to B15 soil bacteria CFU ratio selected on the same Zn concentration.

4.1.4.1.3. Plates with Mercury Additive

Three-way ANOVA analysis showed there were lower Hg 0.001mM-resistant/total bacterial CFU ratios of B10-B14 sub-sites in comparison with those from B17 sub-site. In addition, there were lower Hg 0.001mM-resistant/total bacterial CFU ratios of B10-B13 sub-sites compared to those from B16 sub-site. Moreover, there were significant differences between Hg 0.001mM-resistant/total bacterial CFU ratios of B10-B12 sub-sites compared to those from B15 sub-site. Significant differences between Hg 0.001mM-resistant/total bacterial CFU ratios from B10-B11 in comparison with those from B14 sub-site, were determined ($p < 0.05$) (Figure 4.11).

There were lower Hg 0.01mM-resistant/total bacterial CFU ratios of B10-B12 sub-sites compared to those from B17 sub-site, as well as significant differences between those from B10-B11 compared to those from B16 sub-site (Figure 4.11).

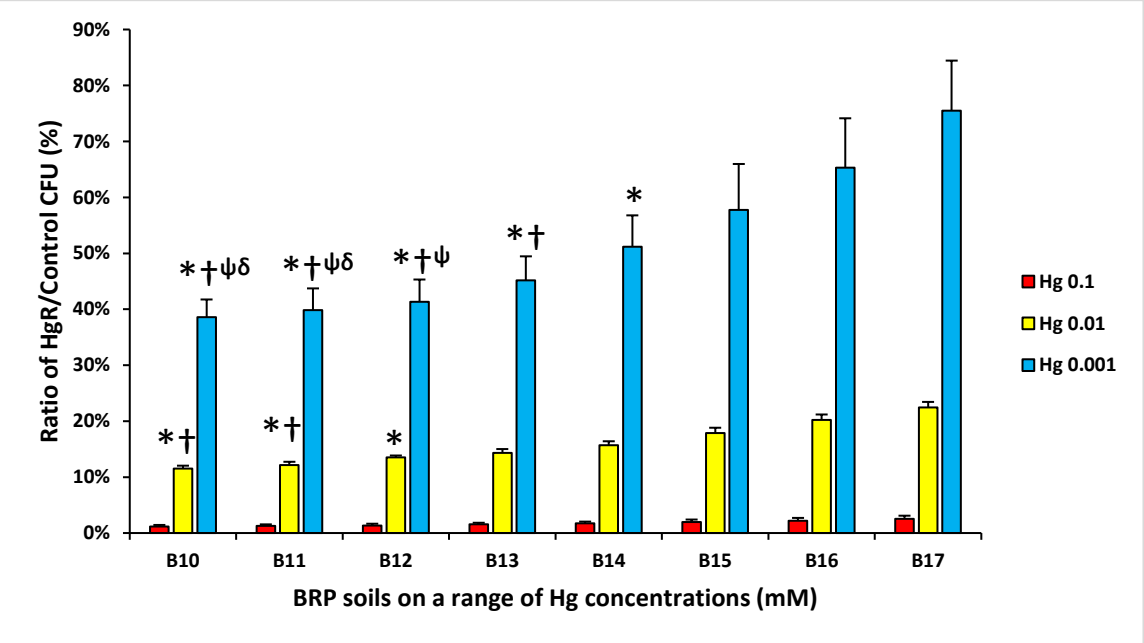


Fig. 4. 11. Mean ratios of HgR/total bacterial CFUs, selected on a range of Hg concentrations, for BRP sub-sites soil samples. *$p < 0.05$ compared to B17 soil bacteria CFU ratio selected on the same Hg concentration; †$p < 0.05$ compared to B16 soil bacteria CFU ratio selected on the same Hg concentration; $^{\psi}p < 0.05$ compared to B15 soil bacteria CFU ratio selected on the same Hg concentration; $^{\delta}p < 0.05$ compared to B14 soil bacteria CFU ratio selected on the same Hg concentration.

4.1.4.2. Resistant Bacterial Counts of Belmont Regional Park Sub-sites' Soil Samples on Plates with Ab additives

Examination of bacterial resistance on a range of Abs (Tc, Cm, Ery, Cb and Amp) was performed for BRP sub-sites soil samples and the results were analysed by three-Way ANOVA (Figure 4.12). Results showed that the soils with higher levels of HMs (B17 and B16) had significantly higher AbR/total bacterial CFUs compared to the soils with lower concentrations of HMs (B11 and B10).

In addition, similar to the HM-contained plates, the lower concentrations of Abs have significantly higher ratios of AbR/total bacterial CFUs compared to the higher concentrations (Figure 4.12).

Statistical analysis showed there were lower ratios of Tc 20 or 100 µg mL^{-1}-resistant/total bacterial CFU ratios from B10-B13 sub-sites compared to those from B16-B17 sub-sites. Moreover, bacterial CFUs from B10-B11 sub-sites soils, which were resistant to Tc 20 or 100 µg mL^{-1}, differed from those isolated from B15 sub-site ($p < 0.05$) (Figure 4.12).

Additionally, there were lower ratios of Tc 200 µg mL^{-1}-resistant/total bacterial CFU from B10-B12 sub-sites compared to those from B17 sub-site. There were also lower Tc 200 µg mL^{-1}-resistant/total bacterial CFU ratios from B10-B11 sub-sites compared to those from the B16 sub-site ($p < 0.05$).

The result of plate culturing for BRP soil samples on a range of Cm showed there were lower CmR/total bacterial CFU ratios from B10-B12 compared to those from B17 sub-site. The plate counts also revealed lower CmR/total bacterial CFU ratios from B10-B11 sub-sites compared to those from the B16 sub-site ($p < 0.05$) (Figure 4.12).

There were significantly lower Ery 20 and 100 µg mL^{-1}-resistant/total soil bacterial CFU ratios of B10-B13 sub-sites compared to those from B16-B17 sub-sites. In addition, the result revealed lower Ery 20 µg mL^{-1}-resistant/total bacterial CFU ratios of B14 sub-site compared to those from

B17 sub-site. Significantly lower Ery 20 µg mL^{-1}-resistent/total soil bacterial CFUs ratios of B10-B12 sub-sites compared to those from B15 sub-site were determined (Figure 4.12).

Statistical analysis showed there were significantly lower Cb 20 µg mL^{-1}-resistant/total bacterial CFUs ratios for B10-B14 sub-sites compared to those from B16-B17 sub-sites, except for those from B14 sub-site which differed only from those relating to the B17 sub-site ($p < 0.05$) (Figure 4.12). Ratios of Cb 100 and 200 µg mL^{-1}-resistant/total bacterial CFU from the B10-B12 sub-sites were lower compared to those from the B17 sub-site, except for those from the B12 which showed significant difference only compared to those from the B17 sub-site ($p < 0.05$).

Three-Way ANOVA revealed significantly lower Amp 20 and 100 µg mL^{-1}-resistant/total bacterial CFUs ratio for the B10-B12 sub-sites compared to those from the B16-B17, except for those from the B12 sub-site, with significant difference only to those from the B17 sub-site. Lower Amp 200 µg mL^{-1}-resistant/total bacterial CFUs from the B10-B11 sub-sites compared to those from the B17 sub-site were also recorded ($p < 0.05$) (Figure 4.12).

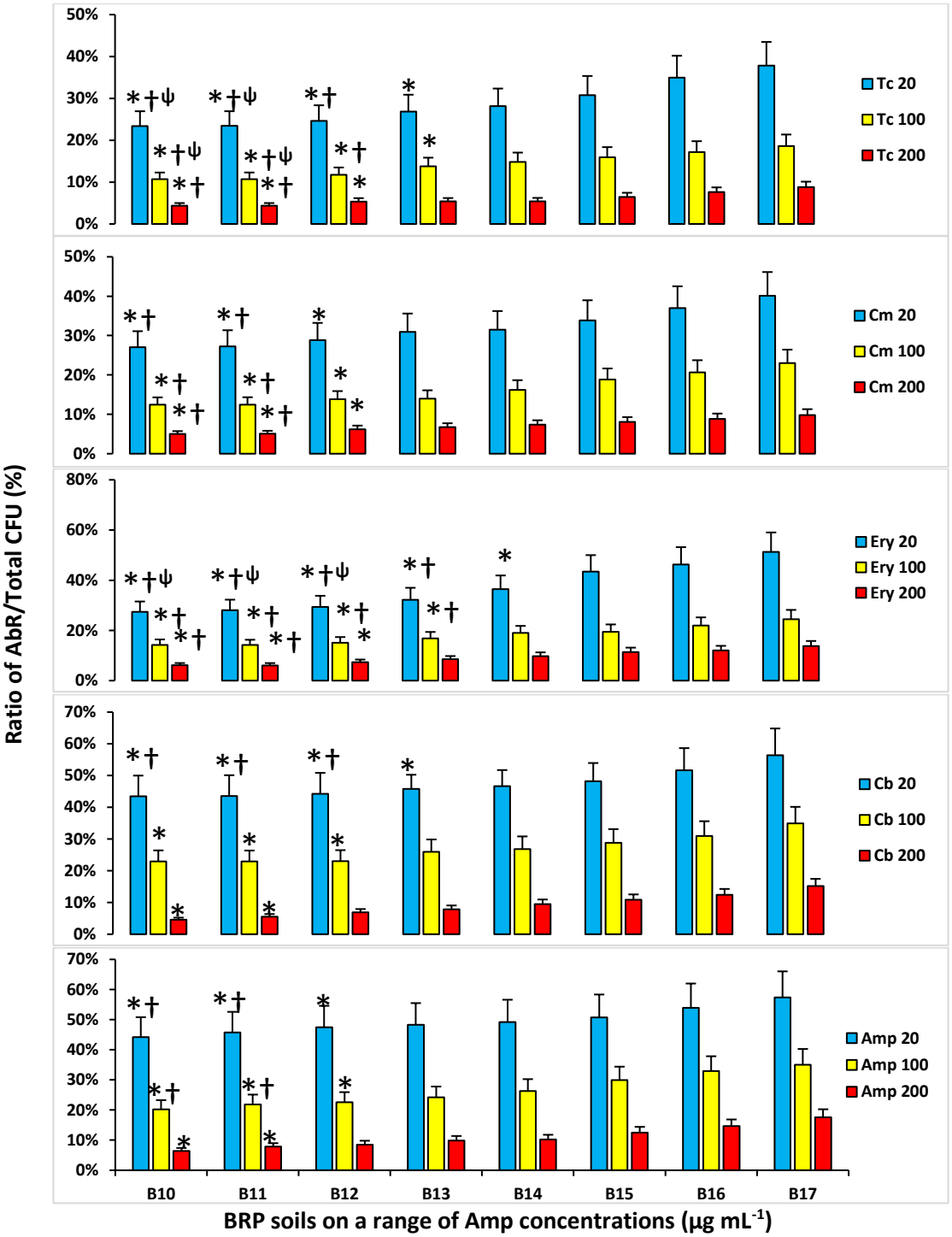


Fig. 4. 12. Mean ratios of AbR/total bacterial CFUs, selected on a range of Ab concentrations, for BRP sub-sites soil samples. The Ab concentrations are per µg mL^{-1}. *$p < 0.05$ compared to B17 soil bacteria CFU ratio selected on the same Tc concentration; †$p < 0.05$ compared to B16 soil bacteria CFU ratio selected on the same Tc concentration; $^{\Psi}p < 0.05$ compared to B15 soil bacteria CFU ratio selected on the same Tc concentration.

4.1.5. Pollution Induced Community Tolerance (PICT)

Samples of bacterial communities isolated from the WR soil sample sets and the BRP sub-sites were subjected to PICT analysis as described in Section 3.10 using a range of Cd, Zn, Hg, Tc, Cm,

Ery, Cb and Amp concentrations. The exponential growth rate of bacteria at 12-hour of incubation was recorded and was used to calculate MIC and EC50 –by the Log (inhibitor) vs. response, Variable slope (four parameters) method– for the HMs and Abs used in this experiment.

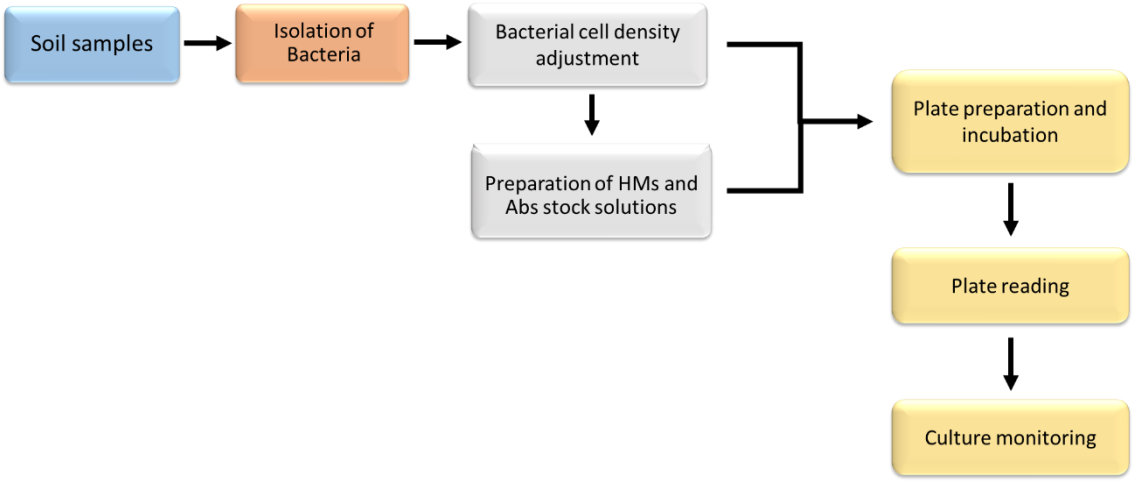


Fig. 4. 13. Flow chart illustrating steps of PICT assay.

4.1.5.1. Waikato Region soils

PICT analysis of MIC and EC50 for Cd, Zn and Hg for bacterial consortia isolated from WRSS1 indicated MIC and EC50 values were greater for HMs for bacterial consortia from pastoral soil compared to those from background soil (Figures 4.14 and 4.15).

In addition, PICT analysis of MIC and EC50 for Tc, Cm, Ery, Cb and Amp for bacterial consortia isolated from WRSS1 revealed larger MIC and EC50 values for Abs for bacterial consortia from pastoral and arable soils, compared to those from background soil (Figures 4.16-4.17). The MIC values determined for bacterial isolates (according to EUCAST ECOFF recommendations [218]) from pastoral soil were higher than the 20 µg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs [217, 220], while MICs were lower than this threshold for bacteria from arable soil for Tc, and also for background soil for Tc and Cm.

PICT analysis of MIC and EC50 for the HMs for bacteria from WRSS2 and WRSS3 showed there were significant differences, similar as for WRSS1, between the HMs' MIC and EC50 values for pastoral soils compared to those from background soils. PICT analysis of MIC and EC50 with the

five Abs for bacteria isolated from WRSS2 and WRSS3 also produced greater Abs' MIC and EC50 values for bacterial communities from pastoral and arable soils compared to those from background soils (Figures A2.6-A2.13).

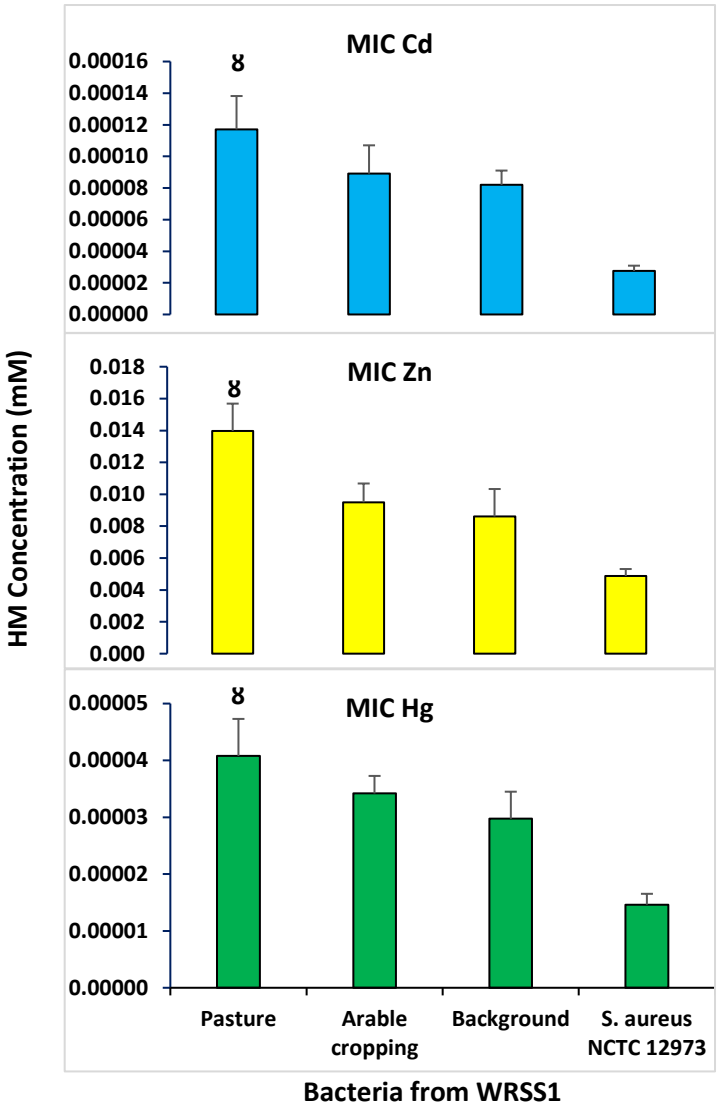


Fig. 4. 14. Mean MIC values of PICT assay with Cd, Zn and Hg for bacteria from WRSS1. ϑ$p < 0.05$ compared to HM MIC values for bacteria from background soil.

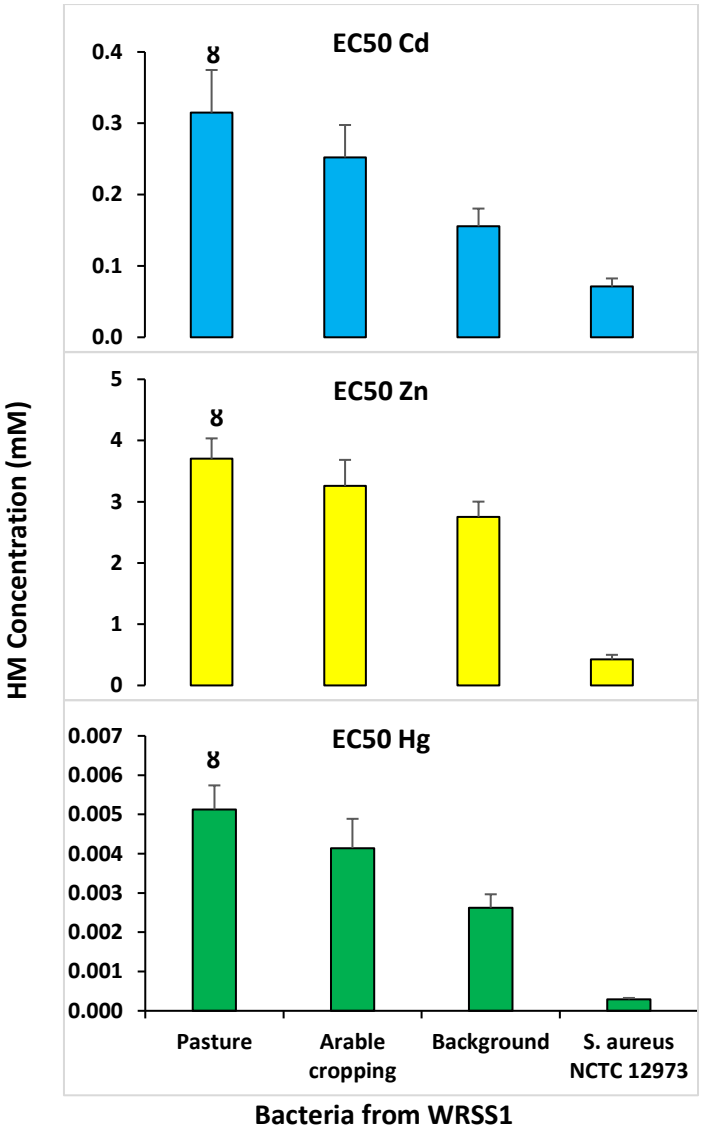


Fig. 4. 15. Mean EC50 values of PICT assay with Cd, Zn and Hg for bacteria from WRSS1. ϑ$p < 0.05$ compared to HM MIC values for bacteria from background soil.

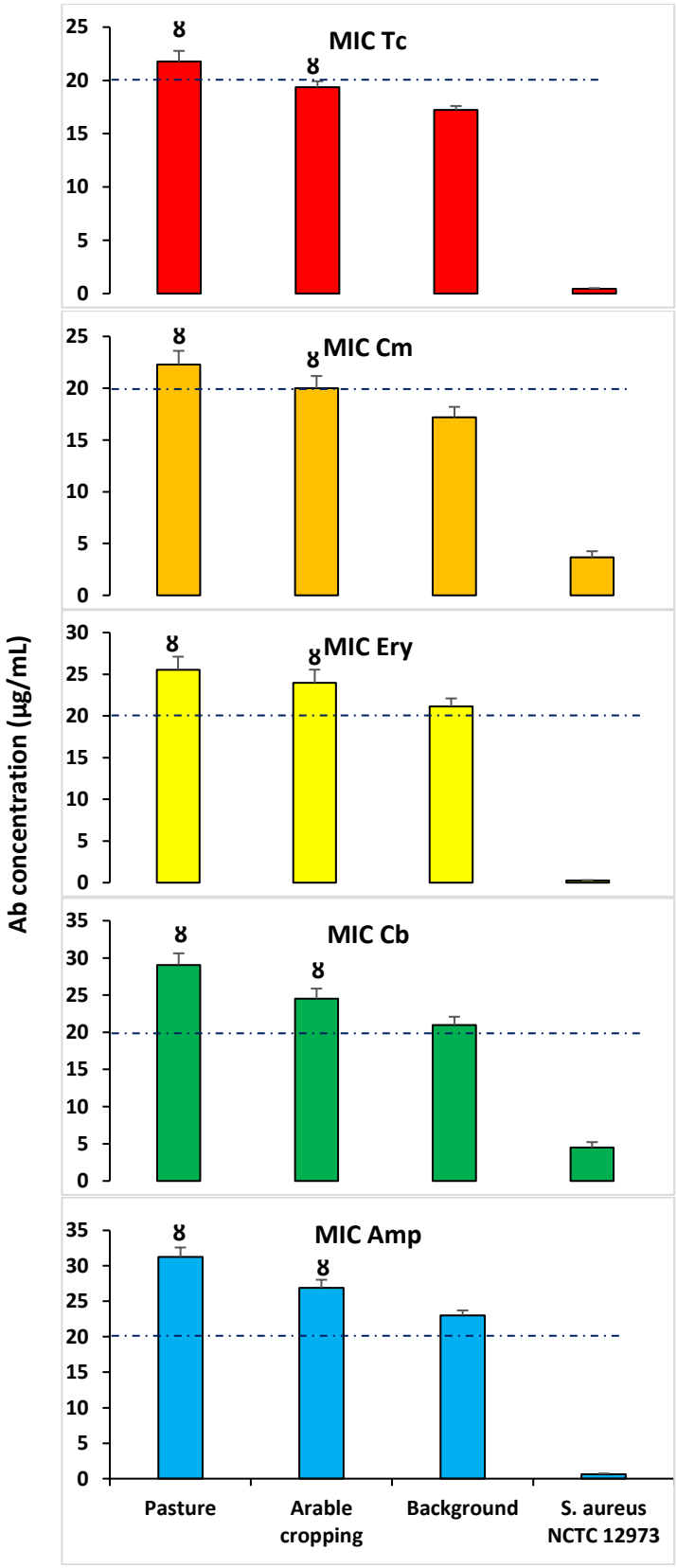


Fig. 4. 16. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS1. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from background soil. The dash line defines AbR level of soil bacteria.

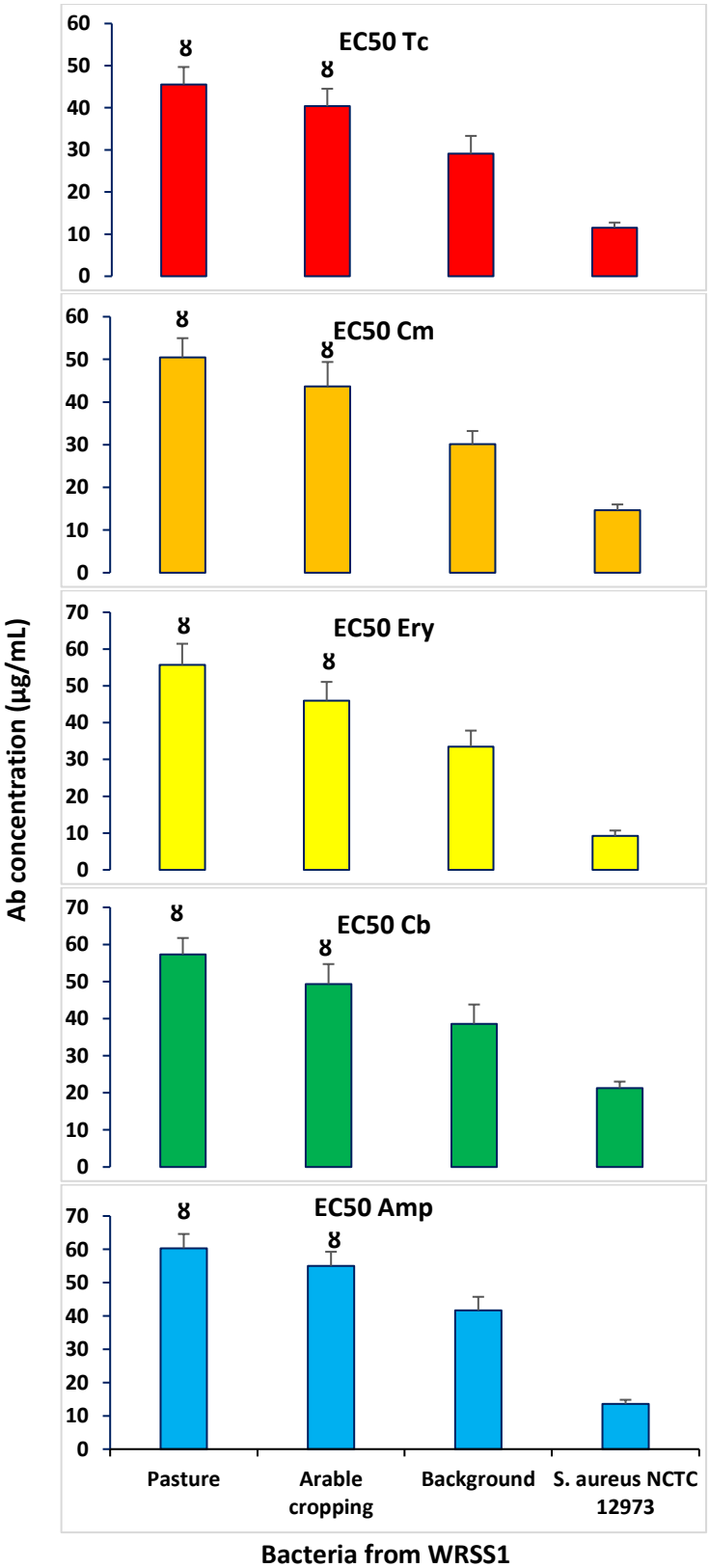


Fig. 4. 17. Mean EC50 values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS1. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from background soil.

4.1.5.2. PICT Analysis of Bacteria Consortia from Belmont Regional Park Soils

The result of PICT analysis for bacteria isolated from three sub-sites of BRP (B17, B14 and B10) are shown here as these soil samples were collected from start (B17), middle (B14) and end (B10) of the BRP airstrip and represented the highest, intermediate and lowest concentrations of HMs amongst the eight BRP sub-sites soils.

PICT analysis of HM's MIC of Cd, Zn and Hg for bacterial consortia from BRP sub-sites samples B17, B14 and B10 showed there were significant differences between these HMs MIC and EC50 values for bacterial consortia from B17 (highest *in situ* concentrations of Cd and Zn) and B14 soils compared to those from B10 soil (lowest *in situ* concentrations of Cd and Zn). Furthermore, it was revealed that there were significant differences between the HMs' MIC values of bacterial consortia from B17 soil compared to those from B14 soil (Figures 4.18).

The EC50 values for HMs for bacterial consortia from B17 soil were greater than those from B10 soil (Figure 4.19).

PICT analysis of Ab's MIC of Tc, Cm, Ery, Cb and Amp produced MIC and EC50 values for bacterial consortia that differed between all three sub-sites (Figure 4.20 and 4.21). The MIC values determined (according to EUCAST ECOFF recommendations [218]) for bacteria isolated from B17, B14 and B10 soils were higher than 20 μg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs [217, 220].

PICT analysis of the HM's MIC for bacterial consortia from other BRP sub-sites soils, including B16, B15, B13, B12 and B11, showed there were greater HMs' MIC values for bacteria from B16-B15 soils compared to those from B10 soil, except for Hg MIC value for bacteria from B15 soil. In addition, there were greater HM's EC50 values for bacterial consortia from B16 soil compared to those from B10 soil (Figures A2.14 and A2.15, Appendix 2).

There were greater Ab's MIC and EC50 values for bacteria from B16-B15 soils compared to those from B10. In addition, there were higher Tc and Cm MIC values for bacterial consortia from B13-B12 soils compared to those from B10 soil (Figures A2.16 and A2.17, Appendix 2).

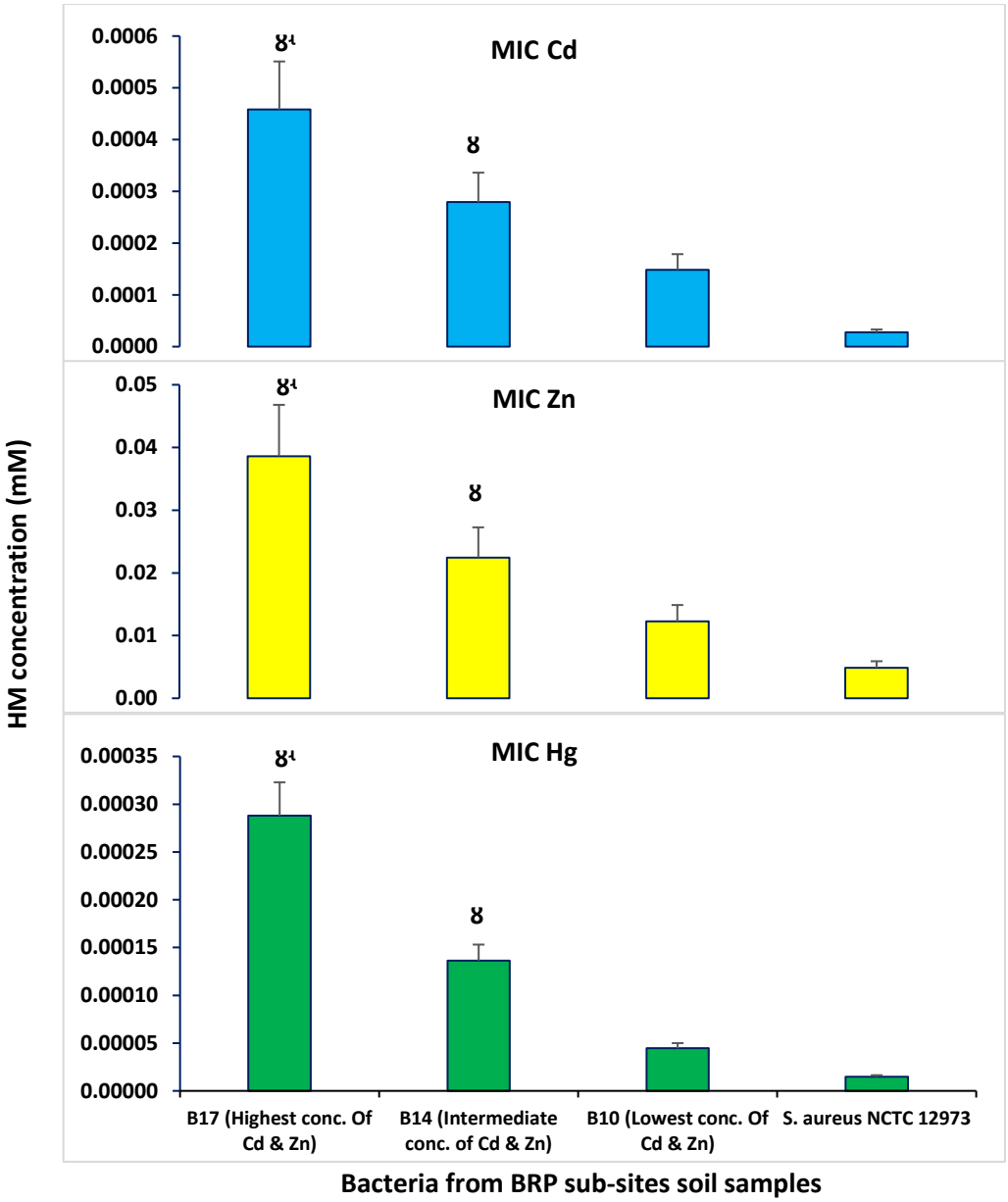


Fig. 4. 18. Mean MIC values of PICT assay with Cd, Zn and Hg for bacteria from BRP B17, B14 and B10 soils. ϑ$p < 0.05$ compared to HM MIC values for bacteria from B10 soil; ¹$p < 0.05$ compared to HM MIC value for bacteria from B14 soil.

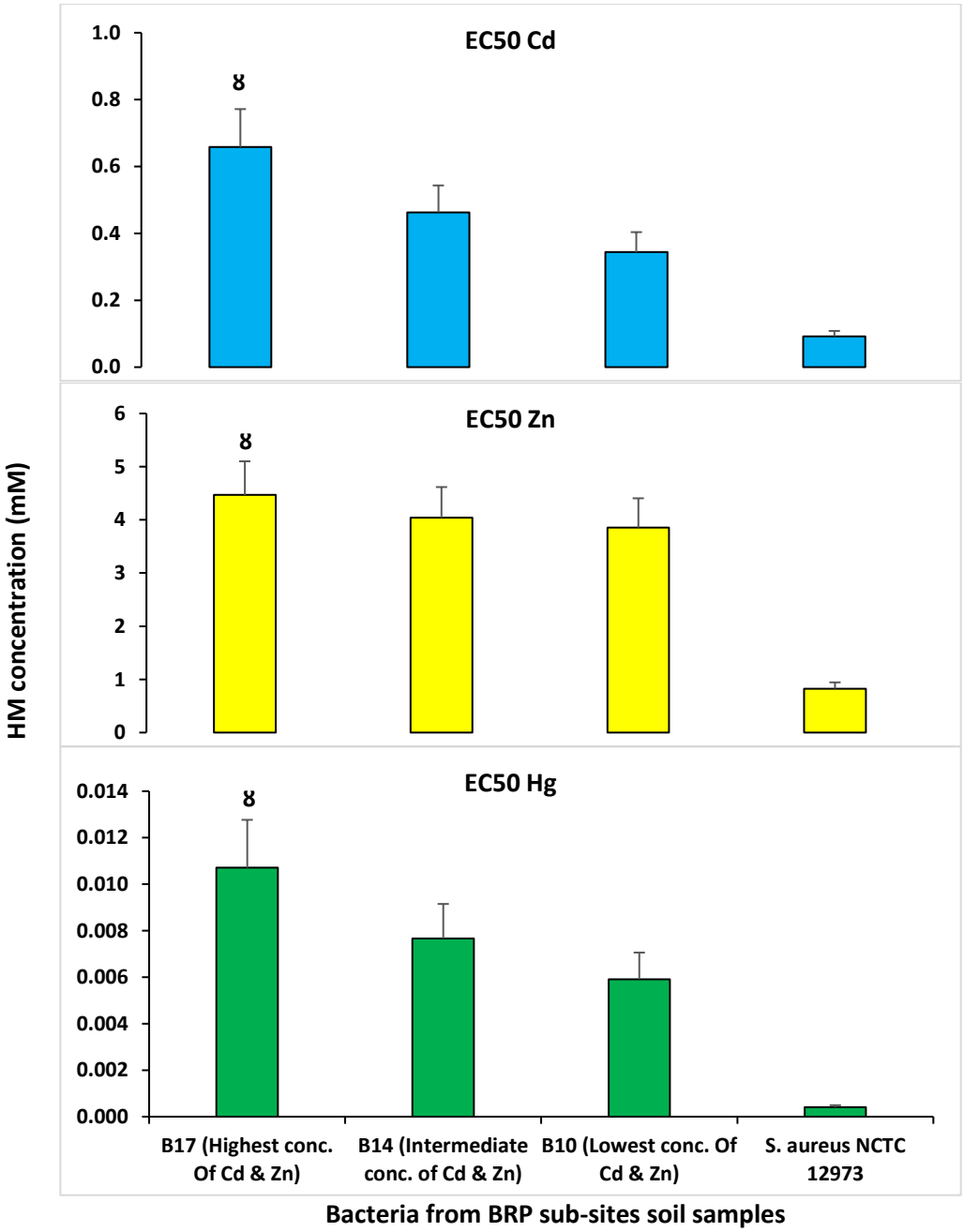


Fig. 4. 19. Mean EC50 values of PICT assay with Cd, Zn and Hg for bacteria from BRP B17, B14 and B10 soils. ȣ$p < 0.05$ compared to HM MIC values for bacteria from the B10 soil.

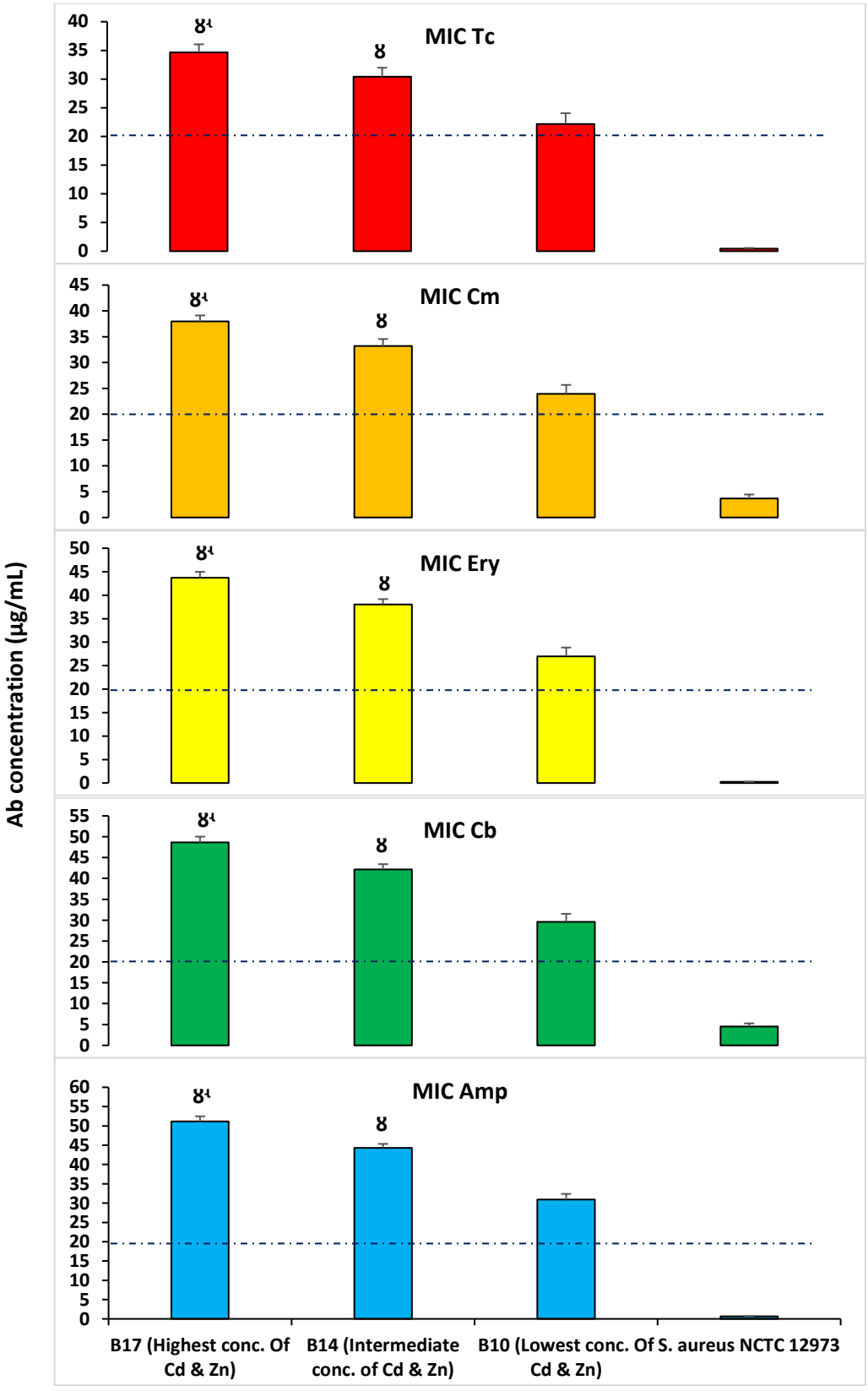


Fig. 4. 20. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from BRP B17, B14 and B10 soils. ȣ$p < 0.05$ compared to Abs MIC values for bacteria from B10 soil; ¹$p < 0.05$ compared to HM MIC value for bacteria from B14 soil. The dash line defines AbR level of soil bacteria.

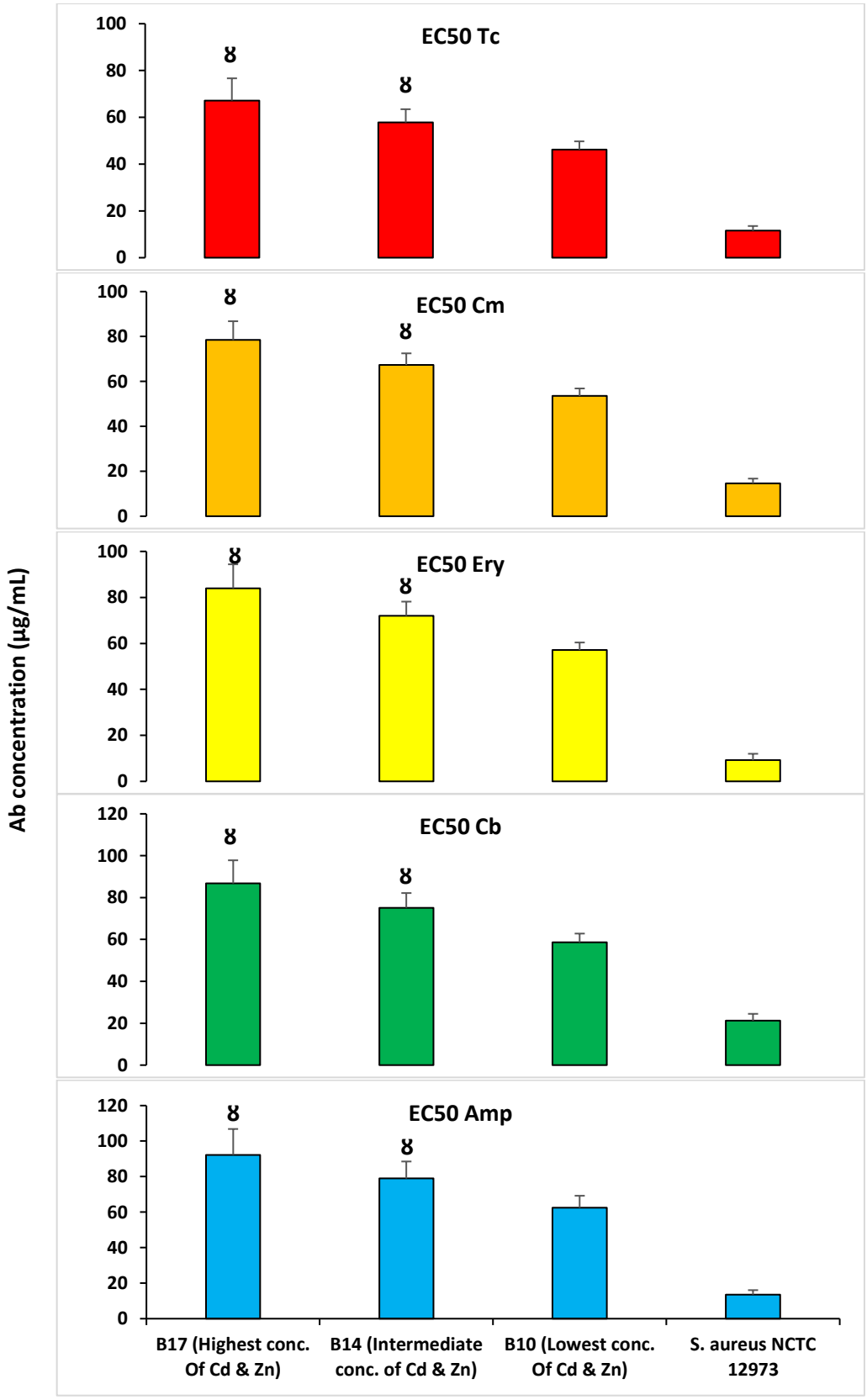


Fig. 4. 21. Mean EC50 values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from BRP B17, B14 and B10 soils. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from the B10 soil.

4.1.6. Quantitation of Susceptibility to HMs and Abs by Broth Microdilution (BM) Analysis

Determination of susceptibility to HMs and Abs by BM assay for HMR bacterial isolates from WR soil sample sets and BRP sub-sites soil samples was performed using Cd, Zn, Hg, Tc, Cm, Ery, Cb and Amp. The exponential growth rate of bacteria after 12 h of incubation was recorded and was used to calculate MIC and EC50 –by Log (inhibitor) vs. response, Variable slope (four parameters) method– of the HMs and Abs used in this experiment. Three-Way ANOVA was performed to investigate the associations between the MICs and EC50s of different HMs and Abs and different sampling sites. Figure 4.21 illustrates different steps of BM analysis.

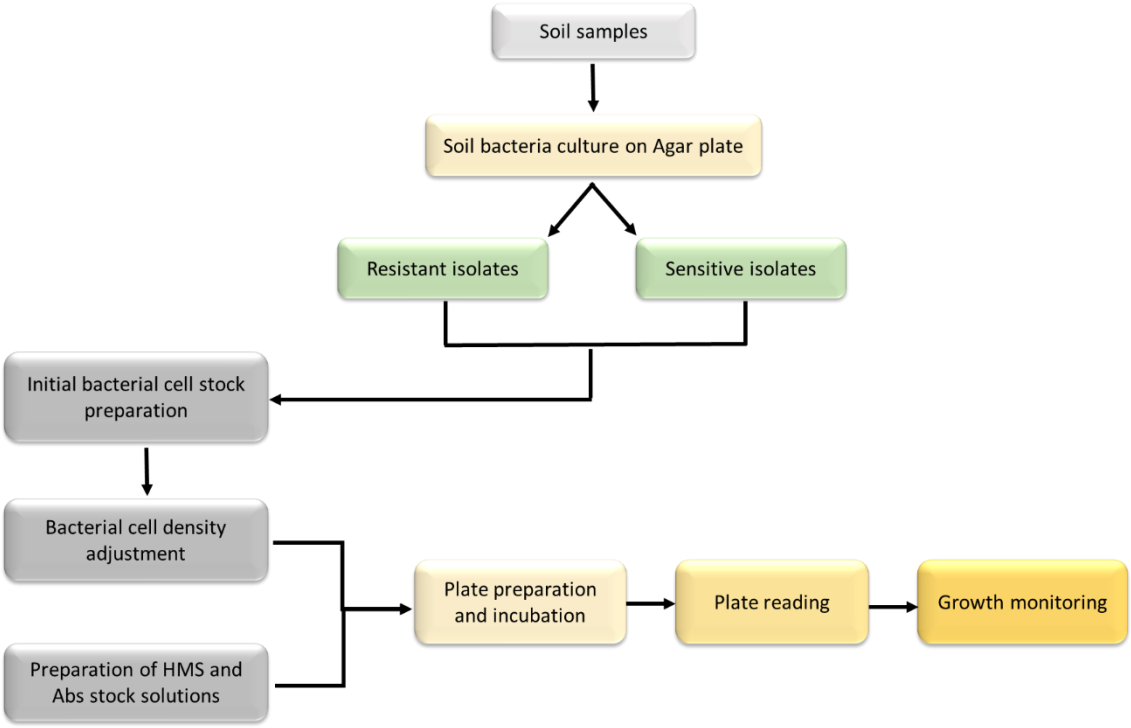


Fig. 4. 22. Flow chart illustrating BM analysis steps.

4.1.6.1. BM Assay for Waikato Region (WR) Soils

The BM assays indicated greater MIC values for Cd, Zn and Hg for the HMR bacteria from WRSS1 pastoral and arable soils compared to those from background soil. These isolates were selected from the plates with HM concentrations, 0.1 and 1 mM of Cd, 1 and 5 mM of Zn and 0.01 and 0.1 mM of Hg. As would be expected there were higher MIC values for Cd, Zn and Hg for HMR

bacteria from WRSS1 pastoral, arable and background soils compared to the HM-sensitive isolates from each soil sample (Figure 4.23).

Similar to the above-mentioned significant differences determined for MIC values, there were significant differences between the HMs' EC50 values for the isolates from different land uses of the WRSS1 samples (Figure 4.24).

There were lower MIC values of Cd, Zn and Hg for bacteria from WRSS1 background soil and selected on plates containing Cd 0.1mM, Zn 1mM and Hg 0.01mM, compared to those from the same soil sample and selected on plates with the higher concentrations of each HM, including Cd 1mM, Zn 1mM and Hg 0.1mM. (Figure A2.18, Appendix 2).

The result for HM EC50 values showed there were lower HMR bacteria from WRSS1 background soil and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM, compared to those from plates with the higher concentration of each HM. (Figure A2.19, Appendix 2).

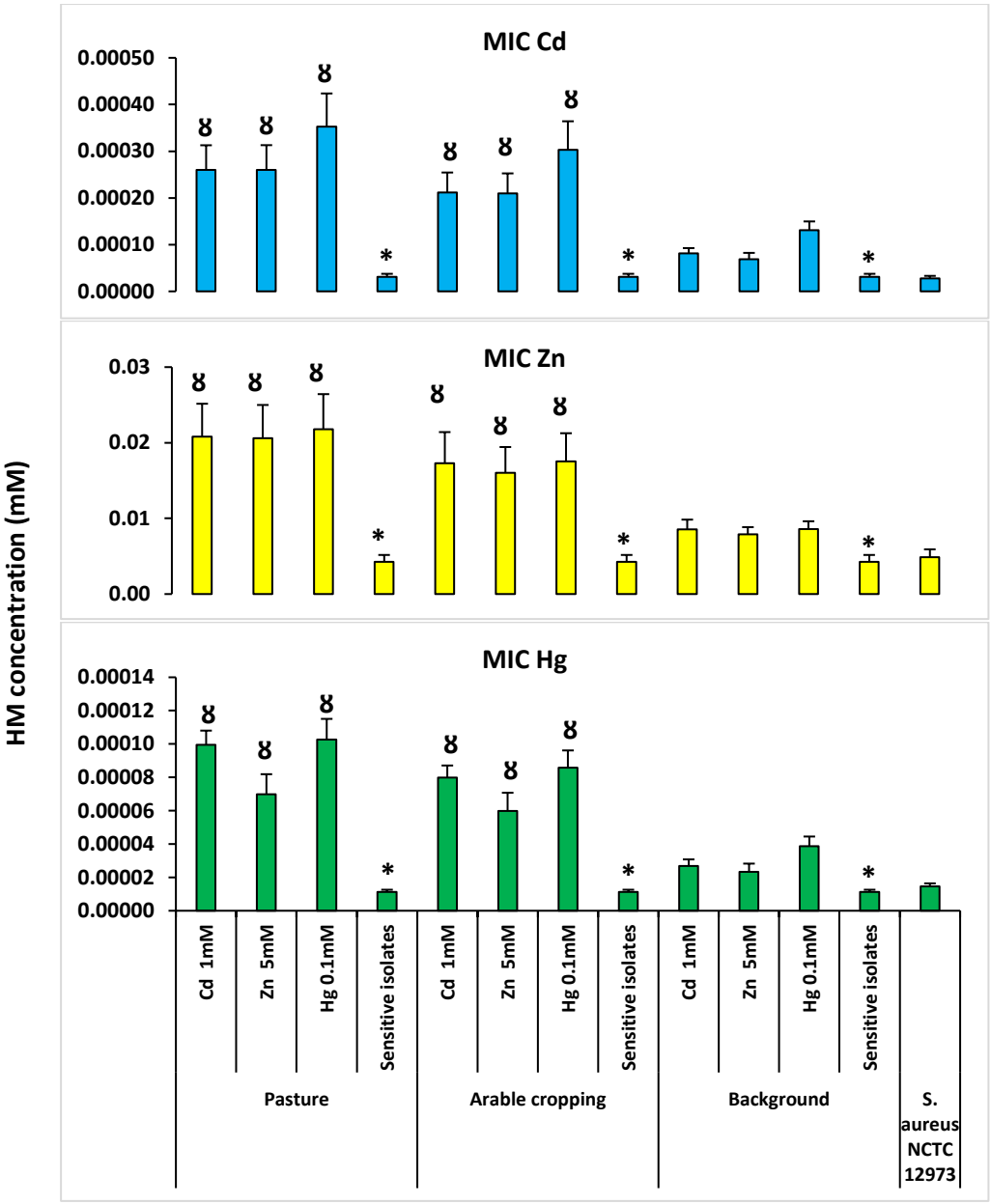


Fig. 4. 23. Mean MIC values from BM assay with Cd, Zn and Hg for HMR isolates from WRSS1. *$p < 0.05$ compared to HM MIC value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to HM MIC value for HMR isolates from background soil.

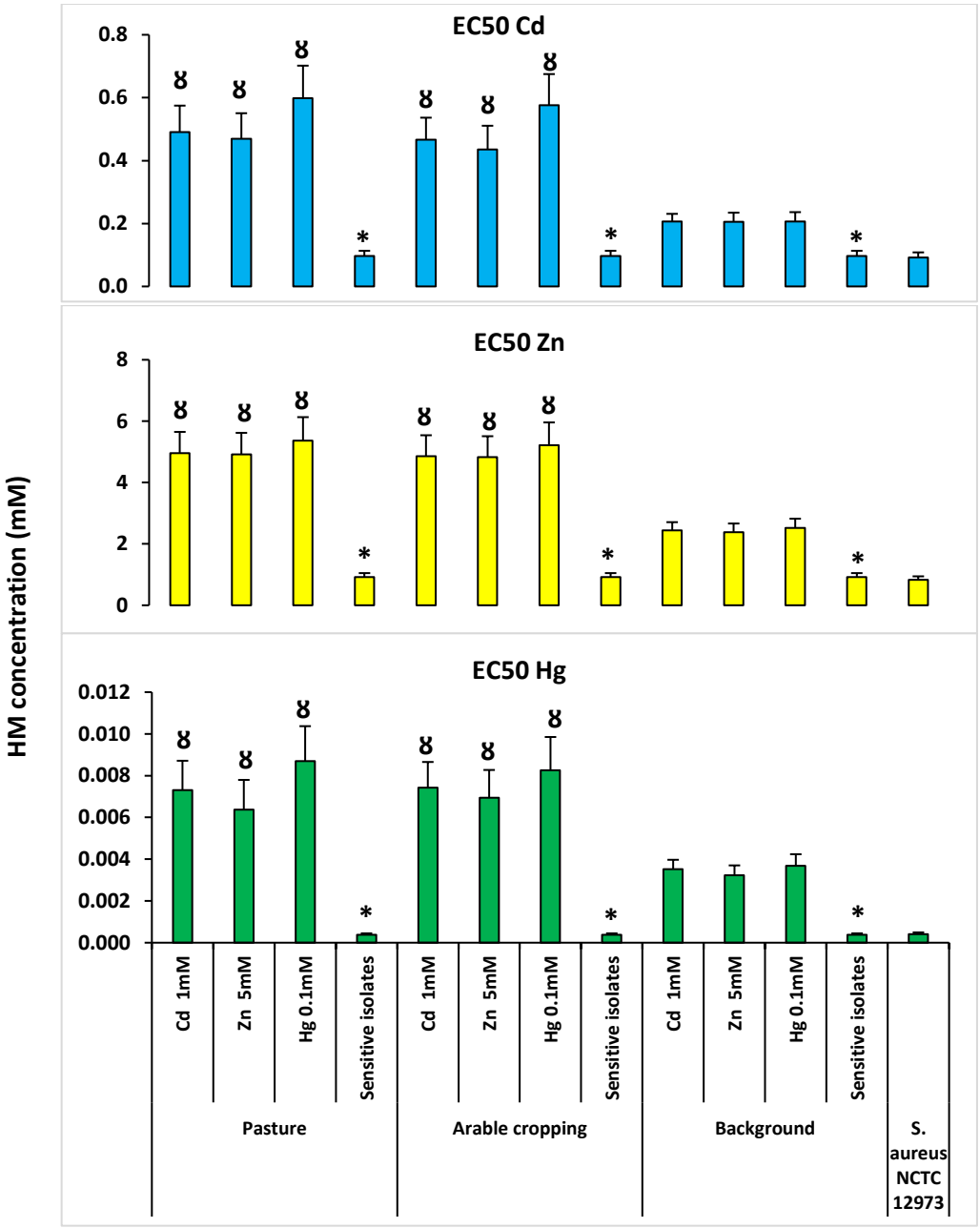


Fig. 4. 24. Mean EC50 values from BM assay with Cd, Zn and Hg for HMR isolates from WRSS1. *$p < 0.05$ compared to HM EC50 value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to HM EC50 value for HMR isolates from background soil.

BM assays for the five Abs showed greater mean MIC values of HMR isolates for Tc, Cm, Ery, Cb and Amp for the bacteria from WRSS1 pastoral and arable soils compared to those from background soil. The MIC values determined (according to EUCAST ECOFF recommendations [218]) for bacterial isolates from pastoral and arable soils were higher than 20 µg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs [217, 220], while these were lower

than this threshold for those bacteria from background soil for Tc and Cm. Furthermore, it was also found the mean MIC and EC50 values for all five Abs for the HMR isolates from WRSS1 pastoral, arable and background soils were greater than those for the HM-sensitive isolates from each WRSS1 soil sample ($p < 0.05$) (Figure 4.25).

Moreover, there were higher Abs' EC50 values for the HMR isolates from WRSS1 pastoral and arable soils compared to those from background soil. Greater Abs EC50 values for the HMR isolates from WRSS1 soils compare to those HM-sensitive isolates from each soil sample were determined (Figure 4.26).

Lower Ab mean MIC values were determined for all five Abs for isolates from WRSS1 background and arable soils and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those selected on plates with the higher concentrations of these HMs (Cd 1 mM, Zn 5 mM and Hg 0.1 mM) (Figure A2.20, Appendix 2). Mean EC50 values' differences for all five Abs were significant between isolates from WRSS1 background and arable soil and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those from plates with the higher concentrations of these HMs (Figure A2.21, Appendix 2).

Similar results were also determined for the HMs and Abs' MIC and EC50 values for HMR isolates from WRSS2 and WRSS3 samples. The data only for the WRSS1 set is presented here and data for WRSS2 and WRSS3 soil sets are shown in Appendix 2. There were no significant differences between the determined HM and Ab's MIC and EC50 values from BM assays in relation to isolates from the three soil samples sets, WRSS1, WRRS2 and WRSS3 (Figures A2.22-A2.37).

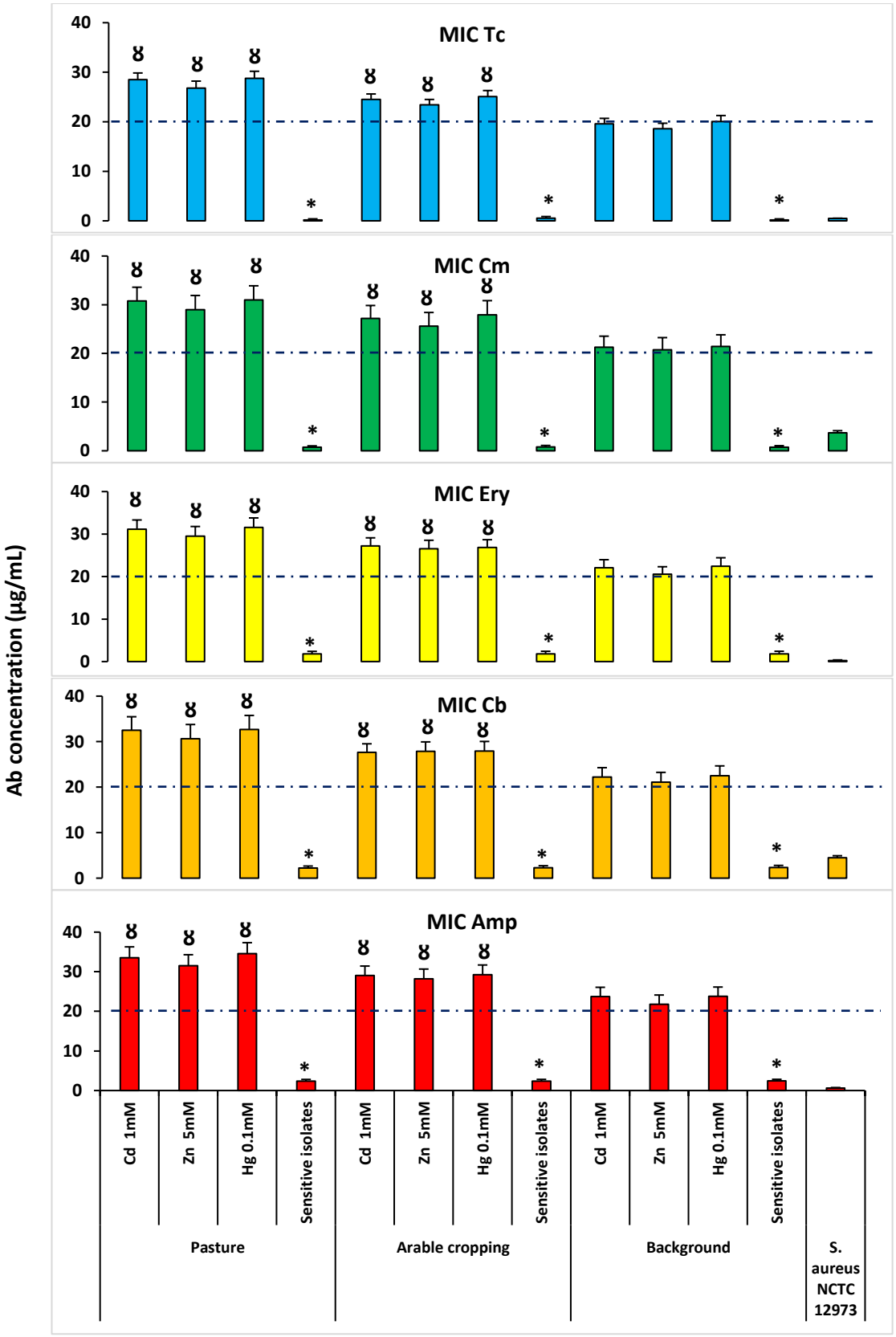


Fig. 4. 25. Mean MIC values for BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS1. *$p < 0.05$ compared to Ab MIC value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for isolates from background soil and selected on the same HM concentration). The dash line defines AbR level of soil bacteria.

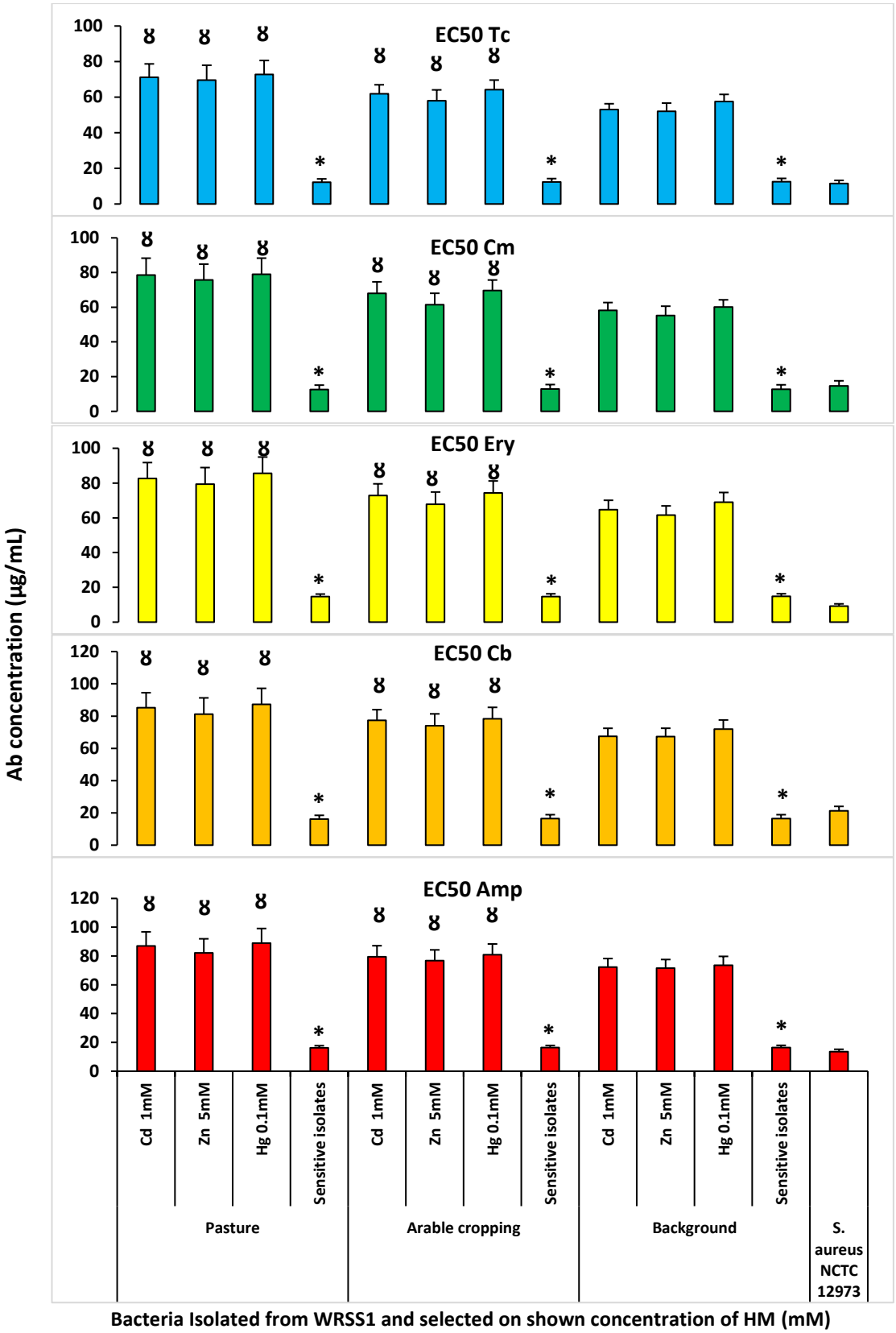


Fig. 4. 26. Mean EC50 values for BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS1. *$p < 0.05$ compared to Ab EC50 value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to Ab EC50 value for isolates from background soil and selected on the same HM concentration.

4.1.6.2. BM Assay for Belmont Regional Park (BRP) Soils

MIC determinations with HMs by BM assay with isolates from three BRP sub-sites (B17, B14 and B10) showed the Cd, Zn and Hg MIC values for HMR isolates differed between all three sub-sites. In addition, there were significant differences between Cd, and Zn and Hg MIC values for HMR bacteria from B17, B14 and B10 sub-sites compared to the HM-sensitive isolates from the same soil samples (Figure 4.27). The purpose of showing results for these three sub-sites here (while the results for other sub-sites are shown in Appendix 2) was that these sub-sites soil samples were collected at the start, middle and end of airstrip at BRP and also the concentrations of HMs were decreased from B17 to B10 and represented the highest (at B17) intermediate (at B14) and lowest (at B10) concentrations of HMs in BRP airstrip sub-site soil samples.

It was determined that there were higher Cd, Zn and Hg EC50 values for HMR bacteria from BRP B17 sub-site compared to those from B10 sub-site. In addition, there were greater Zn EC50 values for CdR and ZnR bacteria from B17 soil compared to those from B14 soil. There were greater Cd, Zn and Hg EC50 values for HMR bacteria from B17, B14 and B10 soils compared to the HM-sensitive bacteria from the same soil samples (Figure 4.28).

No significant differences were determined between the HMs MIC and EC50 values of BM test for HMR bacteria from B17, B14 and B10 sub-sites compared to those from the same samples and selected on plates with the higher concentrations of HMs, including Cd 1mM, Zn 5mM and Hg 0.1mM (Figures A2.38-A2.39, Appendix 2).

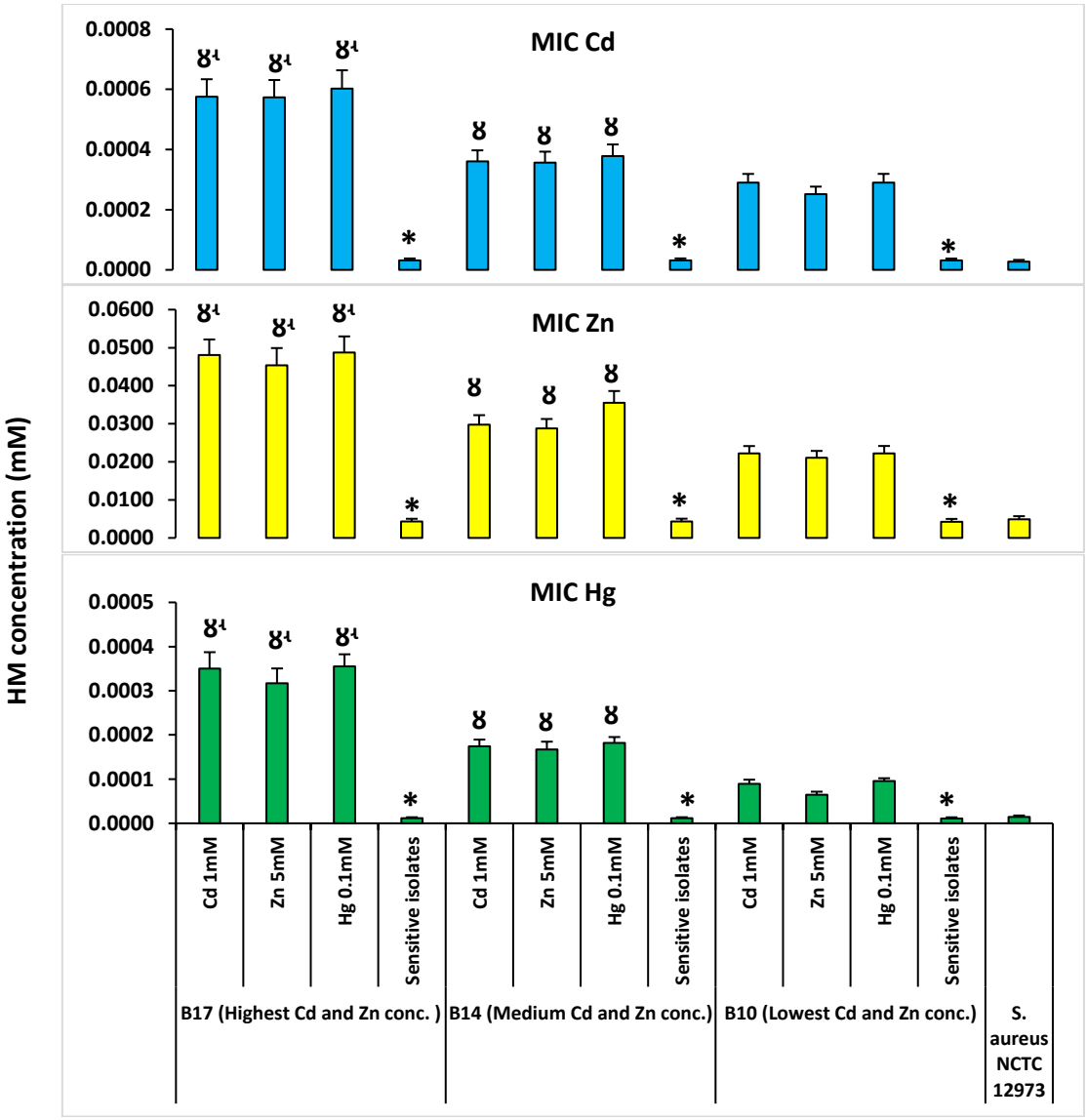


Fig. 4. 27. Mean MIC values of BM assay with Cd, Zn and Hg for the HMR isolates from BRP. *$p < 0.05$ compared to HM MIC value for HMR bacteria from the same soil; ϐ$p < 0.05$ compared to HM MIC value for the bacteria from B10 soil and selected on the same HM; ϟ$p < 0.05$ compared to Ab MIC value for the bacteria from B14 soil and selected on the same HM concentration.

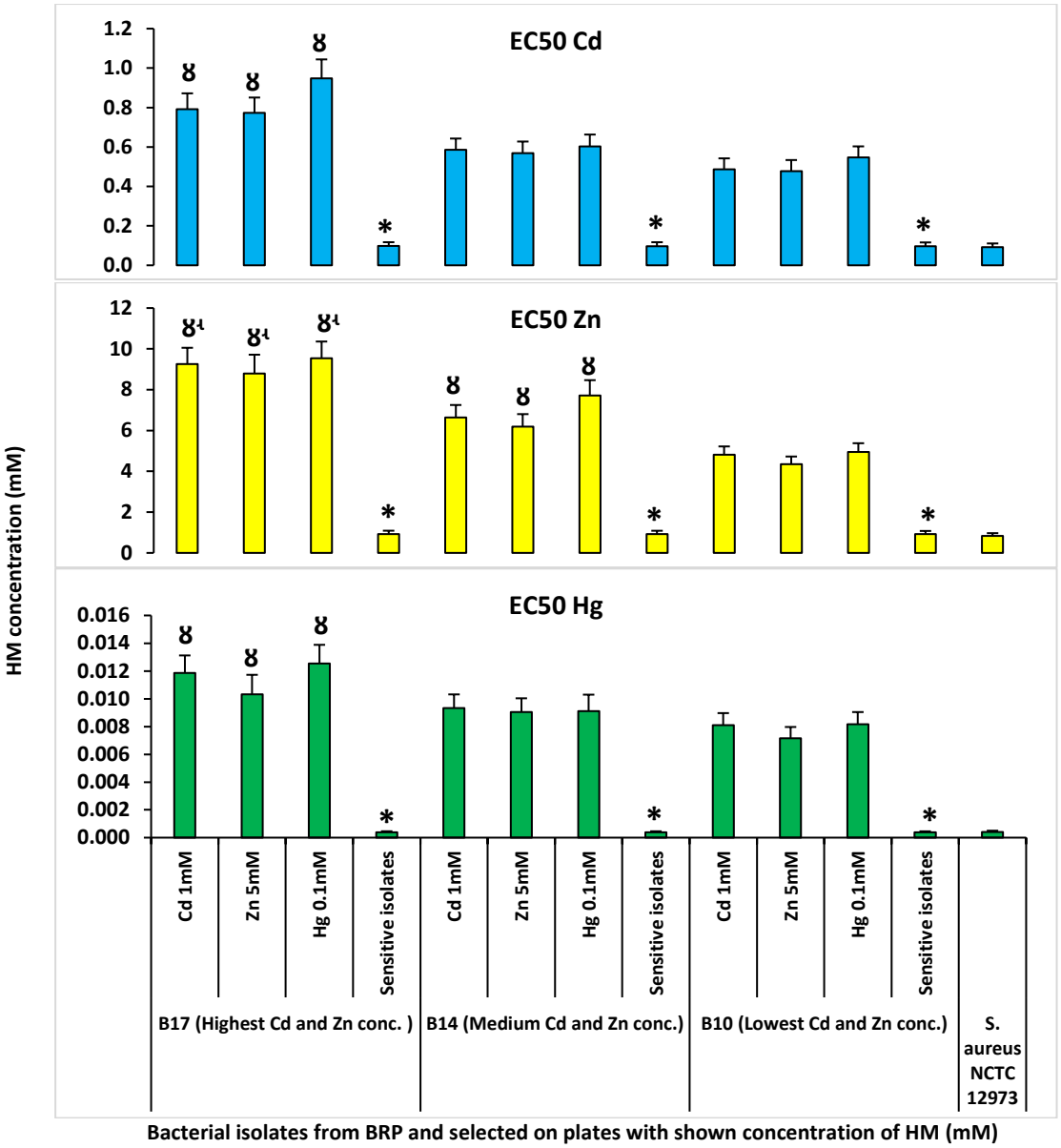


Fig. 4. 28. Mean EC50 values of BM assay with Cd, Zn and Hg for the HMR isolates from BRP. *$p < 0.05$ compared to HM MIC value for the HMR bacteria from the same soil; ϑ$p < 0.05$ compared to HM MIC value for the bacteria from B10 soil and selected on the same HM; ¹$p < 0.05$ compared to Ab MIC value for the bacteria from B14 soil and selected on the same HM concentration.

The result of Abs MIC and EC50 values determination from BM test with TC, Cm, Ery, Cb and Amp showed there were greater values for HMR bacteria from B17 and B14 soils compared to those from B10 soil. It was revealed that there were greater Abs MIC values for HMR bacteria from B17 soil compared to those from B14 soil. For TC and Cm EC50 values it was revealed that there were higher values for HMR bacteria from B17 soil compared to those from B14. The MIC values determined (according to EUCAST ECOFF recommendations [218]) for bacterial isolates

from B17, B14 and B10 soils were higher than 20 µg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs [217, 220], while these were lower than this threshold for those sensitive bacteria from each soil sample. Additionally, higher Abs MIC and EC50 values for HMR bacteria from B17, B14 and B10 compared to the HM-sensitive bacteria from the same soil samples were determined (Figures 4.29 and 4.30).

There were no significant differences between the Abs MIC and EC50 values for HMR bacteria from B17, B14 and B10 soils and plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared those from plates with the higher concentrations of the HMs (Figures A2.40 and A2.41, Appendix 2).

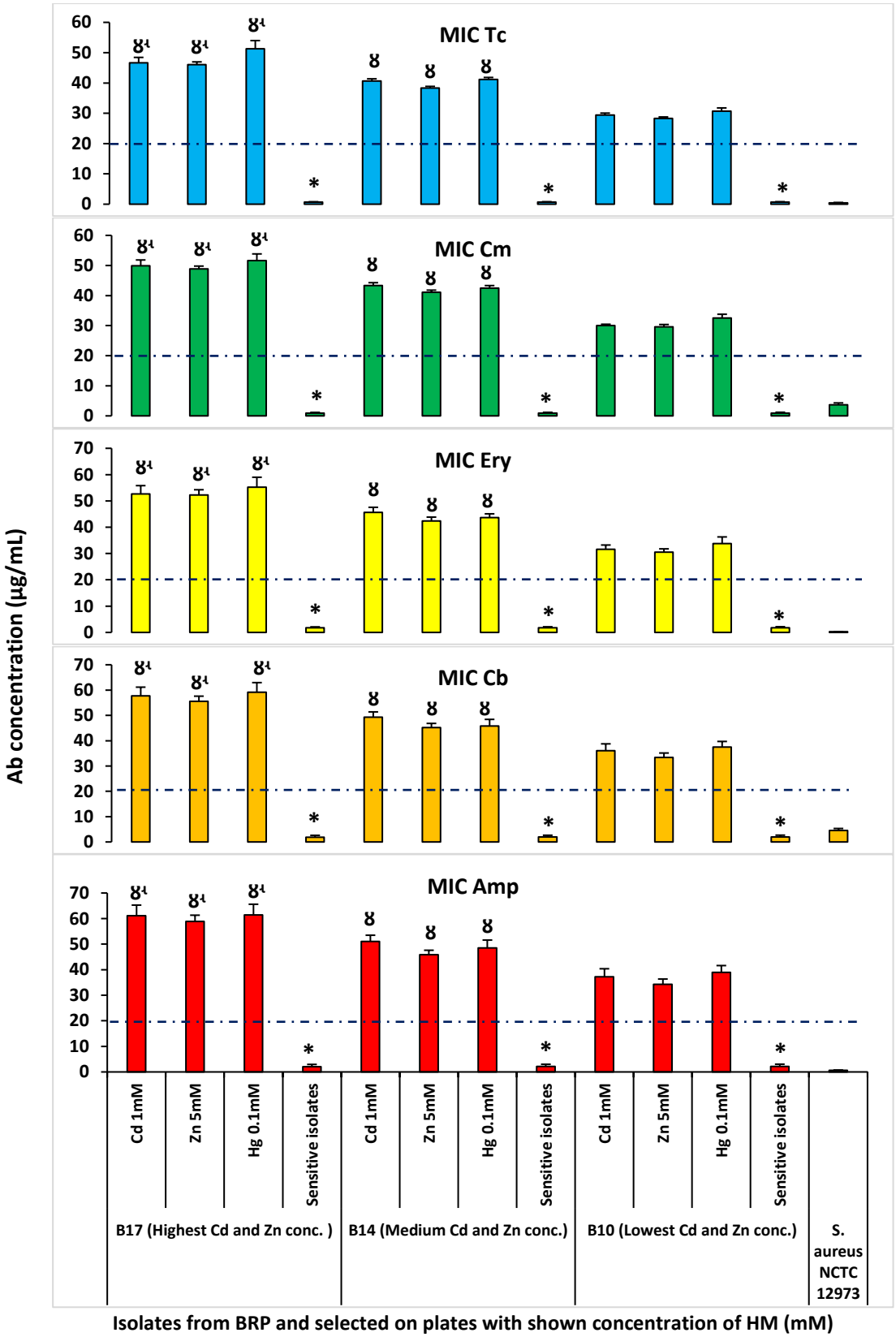


Fig. 4. 29. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for the HMR isolates from BRP. *$p < 0.05$ compared to Ab MIC value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for the bacteria from B10 soil and selected on the same HM concentration; ⸸$p < 0.05$ compared to Ab MIC value for the bacteria from B14 soil and selected on same HM concentration. The dash line defines AbR level of soil bacteria.

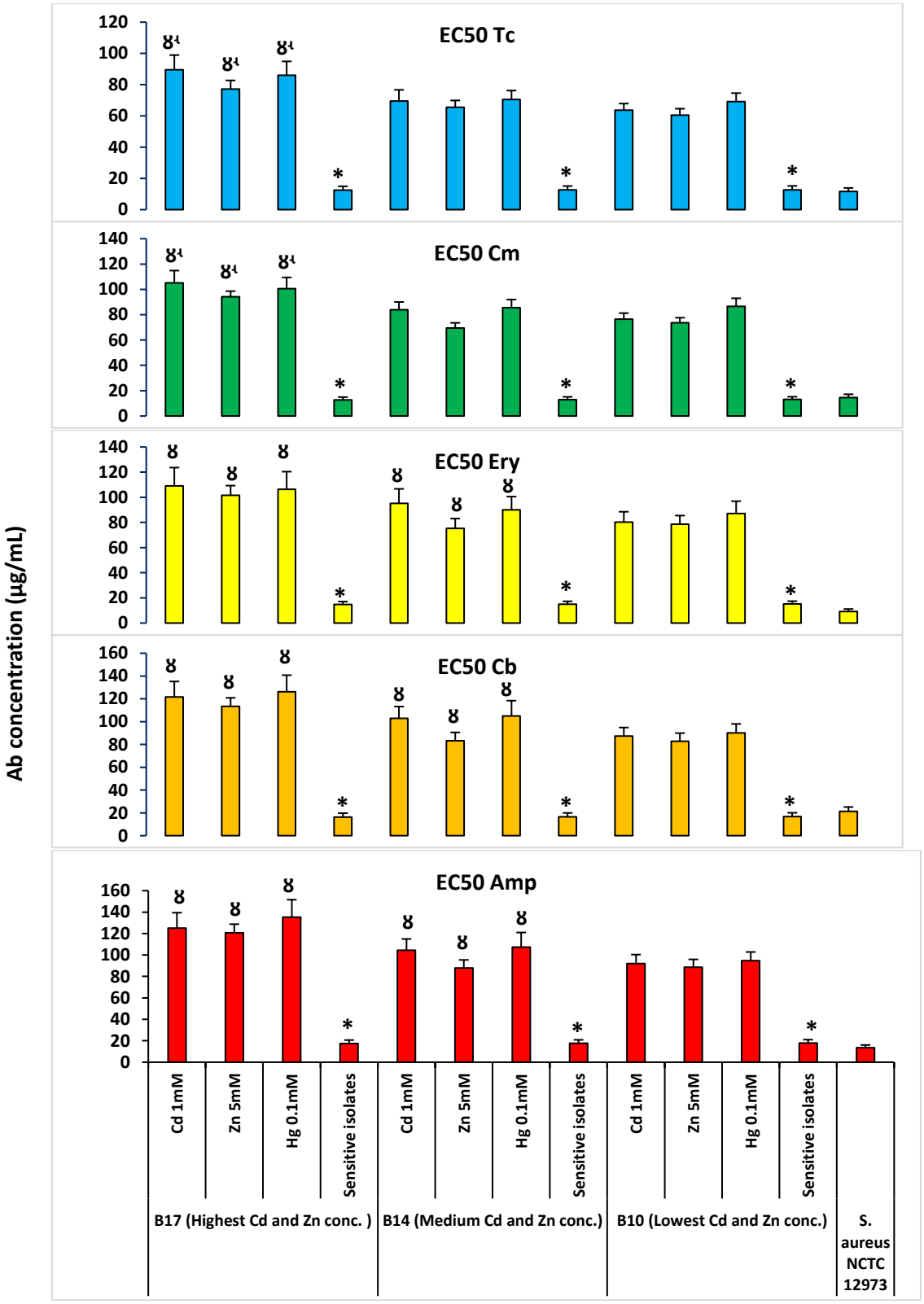


Fig. 4. 30. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for the HMR isolates from BRP. *$p < 0.05$ compared to Ab MIC value for HMR bacteria from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for the bacteria from B10 soil and selected on the same HM concentration; ¹$p < 0.05$ compared to Ab MIC value for the bacteria from B14 soil and selected on the same HM concentration.

MIC and EC50 determination of HMs BM assay for other sub-sites of BRP soil samples, including B16, B15, B13, B12 and B11, showed there were greater HMs MIC and EC50 values for HMR bacteria from soils with higher HM concentrations (e.g. B16) compared to those from soils with lower HM concentrations (B10). There were greater HMs MIC and EC50 values for HMR bacteria from all of these BRP soils compared to the HM-sensitive bacteria from each soil sample. In addition, no significant differences was determined between HMs MIC and EC50 values for bacteria from B16, B15, B13, B12 and B11 soils and plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those from the same soils and plates with the higher concentrations of HMs (Figures A2.42-A2.45, Appendix 2).

MIC and EC50 determination of the Abs BM test for BRP B16, B15, B13, B12 and B11 soil samples showed there were greater values for HMR bacteria compared to the HM-sensitive bacteria from each soil sample. There were greater Abs MIC and EC50 values for HMR bacteria from B16, B15, B13 and B12 soils compared to those from B10 soil. Additionally, it was shown that there were no significant differences between Abs MIC and EC50 values for bacteria from B16, B15, B13 and B12 soils and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those from plates with the higher concentrations of the HMs (Figures A2.46-A2.49, Appendix 2). Overall, there were higher MIC and EC50 values determined for bacterial isolates from soils with higher HM concentrations (e.g. B17) compared to those for isolates from soil samples with lower HM concentrations (e.g. B10). It means soils with higher HM concentrations produce HMR bacteria with higher Ab resistance than soils with lower HM concentrations. In addition, there were higher MIC and EC50 values determined for HMR bacterial isolates compared to sensitive bacterial isolates.

4.1.7. Bacterial Colony Morphological Groups

Isolates from WR and BRP soil samples were selected and categorised according to their colony morphological features.

4.1.7.1. Waikato Region Soil Samples' Bacterial Morphology

HMR bacterial isolates (n=900 for soil sample set) from WR three soil sample sets were categorised into 22 different groups based on their morphological characteristics and Gram staining (Figures 4.31 and 4.32). The 22 morphological groups are described in the Table 4.3. Morphological category number 1 consists the largest fraction of CdR and ZnR isolates from pastoral soils. The most common colony morphology group of ZnR and HgR isolates from arable soils, belongs to two morphological groups (numbers 1 and 2), and this was also true for HgR isolates from pastoral soils. Morphological groups numbers 1 and 5 include the most common colonies for CdR isolates from arable soil, as well as ZnR isolates from background soil (Figure 4.31).

Table 4. 3. Description of bacterial colony morphologies.

	Gram Reaction	Colour	Whole colony	Edge	Elevation	Surface
1	Negative	yellow	circular	entire	pulvinate	smooth
2	Negative	pale yellow	circular	entire	pulvinate	smooth
3	Negative	pink	circular	entire	pulvinate	smooth
4	Negative	white	circular	entire	pulvinate	smooth
5	Negative	cream	circular	entire	pulvinate	smooth
6	Positive	cream	circular	entire	pulvinate	rough
7	Negative	yellow	circular	entire	flat	rough
8	Negative	cream	circular	entire	flat	smooth, glistening
9	Positive	white	circular	entire	pulvinate	dry, powdery
10	Negative	yellow	rhizoid	lobate	flat	smooth
11	Negative	white	irregular	curled	flat	smooth, glistening
12	Positive	cream	circular	undulate	flat	dry, powdery
13	Negative	cream	rhizoid	lobate	flat	smooth
14	Positive	cream	rhizoid	undulate	raised	rough
15	Negative	cream	rhizoid	lobate	convex	smooth, glistening
16	Negative	white	punctiform	curled	flat	smooth
17	Negative	reddish	circular	entire	flat	smooth
18	Negative	reddish	circular	entire	umbonate	smooth, glistening
19	Negative	white	irregular	undulate	umbonate	wrinkled
20	Negative	yellow	irregular	undulate	umbonate	wrinkled
21	Negative	Yellow, red top	circular	entire	umbonate	smooth, glistening
22	Negative	cream	irregular	undulate	flat	wrinkled

4.1.7.2. Belmont Regional Park Sub-Sites Soil Samples' Bacterial Morphology

HMR isolates (n=300 for each sub-site soil sample) from BRP sub-sites soil samples were categorised into 22 different groups based on their morphological characteristics and Gram stain reaction (Figures 4.33 and 4.34). The 22 morphological groups are described in Table 4.3.

The highest proportion of bacterial isolates belonged to group 1 (Table 4.3), and colony morphology groups of 2 and 3 were the next most common (Figure 4.33).

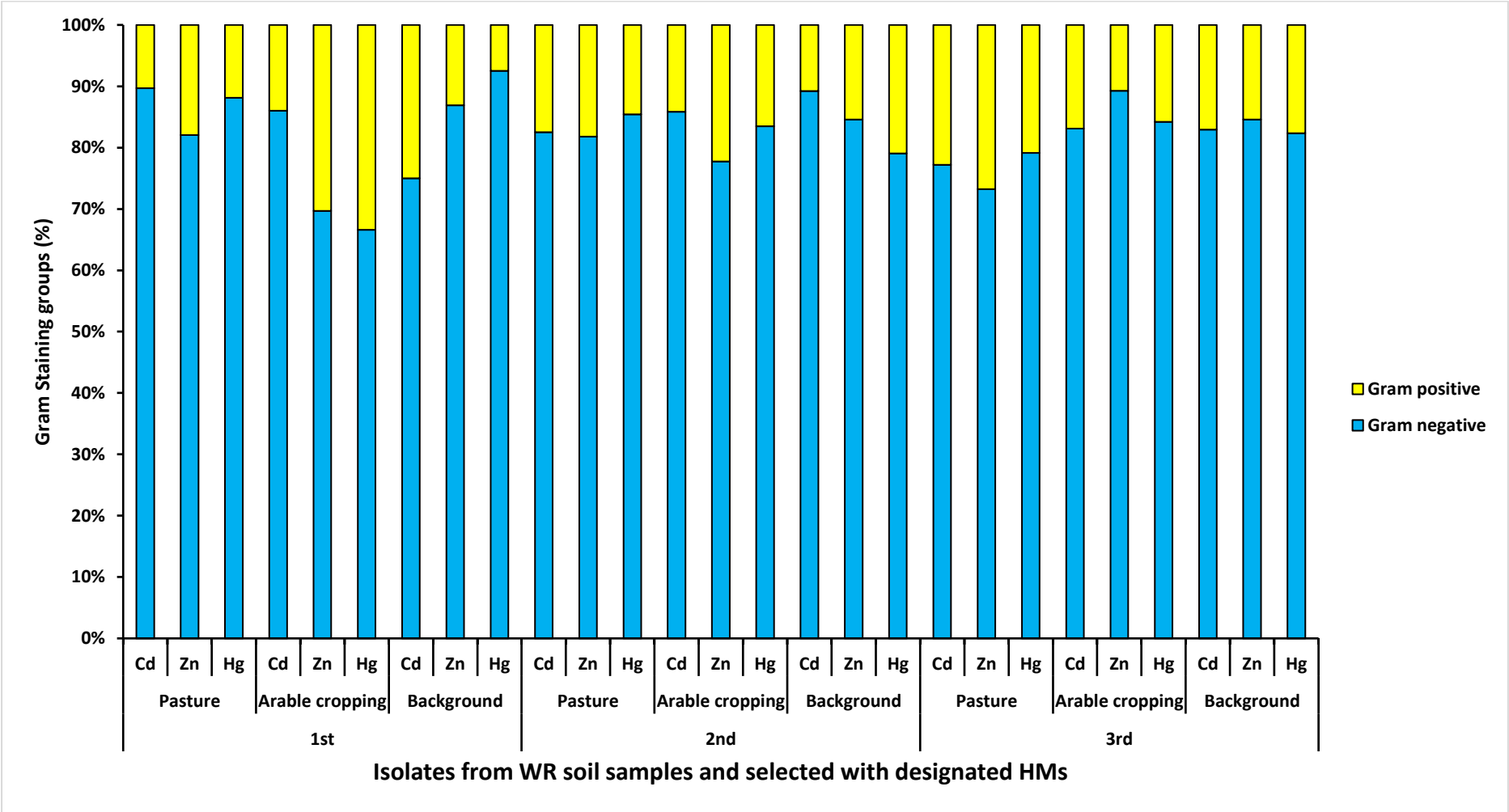


Fig. 4. 31. Proportion of WR soil sample sets' bacterial isolates stratified by Gram stain reaction.

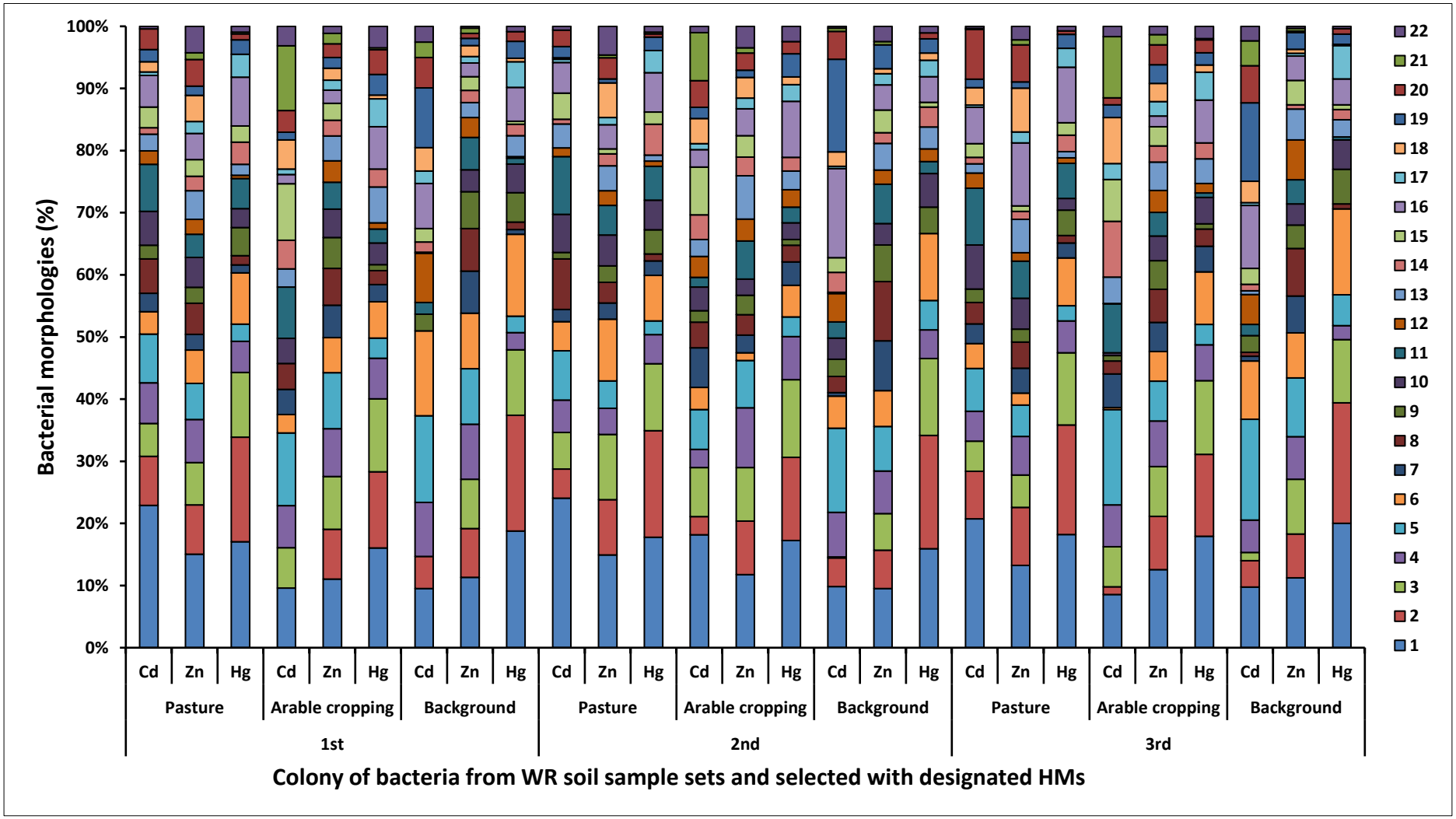


Fig. 4. 32. Percentage of WR soil sample sets' colony morphology groups selected on R2A agar with designated HMs.

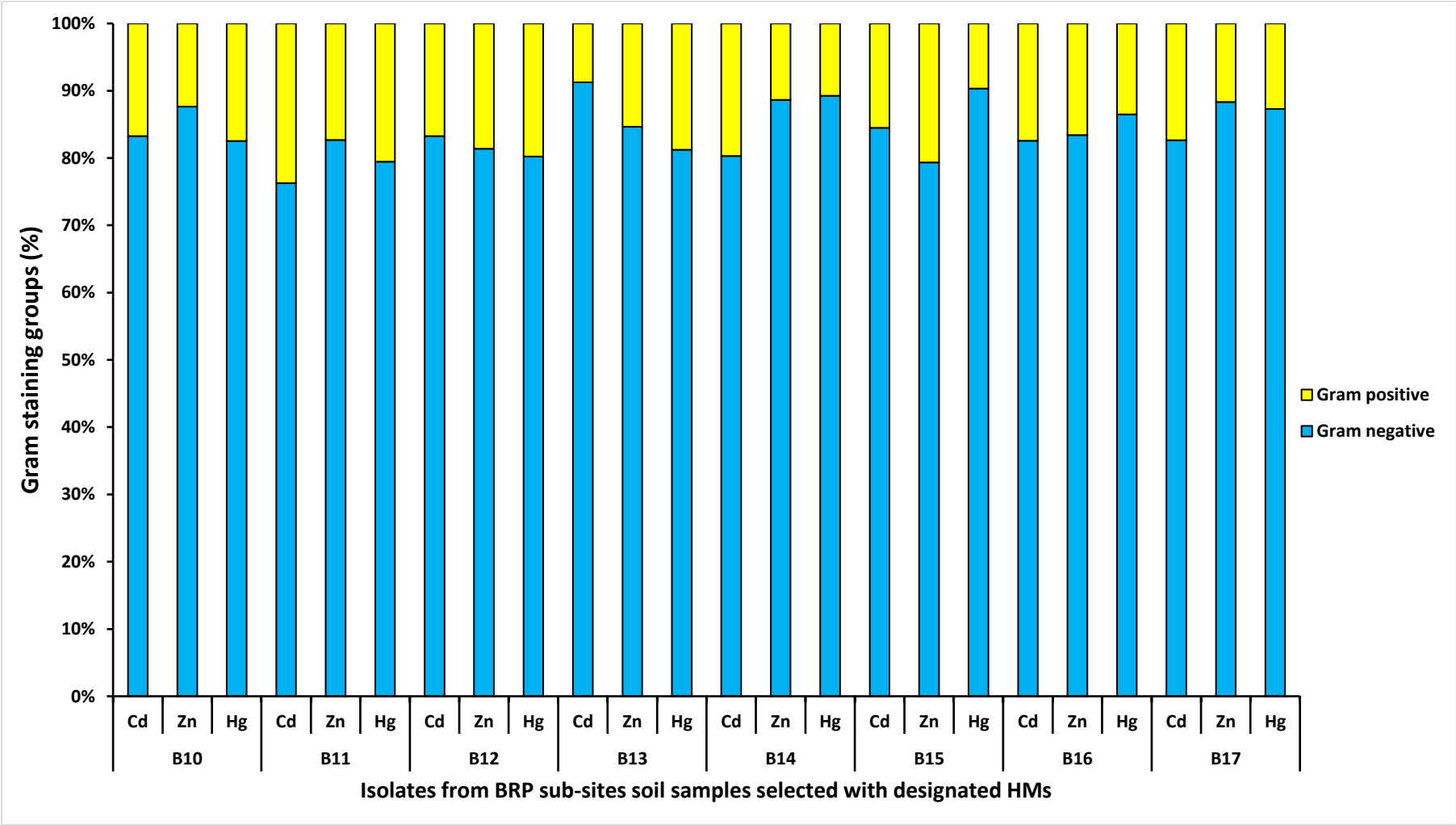


Fig. 4. 33. Proportion of BRP sub-sites soil samples' bacterial isolates stratified by Gram stain reaction.

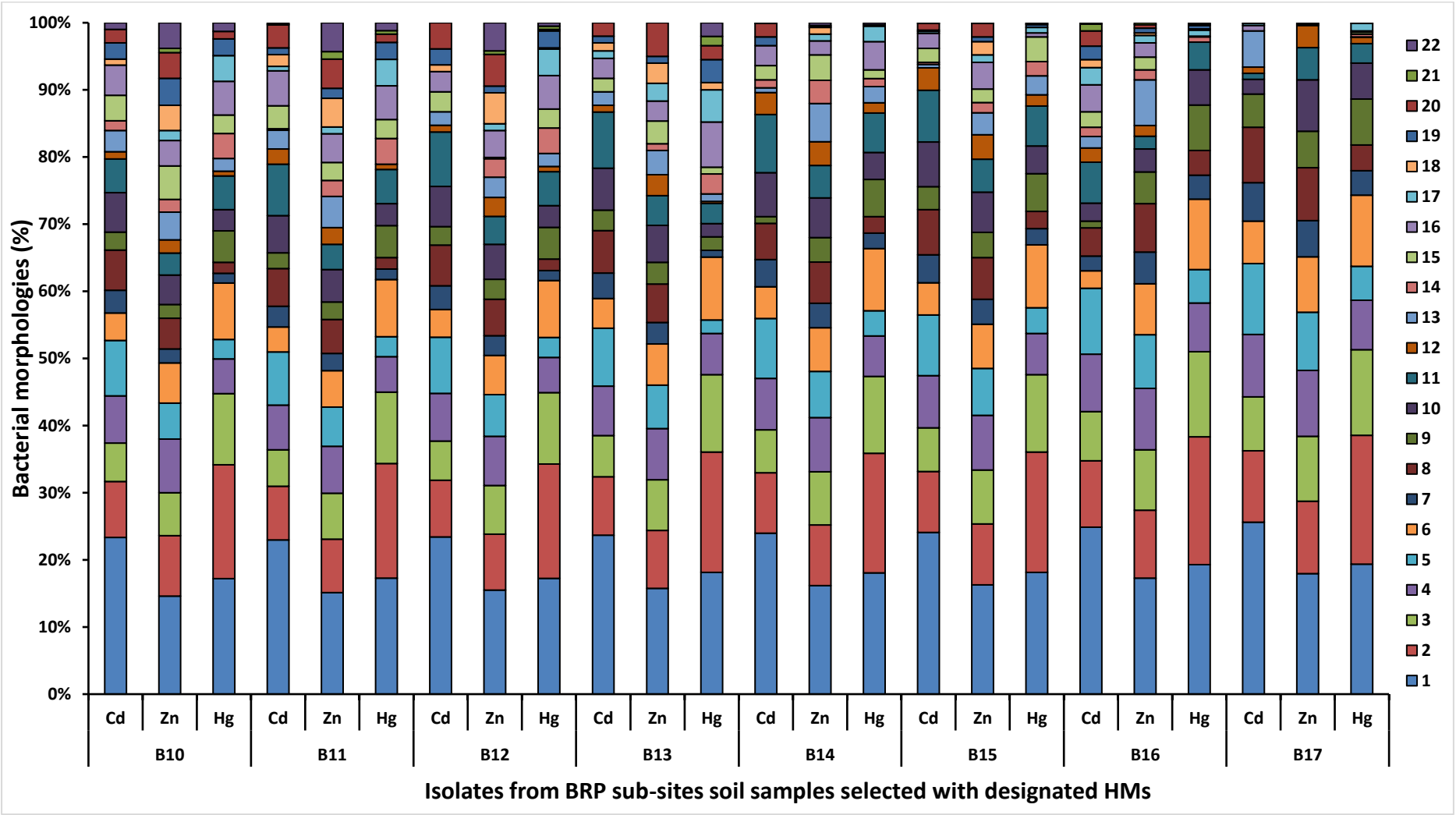


Fig. 4. 34. Percentage of BRP soil samples colony morphology groups selected on R2A agar with designated HMs.

4.1.8. Discussion

The physicochemical properties of the soil samples collected from WR pastoral and arable cropping farms and the background site are listed in the Table 4.1. For soil types and major variables, the three sites are more similar than different, because they were specifically selected as nearby sites with the same soil type, but different land uses. Levels of organic matter, total P and trace elements reflect the histories of land management practices that have occurred on the three properties since land clearance. One property stayed as an undisturbed 'background' site (still covered with native bush), one has been used as a pastoral farm, and the third has been used as an arable (cropping) farm.

The concentration of heavy metals including Cd and Zn increased from native bush soils to pastoral soils. For pastoral soils the two main sources are superphosphate fertilisers which contain elevated P, S, Ca, Cd, F and U; and facial eczema remedies for curing sporodesmin toxicity, which contain high Zn content [251, 252]. In arable soils the main sources of Cd and Zn are superphosphate fertilisers (for Cd, as for pastoral soils), and Zn-containing thiocarbamate fungicide sprays. Use of superphosphate is usually lower on arable compared to pastoral soils, and the elemental analysis of these samples reflect this typical situation.

The Belmont Regional Park (BRP) site was different. Samples from this site (all the same soil type) were collected from near a fertiliser storage bin and along a farm airstrip, used for aerial topdressing. Loading and take-off operations involve spillage, and the farm airstrip site reflects this history.

At the BRP site there is a strong concentration gradient in superphosphate-related contaminants moving from subsites nearest the loading area (B17 and B16; maximum levels) and down the runway (decreasing levels). However, even at a distance from the loading area Cd and Zn levels are substantially higher than levels on broad-acre farmland sites including the WR sub-samples

[262]. The levels of total carbon (%) from B17 to B10 sub-sites are slightly reducing, however, the levels of Cd and Zn in B17 and B16 soils are dramatically higher than other sub-sites [251].

In addition to total concentrations there are various factors that may influence bioavailability of the contaminants to various organisms. Some of these are general soil factors. In this work the WR soil types were matched, and so the main differences would come down to soil factors that have changed with different land management regimes. These include soil fertility and organic matter, of which the microbial content itself is a part. Soil fertility is linked to the fertilisation history, additional nitrogen inputs from clover fixation and animal effluent, and physical factors such as soil aeration or compaction. The levels of P (phosphorus) (as a factor which may increase the bioavailability of Cd and Zn in soil thorough an increase in soil fertility) in pasture soils (sites no. 69 and 135) is higher compared to arable soil (sites no. 85 and 86) [262]. The levels of organic material in Waikato region pastoral soils are higher than the arable soils, reflecting organic matter depletion in the latter [251]. Impacts on availability are harder to determine for several reasons. Some soil-related factors could work in opposition to each other. Cd and Zn bind to organic matter, usually reducing their bioavailability. Pastoral soils have more organic matter, but also have higher total Cd and Zn. Higher levels of Cd and Zn in pastoral soil could compensate for the higher levels of organic material [251].

However, more importantly, bioavailability is partly determined by interactions between the living organism and its soil environment. Bioavailability can also be defined in different ways. The bioavailability measured as the relative proportion taken up within an organism can be significantly different from the bioavailability determined by a weak chemical extraction.

Overall, no specific assumptions could be made about the potential influence of bioavailability at the outset in this work. However, results consistently suggest that effects on microbial populations follow the order pastoral soils > arable soils > background soils. This order is the same as for the total contaminant concentrations in these soils. This suggests that any

differences in actual bioavailability of contaminants between the three different land uses to their microbial populations were not significant enough to noticeably influence the results.

Plate culturing of bacteria isolated from WR soils, showed the bacterial CFUs in pastoral soils were significantly higher than those from arable cropping and also higher than native bush soils. Theoretically, the higher levels of organic matter, nitrogen and phosphorus levels in pastoral soil may allow a higher bacterial population density. Wortmann et. al., 2008 [263], reported the stability of soil bacteria populations with short term changes of soil physicochemical properties. According to Acosta-Martinez et. al., 2010 [264], we can interpret that, based on the long term history of the land usage for the Waikato region pastoral, arable and native bush sites, bacterial populations have adapted to the different levels of carbon, nitrogen, phosphorus and other contaminants in these soils.

Plate culturing for this project showed there were no significant differences between bacterial numbers in WRSS1 compared to the other two sets, except between the arable soils from WRSS1 and WRSS2 (sites no. 85 and 86). This result could be viewed as an outlier. It was only evident in one pair and at one concentration, and the values of the respective ratios differed by only 8% (less than difference seen for different soil types). However, it may also reflect a seasonal difference in the two microbial populations, as a similar result was found for Hg. These three soil sample sets were collected during different seasons at different temperatures and mean water content, although the acidity (pH) was not significantly different in these soils. The mean temperature and rainfall during February, the month of collection of Waikato 1st set were 18.8˚C and 68.7 mm. Corresponding values were 9.8˚C and 103.4 mm in August (2nd set), and 9.5˚C and 113.2 mm in June (3rd set). It appears that changes in soil bacteria numbers are resistant to periodic changes soil moisture and temperature [265, 266]. Hermans et.al. (2017) [267], and Fierer and Jackson (2005) [268], reported that variation in soil environment has a more substantial effect on soil bacterial communities than climate changes. It also has been suggested that soil moisture content and pH are likely the main factors affecting bacterial community

structures [269], along with the different levels of metal contaminations in soils as one of the main factors affecting bacterial communities diversity [270].

To reflect the higher levels of Cd and Zn and other factors affecting the bioavailability of these HMs in pastoral soils compared to arable soils, plate culturing on media with a range of Cd, Zn and Hg concentrations and control plates (with no added HMs) was performed. There were higher ratios of HMR:total CFU from pastoral soils compared to arable and background soils. This observation suggests that while the long term usage of Cd and Zn-contained compounds increase levels of heavy metals in soil, bacterial resistance or tolerance to specific metals occurs. It has been suggested that bacterial resistance or tolerance to Cd would result in higher levels of resistance to Zn and Hg too, due to pleiotropic mutations and/or resistance genes encoding proteins involved in causing resistance to multiple metals (e.g. *czc* gene encoding cellular efflux pumps for Cd, Zn and Co) [23, 271-275]. The data indicates there were more HgR bacteria in pastoral soils compared to those from arable and background soil, although there is no significant differences in the levels of Hg in pastoral soils compared to background soil; and Hg levels, in fact, were found to be highest in arable soils. According to the review of Harrison et. al., 2007 [16] about the effects of multidrug resistance among bacterial communities, it can be suggested that the main factor for the higher level of bacterial resistance to Hg –based on plate colony counts– for pastoral soils' bacterial isolates compared to arable, is the higher levels of Cd and Zn in these soils compared to arable soil. A repeat of the pattern of the relative resistance following the order pastoral > arable > background is interesting, because in the case of Hg, levels in all soil samples were within their normal background ranges (Table A2.4, Appendix 2). there is no specific evidence for significant enrichment of Hg in farmed soils [276]. This may indicate that resistance to Hg seen in these samples has been co-selected through exposure to other contaminants. In addition to Cd (phosphate fertiliser) and Zn (facial eczema remedies) other enriched elements in farmed soils include the nutrient elements N, P and S, the major Ca, and the trace of elements F, U, La, Ce, Y, Tl, Mn, Mo and Ag [251].

Plate culturing of bacteria isolated from WR soil samples showed higher levels of bacterial AbR in pastoral soils compared to arable and background soils. According to the higher levels of Cd and Zn in pastoral soils compared to the two other soils, it can be interpreted that the higher levels of resistance to these HMs can induce the co-selection of resistance to these Abs. This observation is similar to the observations reviewed by Baker-Austin et. al., 2006, and the findings reported by Li et. al., 2017, Zhou et. al., 2016, and Zhao et. al., 2019 [36, 253, 277, 278], although Hau et. al., 2017 [279], suggested that multilocus sequence type lineage is more likely to be the reason for methicillin resistance in *Staphylococcus aureus* (LA-MRSA) ST5 than Zn co-selection in livestock farms. They suggest that likely there is a direct relationship between the multilocus genetic lineage and transfer of HMs resistance determinants.

Plate culturing for bacteria isolated from BRP soil samples was performed and the result showed the number of bacterial CFU was more in soils with the lower levels of accumulated Cd and Zn compared to the sub-sites with higher levels of HMs (Figure 4.10). High levels of HMs accumulation in B17 soils can result in higher HMR bacterial population as well as bacterial communities diversity, especially in soils with HMs levels much higher than bacteria's threshold of tolerance to HMs [269, 270]. This is more likely to occur in soils with huge levels of non-essential HMs with no biological functions, e.g. Cd and Hg; although it has been reported that Cd is categorised as an essential metal for a few bacterial species [10, 280].

Plate counts from BRP soils revealed that the proportions of HMR and AbR bacteria amongst total CFU from the sub-sites with higher levels of Cd and Zn were greater than at the sub-sites with lower levels of HMs (Figures 4.11-4.18). In addition to the higher levels of Cd and Zn in soils closer to the fertiliser storage at BRP (B17 downward), the higher level of P (phosphorus) maybe a factor increasing the bioavailability of Cd and Zn [251]. However, no major differences in the levels of organic materials (as a factor reducing the bioavailability of Cd and Zn in soil) were determined through the BRP sub-sites soil [262]. The higher levels of extractable and bioavailable Cd and Zn can be lead to higher levels of bacterial resistance to these HMs, as well

as other HMs, e.g. Hg, and a range of antibiotics owing to the various resistance mechanisms, due to the same cellular efflux pumps and presence of resistance genes on the same genetic elements [16, 36, 253, 277, 278].

The PICT assays of bacterial communities from WR and BRP soils revealed there were greater MIC and EC50 values for HMs and Abs in WR pastoral soils consortia compared to those from background soil. Similarly higher MIC and EC50 values for BRP soils with higher HMs compared to B10 soils (with the lower concentrations of HMs). According to the determined higher levels HMs in pastoral soils and previous reports [281-283], it is interpreted that higher levels of HMs in soils can lead to higher levels of HM resistance in bacterial isolates. This co-resistance for Ab can occur in bacterial isolates with different levels of Hm resistance [284]. The determined Ab MIC (recommended by EUCAST ECOFF [218]) for the bacteria isolated from WR pastoral and arable soils was higher than those determined for background soil, these MIC values where higher than 20 μg mL^{-1} threshold for all of Abs MIC values for pastoral soil bacteria and for Ery, Cb and Amp for arable and background soils. The higher MIC and EC50 values determined for pastoral and arable soils compared to background soils reflected the effects of higher levels of HMs in these soils and the subsequent induced Ab resistance in the bacterial isolates present in these soils [285]. The higher levels of Cd and Zn in WR pastoral soils and their higher bioavailability due to higher levels of P in pastoral soils may be the main factors involved in co-selection of Abs resistance in presence of the HMs resistance and higher HMs and Abs MIC and EC50 values [255, 283]. There were higher Abs MIC and EC50 values determined for bacteria isolated from BRP soil samples with higher levels of HMs (B17) compared to those with lower levels of HMs (B10), however, all of the Abs MIC were higher than 20 μg mL^{-1} threshold for soil bacteria resistance. This finding supports the observation of higher bacterial Ab resistance associated with higher levels of resistance to HMs [283, 286]. In addition to the higher levels of the HMs at sub-sites B17 and B16, the higher levels phosphorous in these soils led to more

bioavailability of the HMs involved in bacterial resistance development and increases the potential of subsequent Abs MIC and EC50 [255].

HMs' MIC and EC50 values determined by the BM method for HMR isolates from WR soils showed there were significant differences between those for bacteria from pastoral and arable cropping soils compared to those for bacteria from background soils. Isolates from pastoral and arable soils, which contain higher levels of HMs, were more resistant to the HMs with higher MIC and EC50 values [282, 287, 288]. The levels of Ab resistance in HMR isolates is likely higher due to the potential Ab co-selection. This may occur because of various mechanisms, e.g. co-location of resistance gene [254, 282, 287]. Henriques et. al., 2016 [283], suggest that the levels of Abs resistance occurrence in bacteria are significantly related to the levels of HMs in environment. The higher levels of Cd and Zn in pastoral soils and their higher bioavailability due to higher levels of P in pastoral soils may explain the subsequent co-selection of Abs resistance in presence of the HMs resistance [255, 283].

Greater MIC and EC50 values for bacteria from WR's pastoral and arable soils compared to those for bacteria from background soil were determined. The higher levels of HM resistance in bacteria from pastoral and arable soils (with higher initial levels of HMs) can lead to higher levels of Ab resistance in bacteria [283, 287]. According to D'costa et. al. (2007) and Bhullar et.al. (2012) [220, 221], the percentage of bacteria categorised as Resistant amongst HMR isolates were significantly higher than the HM sensitive bacteria, which this observation supports the interpretations for HM and Ab co-resistance phenomenon.

BM assay with bacterial isolates from BRP soils showed there were significantly higher MIC and EC50 values for both HM and Ab for isolates from the sub-sites with *in situ* higher levels of HMs (B17 and B16) compared to the sub-sites with lower concentrations of HMs. In addition, according to bacterial resistance definition as growth at 20 μg mL^{-1} [220, 221], there were significantly higher proportion (%) of HMR and AbR bacteria in B17 and B16 soils compared to those from soils with lower HMs. As with WR soils bacteria, higher levels of HMs in soils and

subsequent higher bacterial HM resistance may lead to higher levels of Ab resistance in bacteria according to the previous surveys [255, 283, 286, 287] (Figures 4.32-4.36; and Figures A1.35-A1.48, Section A1.5, Appendix 1). The MIC and EC50 values of Abs were higher in the bacteria from the sub-sites with the higher levels of Cd and Zn accumulations. The higher levels of the HMs at B17 and B16 sub-sites, in addition to the higher levels phosphorous in these soils resulting in more bioavailability of the HMs, explains bacterial resistance development and subsequent Abs resistance [255, 283].

The bacteria isolated from WR and BRP soils which showed initial resistance to HMs, were examined to determine their Gram staining reactions and colony morphologies. Based on these examinations bacteria collected from these soils were categorised into 22 different groups (Table 4.32). Of these 18 out of 22 groups of bacteria were determined as Gram-negative. According to the previous surveys [289, 290], the outer membrane in Gram-negative bacteria is actively involved in excluding antimicrobial agents from penetrating the cells. The interpretation of this observation is supported by the unique feature of Gram-negative bacteria. There were significantly fewer morphological groups for bacteria isolated from BRP B17 and B16 soils compared to those from B10 soil. Higher levels of HMs in these soils could have been the main reason for the lower bacterial diversity detected in these soils. Others have reported fewer groups of bacteria can tolerate these high levels of HMs in soil [291].

In conclusion, the results of the present study showed that the abundance of the bacterial isolates resistant to different levels of HM and Abs is greater in soils with high levels of HMs, compared to the abundance of those bacteria from soil samples with lower levels of HMs. In addition, the isolates from soil samples with high levels of HMs show greater resistance to various concentrations of HMs and Abs compared to those from soil samples with lower levels of HMs.

Three sets of soil samples from Waikato region were used for the current project and each set included three soil samples of pastoral, arable cropping and native bush as background. The results from these three soil sets were very similar and showed very similar trends. Considering that the levels of Cd, for example, in these three soil sets were in the average range of those shown in the map in Figure 4.1 [257]. The results of the investigations on Belmont Regional Park B10 and B11 sub-sites (the sub-sites with the most distance from the fertiliser storage at the start of airstrip) are clearly comparable with the results of pastoral soils from Waikato region with similar trends. It is concluded that the results obtained from the Waikato region and Belmont Regional Park soil samples are transferrable to other farms and soil samples around New Zealand, however, more investigations on more soil samples from wider regions of New Zealand is required to confirm this.

Chapter 5, Results Part 2

5.1. Study 2: 6-weeks and 6-months Microcosms Bacterial Resistance Investigations

5.1.1. Introduction

Many HMs have a fundamental role in the metabolism of microorganisms and act as essential elements in the environment. HMs are added to the environment by a variety of different human-related activities (such as industrial or sewage waste streams) or natural processes (such as volcanic depositions). Accumulation of these HMs in soil can lead to bacterial resistance to HMs and also co-selection for antibiotic resistance [8, 9]. HMs are generally immutable and nondegradable in soil [292]. Soil microbiological factors, e.g. bacterial community structure, are also affected by HMs [16]. Soil microcosms are novel tools to explore, on a small and controlled scale, the factors involved in inducing resistance to HMs in soil bacteria in a small, controlled scale [293].

Bacterial community dynamics were investigated in microcosms, containing soil sources from sites EW-13 native bush (Background) and EW-24 pasture to which various concentrations of Cd, Zn and Hg were added and incubated for either 6-week or 6-month. These two time periods were used to investigate the bacterial community structures changes over a shorter and longer period of time. The objective of this chapter was to investigate whether specific concentration(s) of HMs would induce resistance in bacterial isolates while present in the bacterial biosphere.

5.1.2. Methodology

The methodology used for this chapter is described in Sections 3.2-3.9 and 3.11-3.12 of the Methodology Chapter. In short, preparation of microcosms, plate culturing, analysis of microcosms leachate HMs concentration, BM (Broth Microdilution) and PICT (Pollution Induced Community Tolerance) analyses were applied to investigate various HM concentrations on soil microbial resistance and bacterial communities. In this part of the investigation of bacterial consortia and dynamics in soil in the presence of various levels of HMs, two types of soils with

different initial levels of Cd and Zn were used. The objective of using two different soils was to investigate if there were any differences between the levels of induced HM and Ab resistance in bacteria from microcosms containing pastoral and background soils. Incubation for 6 months was performed to investigate changes in the bacterial consortia and dynamics compared to 6 weeks incubation. The HMs concentrations added to the microcosms were 5, 10, 50, 100 and 200 mM (1.182, 2.236, 11.82, 22.36 and 44.72 mg kg^{-1}) of Cd, 20, 50, 100, 200 and 300 mM (3.78, 9.46, 18.93, 37.87 and 56.79 mg kg^{-1}) of Zn and 0.5, 1, 5, 10 and 50 mM (0.1, 0.2, 1.0, 2.0, 10.02 mg kg^{-1}) of Hg. Figure 5.1 illustrates different experiments and steps for this chapter.

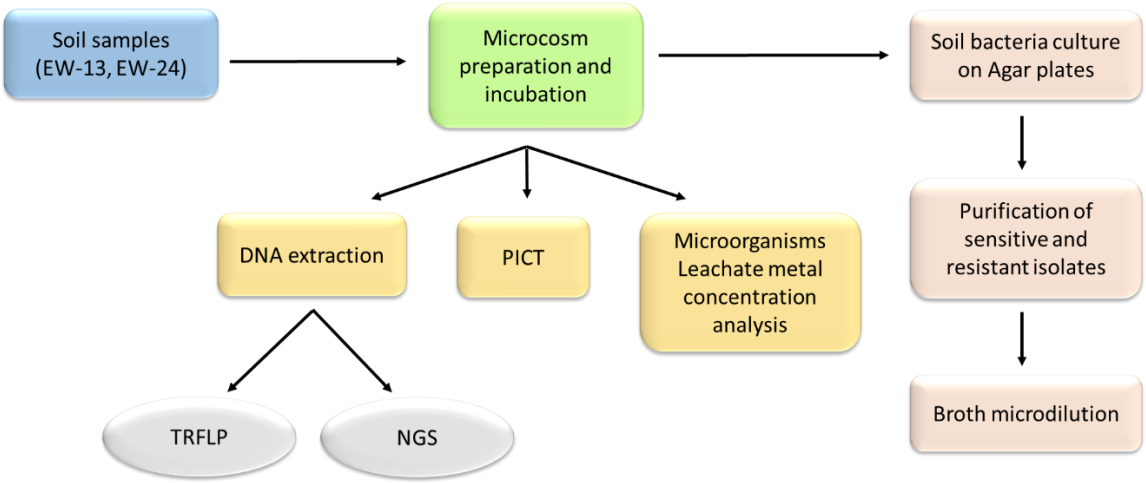


Fig. 5. 1. Flow chart illustrating different steps and experiments of Chapter 5.

5.1.3. Physicochemical Properties of the Soil Sampling Sites.

Soil samples collected from a site near Taupo with a long history of plantation forestry and a sandy loam soil structure (Site EW-13) was used for the 6-weeks and some 6-months microcosms. Pastoral soil from Coromandel (West of Whangapoua) from a property used for dry-stock farming with clay loam soil (M. Taylor, Personal Communication) (Site EW-24) was used for the remaining 6-months microcosms. Chemical analysis of representative samples indicated the concentration of the three HMs of interest in the soil used as starting material for the microcosms (Tables 5.1; and Table A3.1, Appendix 3).

Table 5. 1. EW-13 and EW-24 sites soil samples physicochemical properties.

Soil property	EW-13	EW-24
Annual rainfall (mm)	1750	1887
pH	5.90	5.40
Total C (%)	5.10	8.20
Total N (%)	0.36	0.63
C:N	14.2	13.0
Olsen P *	40.0	14.0
Cd *	0.13	0.29
Hg *	0.05	0.07
Zn *	19.4	32.0
Fe *	4500	38000
P *	1090	830

*mg kg^{-1} of dry soil

5.1.4. Quantification of Bacterial Resistance in Microcosm Trials

5.1.4.1. Microcosms Incubated for 6-weeks (6WM)

5.1.4.1.1. Plate culture for HMR

Bacteria from samples of microcosm soil sampled at time 0 and 2 weeks intervals were plate cultured on plates with a range of HMs and control plates. Total CFUs were calculated for each soil sample, using Equation 3.2.

At time 0, the ratio of CdR/total number of soil bacteria from the 6-week-incubated Cd-spiked microcosms (6WCdM) increased from the control microcosm to Cd 50 mM-spiked microcosm and then decreased in Cd 100 and 200 mM-spiked microcosms. There were significantly smaller CdR/total bacterial ratios for control microcosms compared to those from Cd-spiked microcosms ($p < 0.05$). The ratios of CdR/total bacteria in Cd-spiked microcosms at Time 0 were significantly less compared to those from the same microcosms after 6 weeks. For instance, this ratio was about 1.5 times greater for bacteria from the Cd 50 mM-spiked microcosms at 6-weeks compared to those at Time 0. Similar trends were determined for the 6-week-incubated Zn-spiked and 6-week-incubated Hg-spiked microcosms (6WZnM and 6WHgM) (Figures 5.2-5.4, Appendix 3).

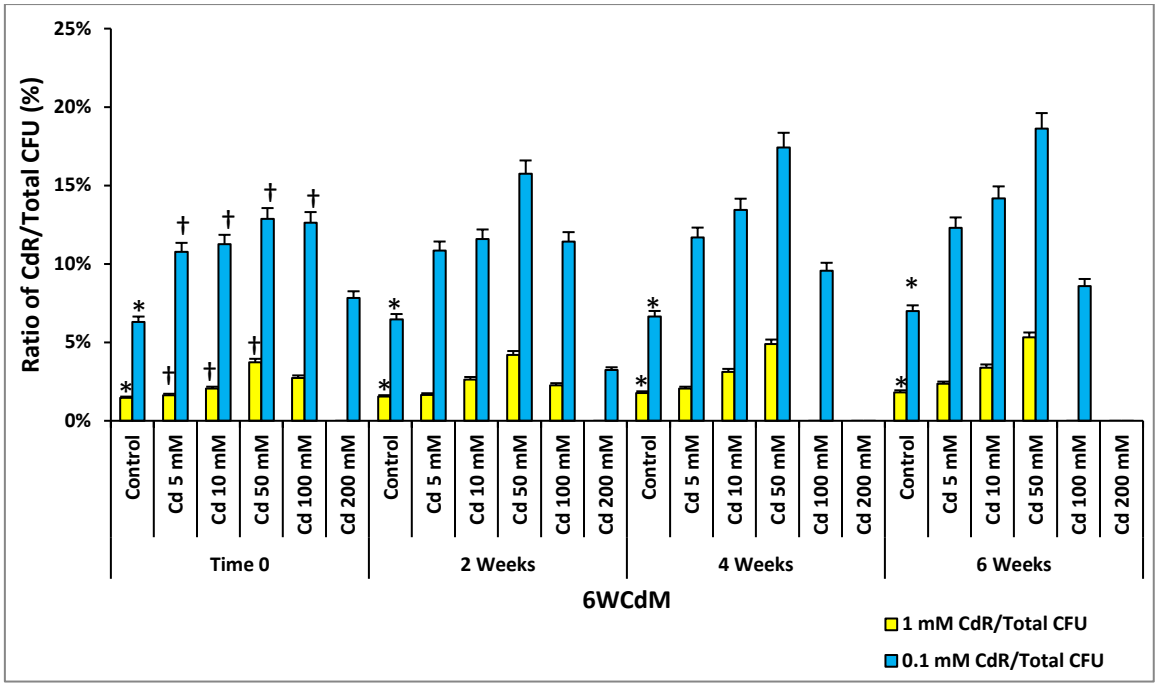


Fig. 5. 2. Mean ratios of CdR/total bacterial CFU, selected on plates with two concentrations of Cd for 6WCdM. *$p<0.05$ compared to the CdR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the CdR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-week interval.

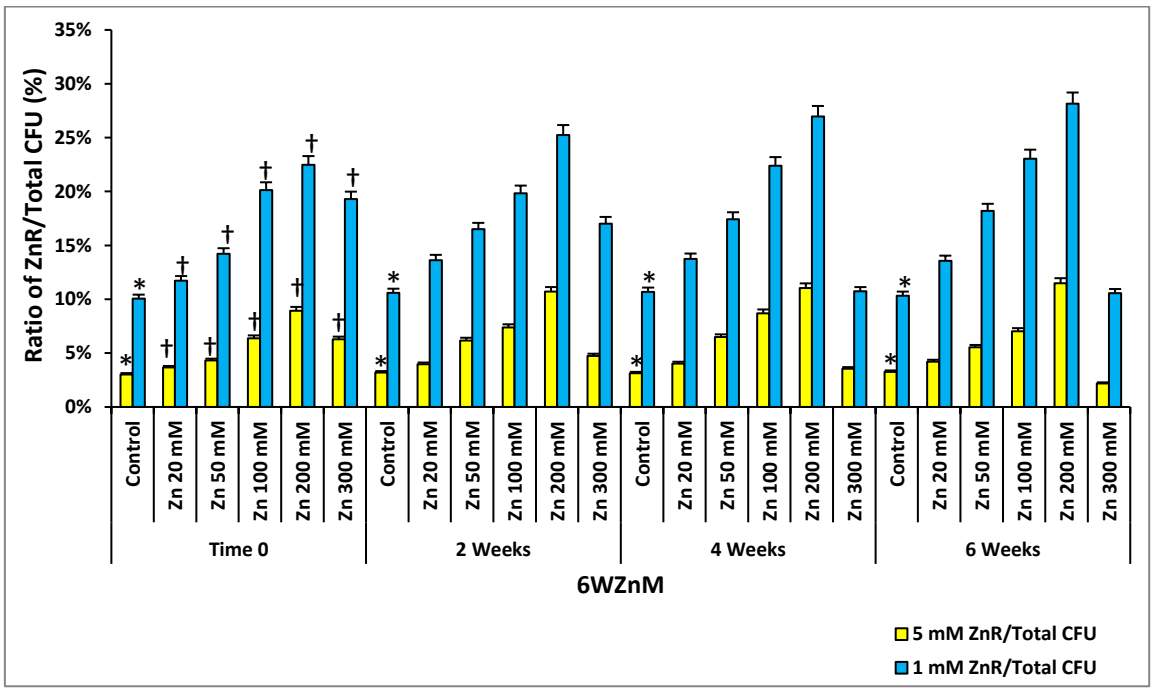


Fig. 5. 3. Mean ratios of ZnR/total bacterial CFU, selected on plates with two concentrations of Zn for 6WZnM. *$p<0.05$ compared to the ZnR/total bacterial CFU ratios in Zn-spiked microcosms; †$p<0.05$ compared to the ZnR/total bacterial CFU ratios in the same Zn-spiked microcosms at the 6-week interval.

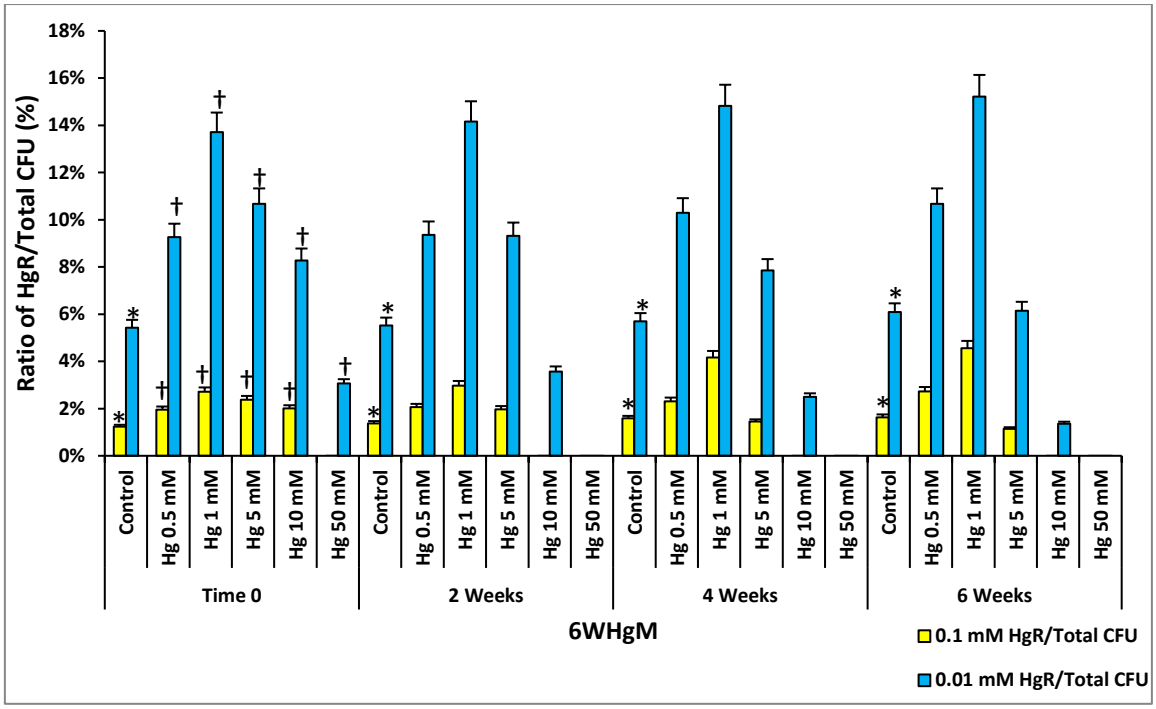


Fig. 5. 4. Mean ratios of HgR/total bacterial CFU, selected on plates with two concentrations of Hg for 6WHgM. *$p<0.05$ compared to the HgR/total bacterial CFU ratios in Hg-spiked microcosms; †$p<0.05$ compared to the HgR/total bacterial CFU ratios in the same Hg-spiked microcosms at the 6-week interval.

5.1.4.1.2. Plate Culture for Abs

The mean ratio of AbR/total number of CFU from the 6WCdM at Time 0 increased from the control microcosms to Cd 50 mM-spiked microcosms and then decreased in the Cd 100 and 200 mM-spiked microcosms (Figure 5.5). There were significantly smaller AbR/total bacterial ratios for control microcosms compared to those from Cd-spiked microcosms ($p < 0.05$). The ratios of AbR/total bacteria in Cd-spiked microcosms at Time 0 were significantly less than those from the same microcosms after 6 weeks. For example, the ratio for TcR bacteria from Cd 50 mM-spiked microcosm after 6 weeks of incubation was two times greater than that from the same microcosm at Time 0 (Figure 5.5). The same trend was observed for the microcosms spiked with a range of Zn and Hg concentrations. The highest ratios for the other Abs (Cm, Ery, Cb and Amp) were recorded from the same HM-spiked microcosms which were Cd 50 mM, Zn 200 mM and Hg 1 mM (Figures A3.1-A3.2, Appendix 2).

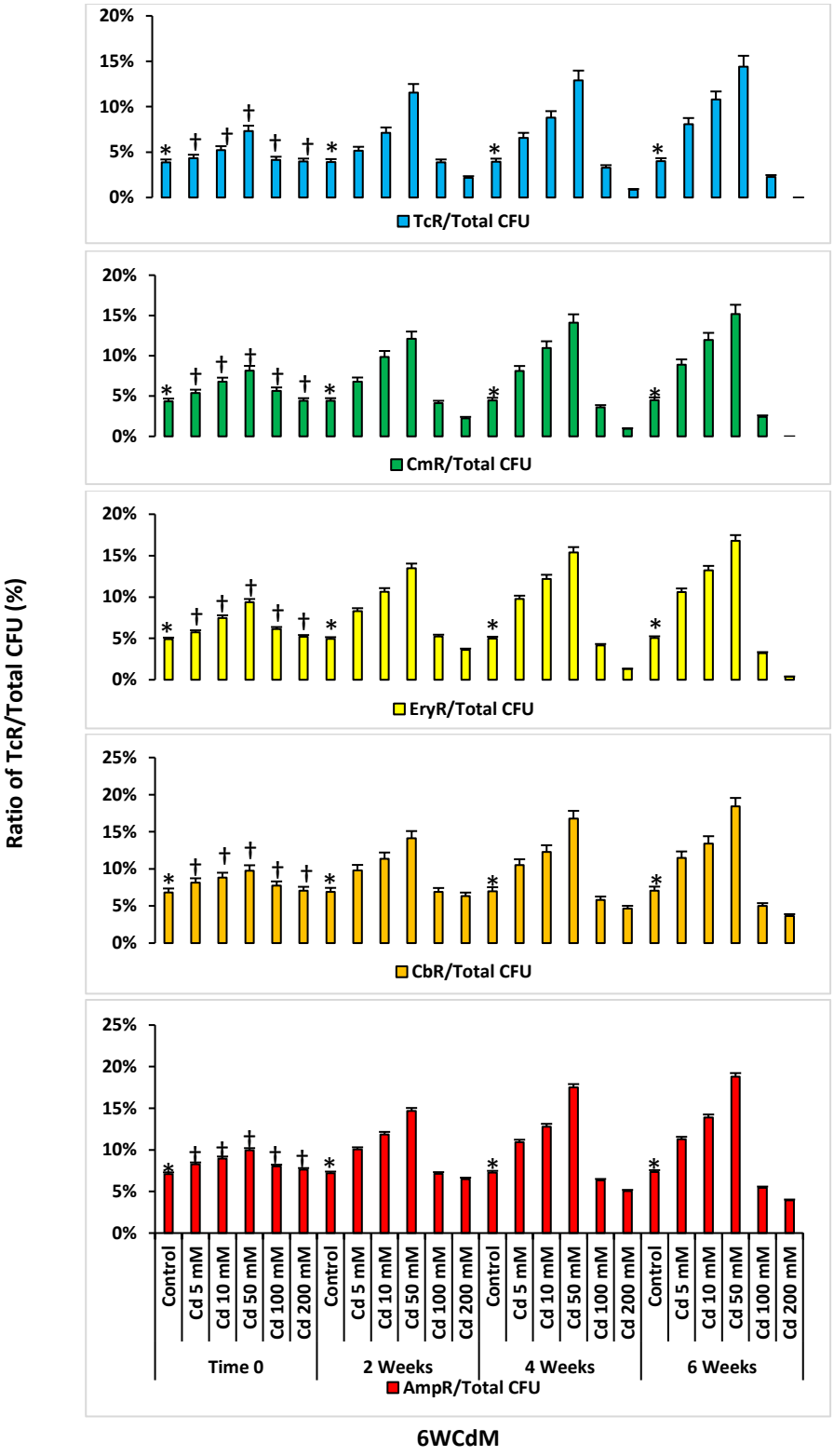


Fig. 5. 5. Mean ratios of AbR/total bacterial CFU, selected on a concentration of Abs for 6WCdM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-week interval.

5.1.4.2. Microcosms containing Background or Pasture Soil Incubated for 6 months (B6MM and P6MM)

5.1.4.2.1. Plates with HMs Additive

Plate culturing of bacteria from B6MM and P6MM from samples taken at Time 0 and 2 months intervals was performed. Samples were cultured on R2A media with a range of HM concentrations. For bacterial CFU calculation Equation 3.2 was used.

The ratios of CdR/total CFUs from both B6MCdM (background soil-contained Cd-spiked 6 months incubated microcosms) and P6MCdM (pastoral soil-contained Cd-spiked 6 months incubated microcosms) at Time 0 increased from the control microcosms (0 mM Cd) to the Cd 50 mM-spiked microcosms and then decreased for the Cd 100 and 200 mM-spiked microcosms (Figures 5.6-5.11). The CdR/total bacterial CFUs ratios from the control microcosms were smaller compared to those from Cd-spiked microcosms ($p < 0.05$). The ratios of CdR/total bacterial CFUs in Cd-spiked microcosms at Time 0 were significantly less compared to those from the same microcosms after 6 months ($p < 0.05$). For example, this ratio was ˜1.5 times greater for Cd 50 mM-spiked B6MM after 6 months compared to Time 0. The ratio of CdR/total CFUs was ˜1.2 times greater for Cd 50 mM-spiked P6MM after 6 month compared to Time 0. A trend of increasing CdR/total CFU ratios was determined in the background soil-contained microcosms at the 2 and 4 month time points, although they were not significantly different to the ratio at 6 months. The trend of increase of CdR/total CFU ratios plateaued in the 2-month to 6-month interval. Similar trends were determined for the microcosms spiked with a range of Zn and Hg concentrations (Figures 5.6-5.11).

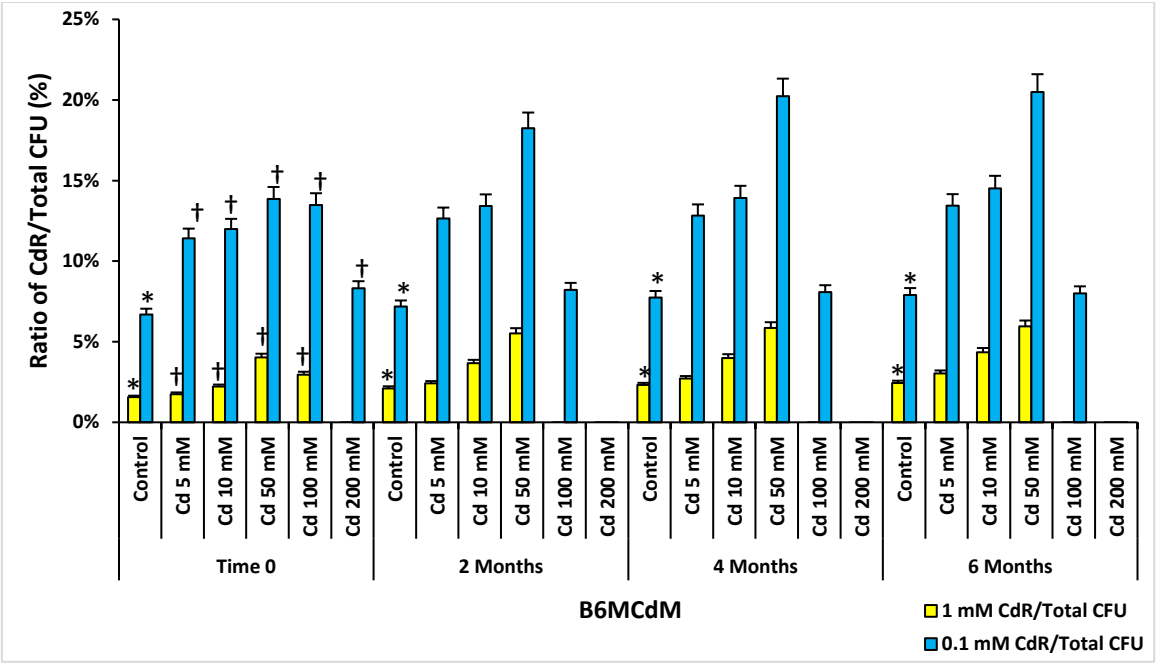


Fig. 5. 6. Mean ratios of CdR/total bacterial CFU, selected on plates with two concentrations of Cd for B6MCdM. *$p<0.05$ compared to the CdR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the CdR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-month interval.

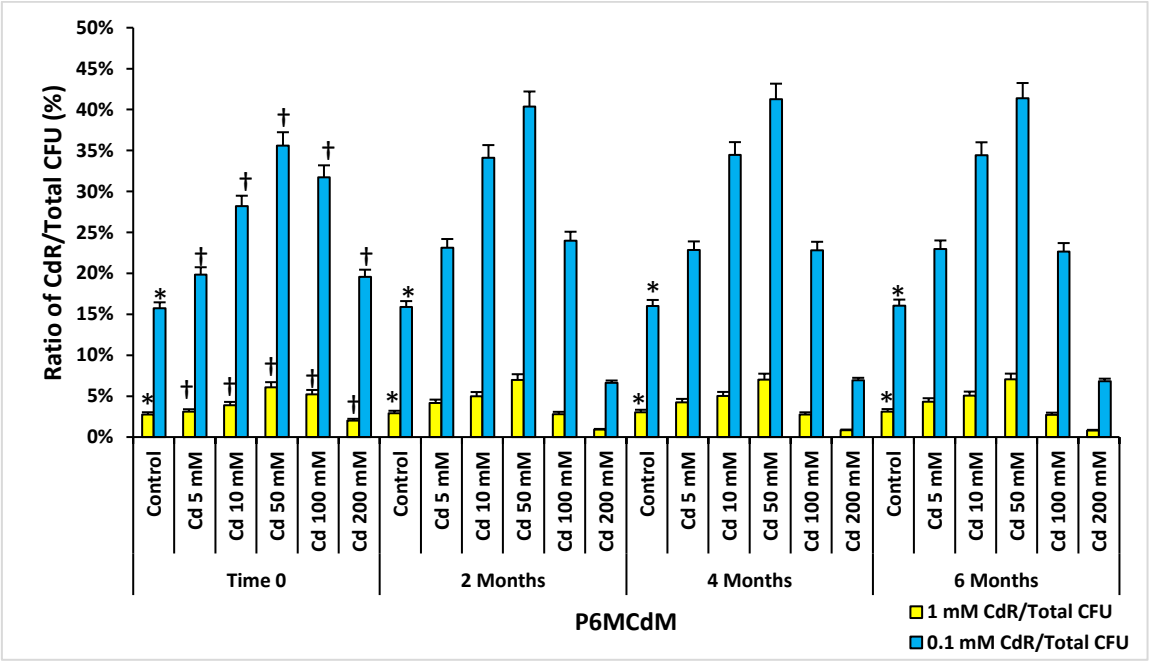


Fig. 5. 7. Mean ratios of CdR/total bacterial CFU, selected on plates with two concentrations of Cd for P6MCdM. *$p<0.05$ compared to the CdR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the CdR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-month interval.

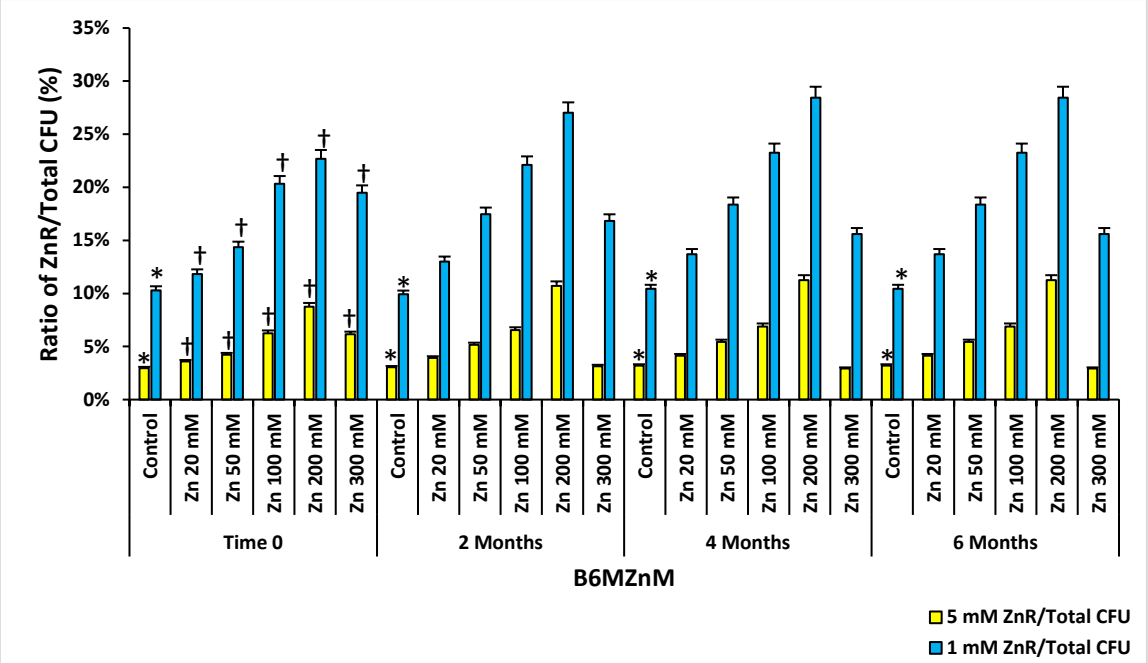


Fig. 5. 8. Mean ratios of ZnR/total bacterial CFU, selected on plates with two concentrations of Zn for B6MZnM. *$p<0.05$ compared to the ZnR/total bacterial CFU ratios in Zn-spiked microcosms; †$p<0.05$ compared to the ZnR/total bacterial CFU ratios in the same Zn-spiked microcosms at the 6-month interval.

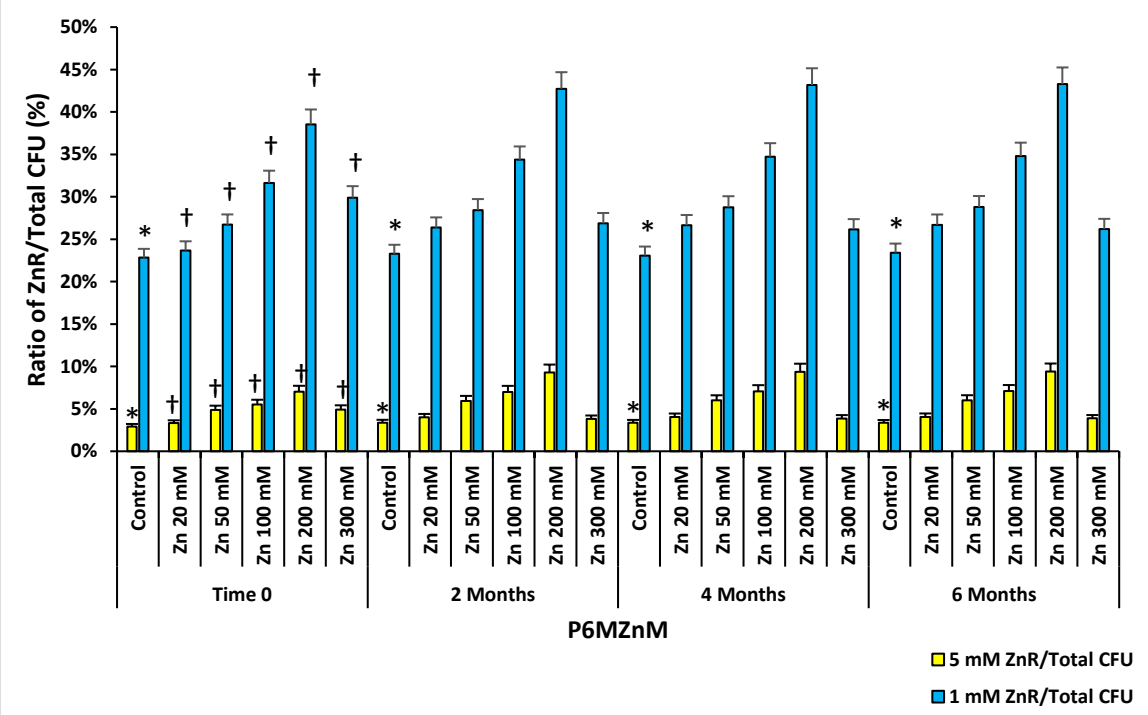


Fig. 5. 9. Mean ratios of ZnR/total bacterial CFU, selected on plates with two concentrations of Zn for P6MZnM. *$p<0.05$ compared to the ZnR/total bacterial CFU ratios in Zn-spiked microcosms; †$p<0.05$ compared to the ZnR/total bacterial CFU ratios in the same Zn-spiked microcosms at the 6-month interval.

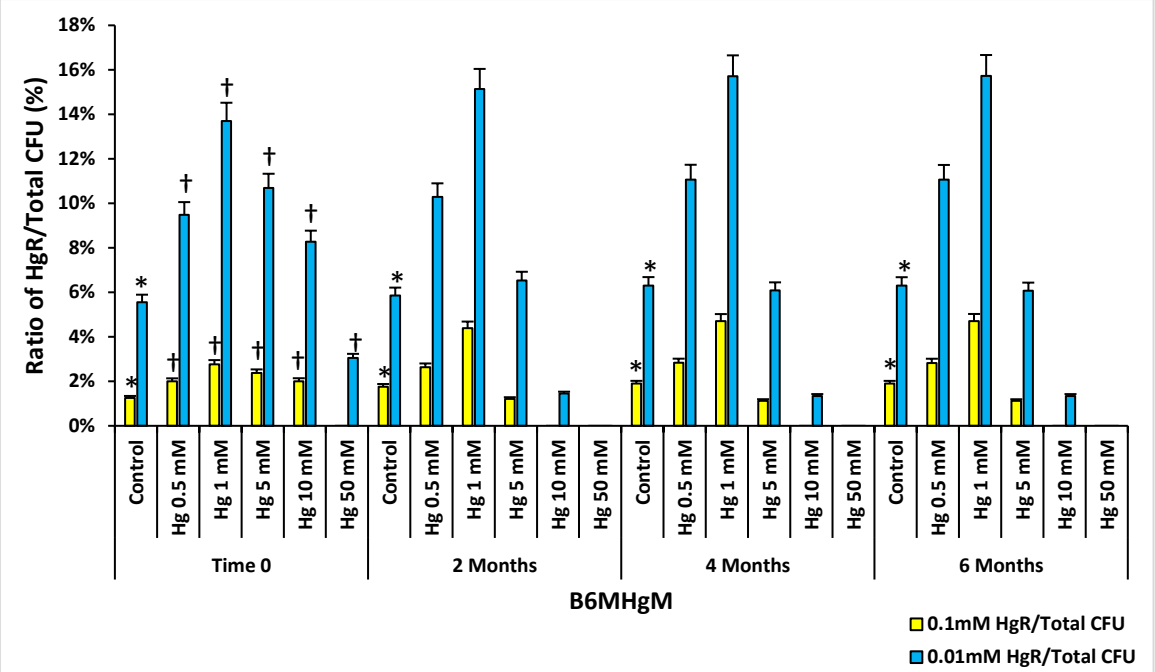


Fig. 5. 10. Mean ratios of HgR/total bacterial CFU, selected on plates with two concentrations of Hg for B6MHgM. *$p<0.05$ compared to the HgR/total bacterial CFU ratios in Hg-spiked microcosms; †$p<0.05$ compared to the HgR/total bacterial CFU ratios in the same Hg-spiked microcosms at the 6-month interval.

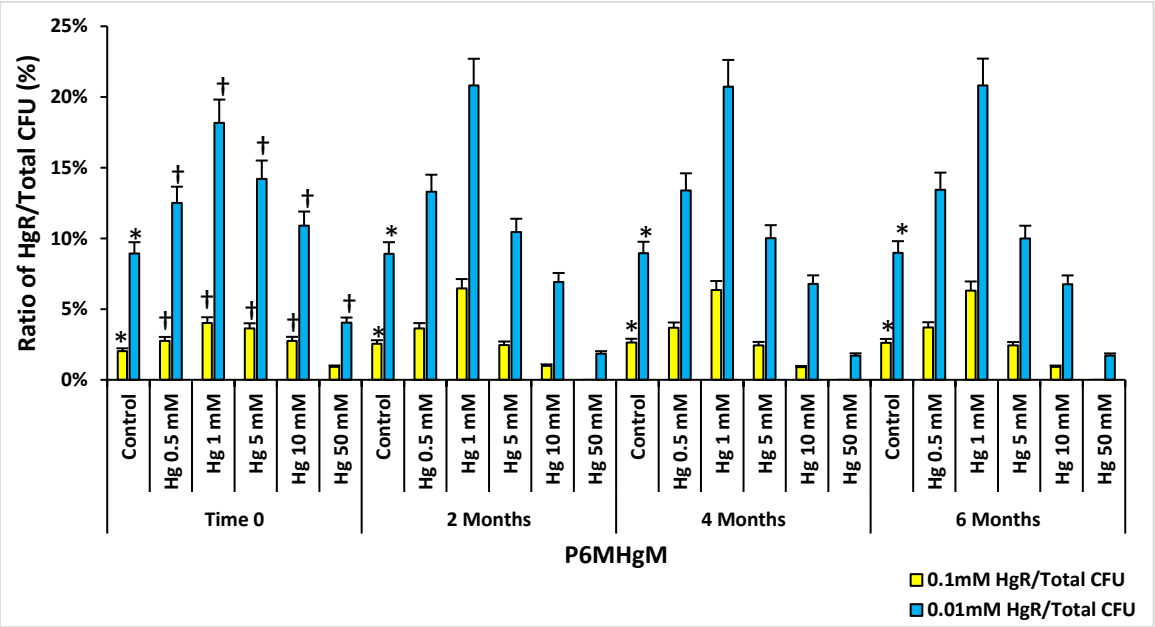


Fig. 5. 11. Mean ratios of HgR/total bacterial CFU, selected on plates with two concentrations of Hg for P6MHgM. *$p<0.05$ compared to the HgR/total bacterial CFU ratios in Hg-spiked microcosms; †$p<0.05$ compared to the HgR/total bacterial CFU ratios in the same Hg-spiked microcosms at the 6-month interval.

5.1.4.2.2. Plates with Abs Additive

The AbR/total bacterial CFU ratios for B6MCdM and P6MCdM increased from those from the control microcosms at Time 0 to those for the Cd 50 mM-spiked microcosms and then decreased in the Cd 100 and 200 mM-spiked microcosms. The AbR/total bacterial ratios for control microcosms were significantly smaller compared to those from the Cd-spiked microcosms ($p < 0.05$). The ratios of AbR/total bacteria in Cd-spiked microcosms at Time 0 were less than those from the same microcosms after 6 months ($p < 0.05$). For example, this ratio was >2 times greater for TcR bacteria in Cd 50 mM-spiked B6MM and ˜1.3 times greater for Cd 50 mM-spiked P6MM at the 6-month endpoint compared to Time 0 (Figures 5.12-5.13). The CdR/total CFU ratios increased in the B6MCdM at the 2 and 4 month time points, but not significantly different compared to those at 6 months (Figures 5.12-5.13). A similar pattern was observed in pasture soil microcosms. Similar trends were determined for the microcosms spiked with a range of Zn and Hg concentrations (Figures A3.3-A3.6).

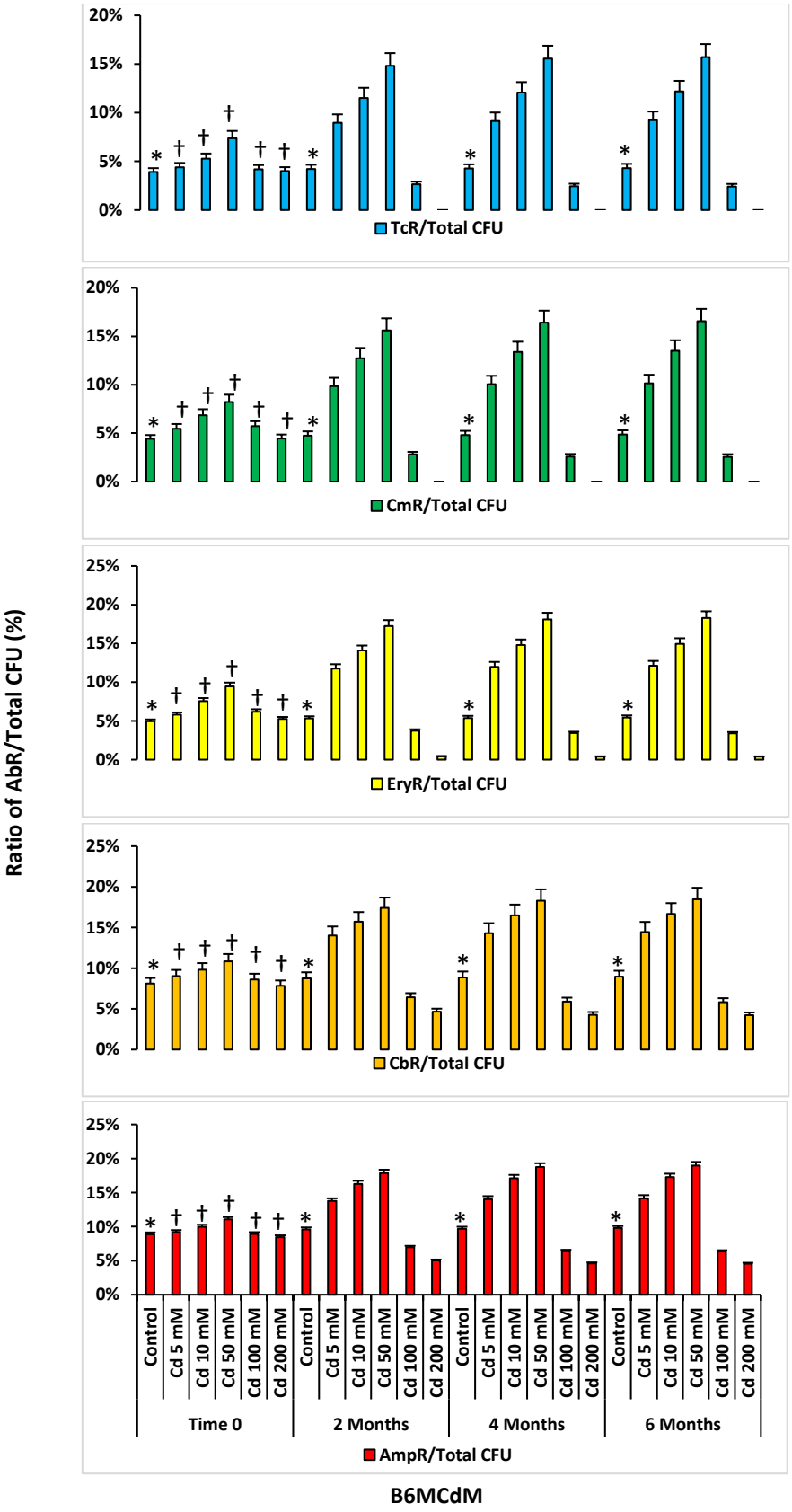


Fig. 5. 12. Mean ratios of AbR/total bacterial CFU, selected on Abs for B6MCdM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-month interval.

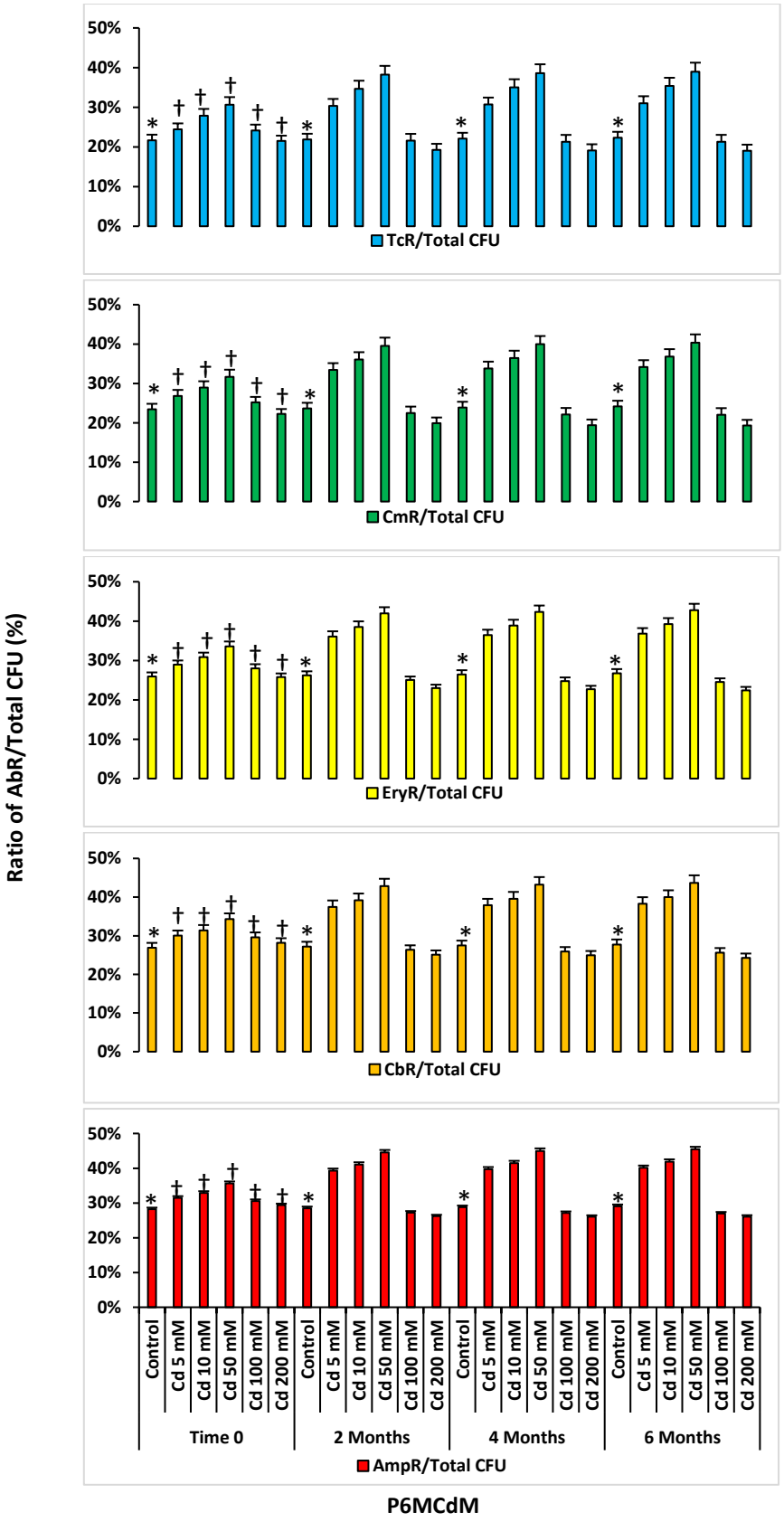


Fig. 5. 13. Mean ratios of AbR/total bacterial CFU, selected on Abs for P6MCdM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in Cd-spiked microcosms; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same Cd-spiked microcosms at the 6-month interval.

5.1.5. Pollution Induced Community Tolerance (PICT) Analysis

The PICT analysis was performed to investigate MIC and EC50 values for HMs and Abs for bacterial consortia in the microcosms' soil samples. Data from PICT analysis can be used as a precise scale to compare with data from the BM analysis which investigated the MIC and EC50 values for HMs and Abs for individual bacterial isolates. The PICT analysis of bacterial consortia isolated from 6WM and B6MM and P6MM soils was performed using a range of concentrations of the 3 three Hms and 5 Abs used previously. The exponential growth rate of bacteria at 12-hour of incubation was recorded and was used to calculate MIC and EC50.

5.1.5.1. PICT Analysis for 6-Weeks Microcosms (6WM)

5.1.5.1.1. Analysis Using HMs

PICT analysis for MICs and EC50s for Cd, Zn and Hg for bacterial consortia from 6WCdM (6-weeks-incubated Cd-spiked microcosms) showed there were larger Cd MIC and EC50 values for bacterial consortia from Cd-spiked microcosms compared to those from control microcosms ($p < 0.05$). Lower MIC and EC50 values for Cd were determined for bacterial consortia from 6WCdM at Time 0 compared to those for consortia from the same microcosms at the 2, 4 and 6 weeks sampling point (Figures 5.14-5.16). Similar trends were observed for MIC and EC50 values of Cd, Zn and Hg for bacterial communities from 6WZnM and 6WHgM (Figures A3.7-A3.12, Appendix 3).

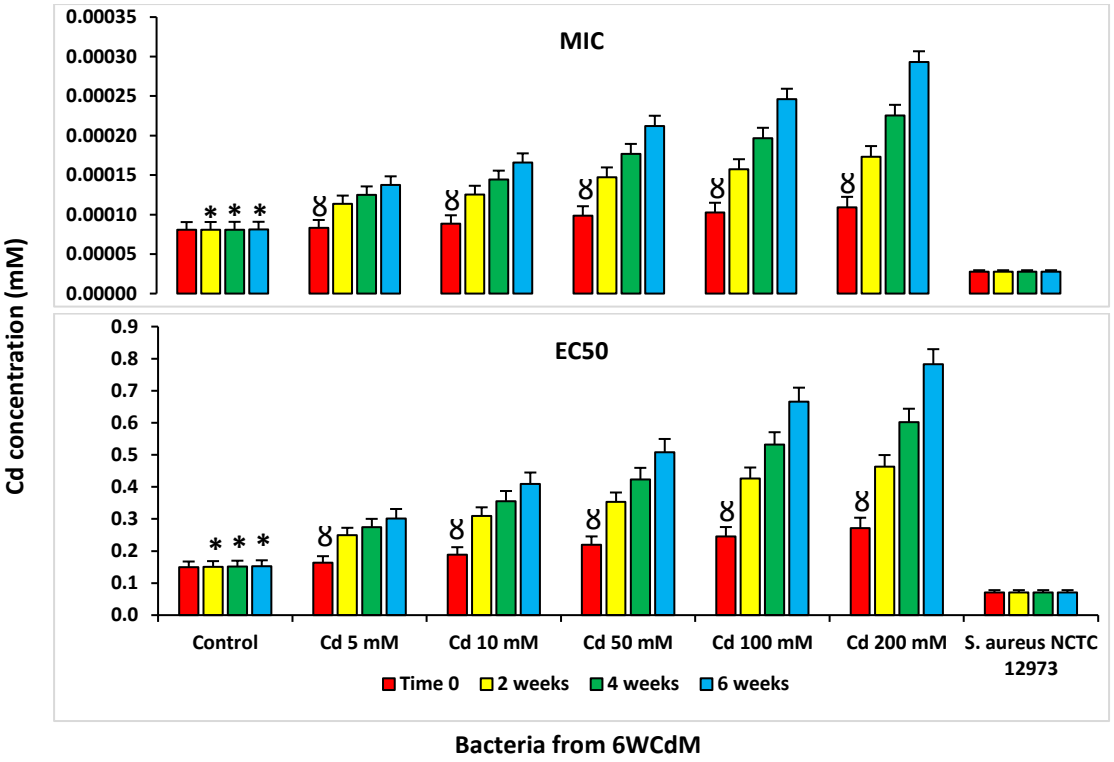


Fig. 5. 14. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from 6WCdM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week.

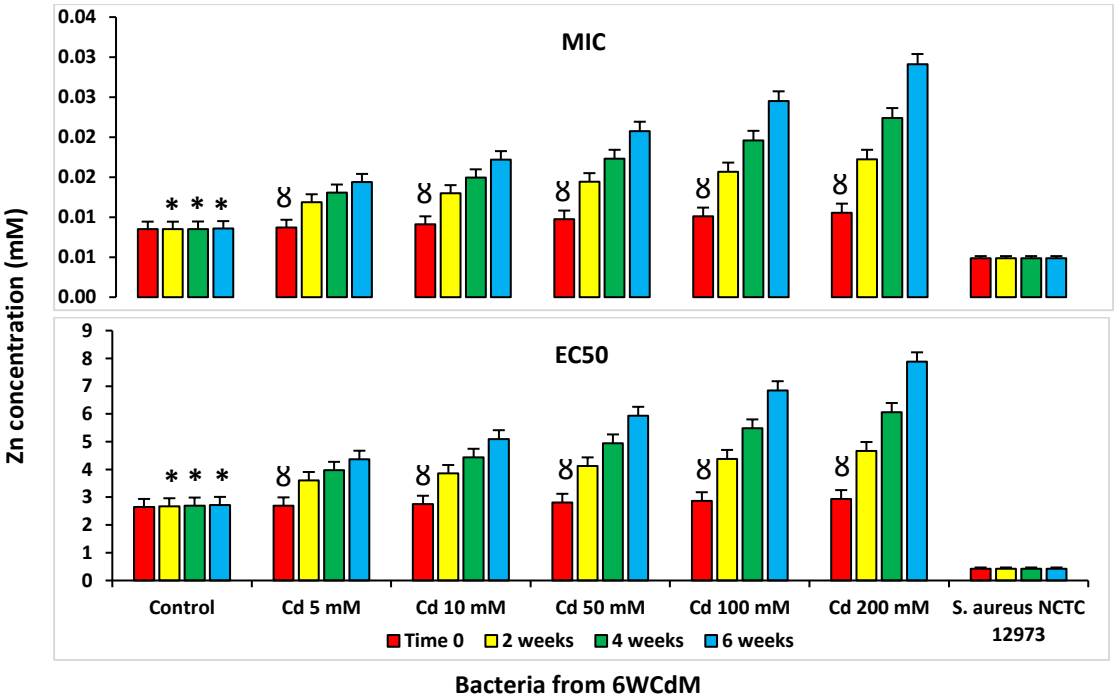


Fig. 5. 15. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from 6WCdM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week.

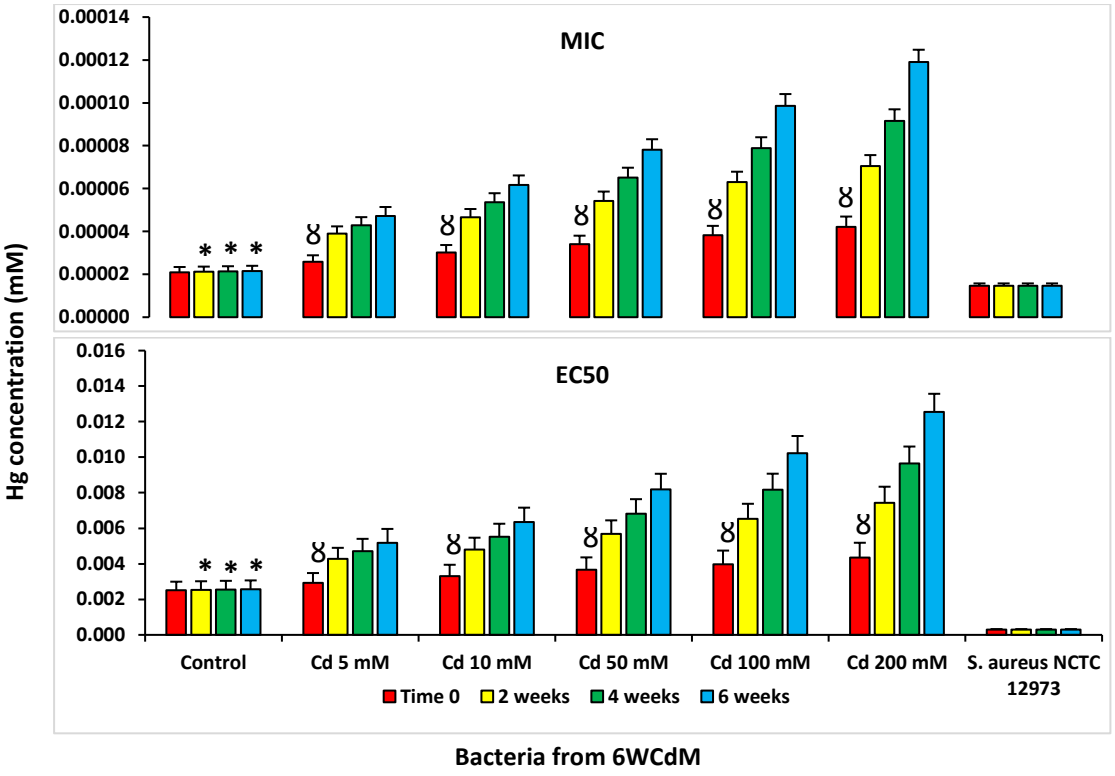


Fig. 5. 16. Mean MIC and EC50 values of PICT analysis with Hg for bacteria from 6WCdM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week.

5.1.5.1.2. Analysis Using Abs

PICT analysis of MIC and EC50 for Tc, Cm, Ery, Cb and Amp for bacterial consortia isolated from 6WCdM showed there were higher MIC and EC50 values for Abs from bacterial consortia from Cd-spiked microcosms compared to those from the control microcosm. In addition, there were lower MIC and EC50 values for bacteria from 6WCdM at Time 0 compared to those isolated from the same microcosms at 2, 4 and 6 weeks intervals (Figure 5.17). The result shown here is for MIC and EC50 values for Tc for bacterial consortia from 6WCdM, 6WZnM (Figure 5.18) and 6WHgM (Figure 5.19), and the results for other Abs are shown in Appendix 3 (Figures A3.13-A3.24, Appendix 3). Similar trends were determined for MIC and EC50 values of Tc, Cm, Ery, Cb and for bacterial consortia from 6WZnM and 6WHgM (Figures 5.18-5.19). The MIC values determined for bacterial consortia (according to EUCAST ECOFF recommendations [218]) from 6WM soil were higher than the 20 µg mL^{-1} threshold defined for Ab resistance in soil bacterial

for all of the five Abs at 2, 4 and 6-week time-points [217, 220], while these were lower than this threshold for Tc and Cm at Time 0.

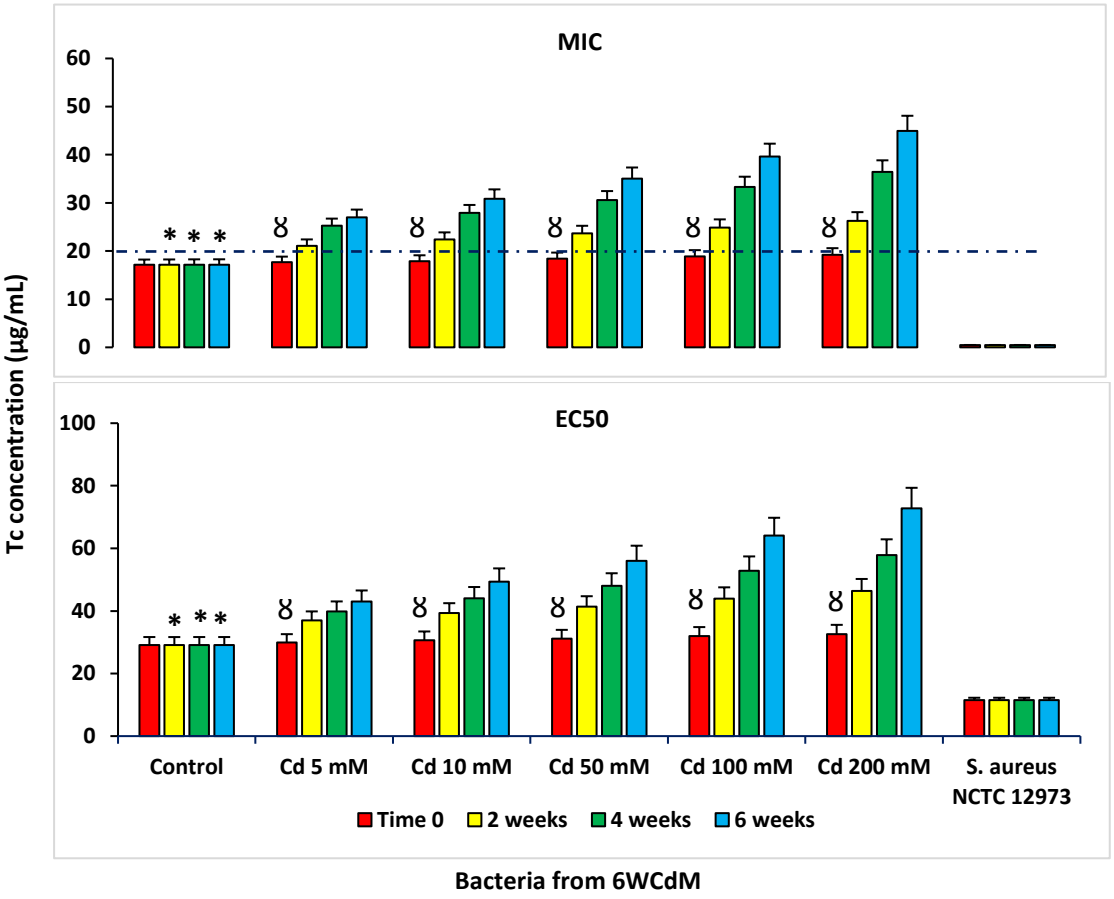


Fig. 5. 17. Mean MIC and EC50 values of PICT analysis with Tc for bacteria from 6WCdM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϐ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

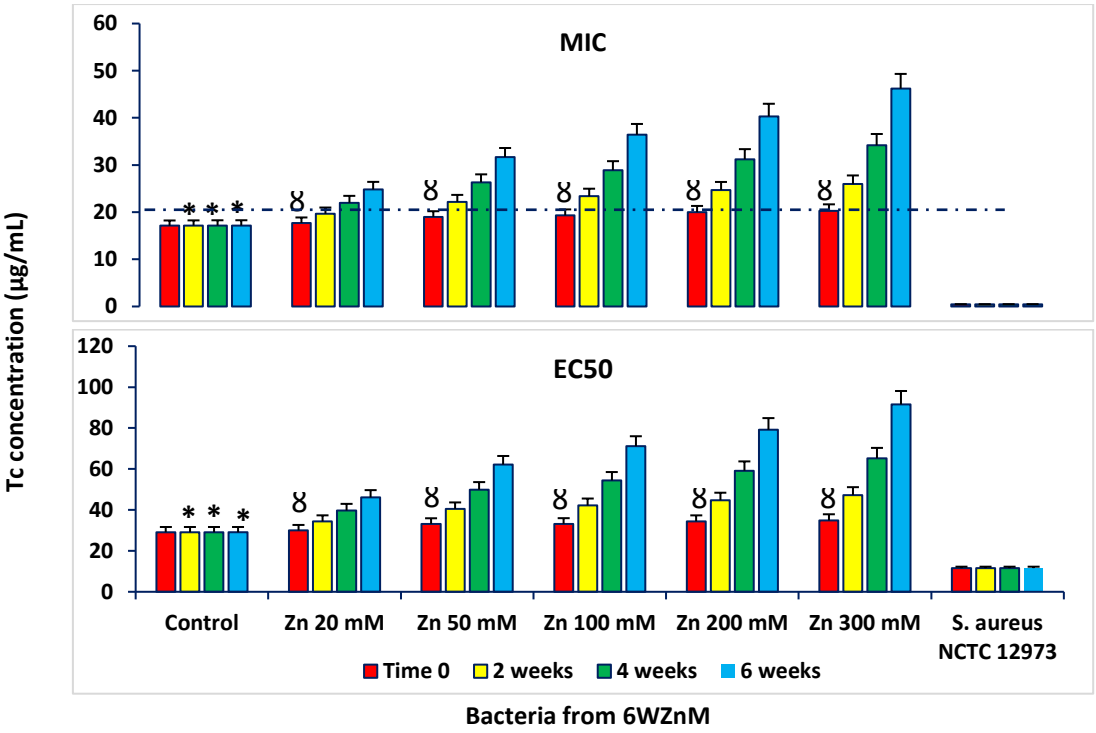


Fig. 5. 18. Mean MIC and EC50 values of PICT analysis with Tc for bacteria from 6WZnM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

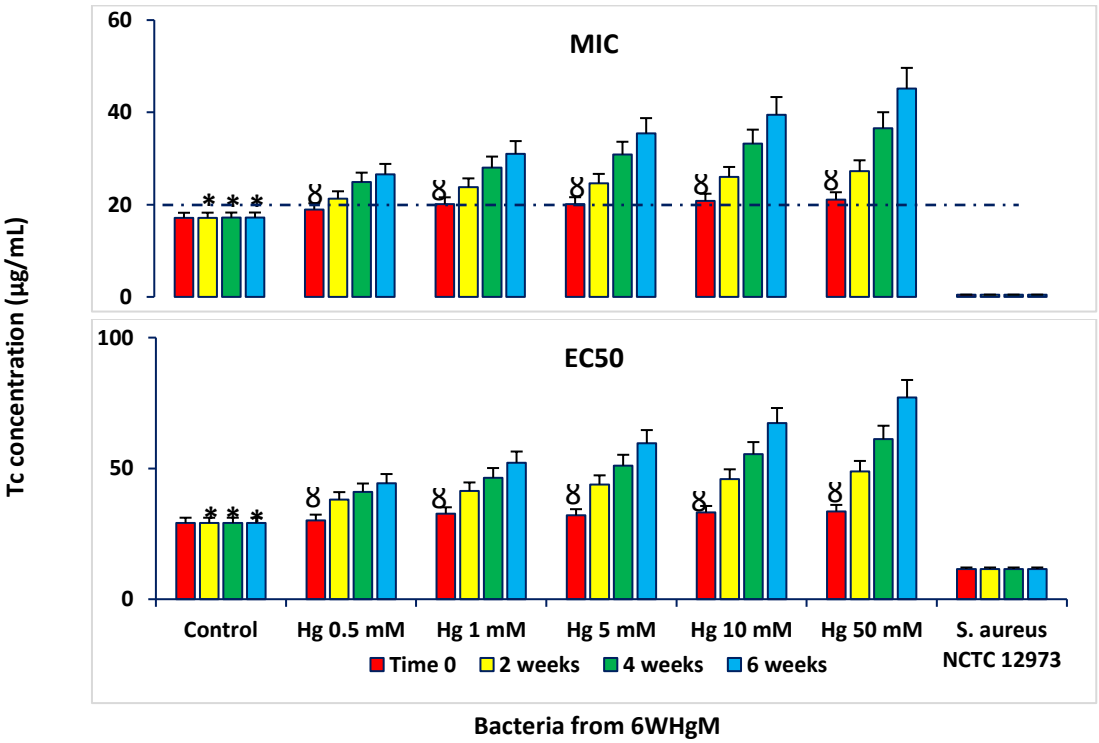


Fig. 5. 19. Mean MIC and EC50 values of PICT analysis with Tc for bacteria from 6WHgM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

5.1.5.2. PICT Analysis of 6-month microcosms Containing Background and Pasture soil (B6MM and P6MM)

5.1.5.2.1. Analysis Using HMs

PICT determination of MICs and EC50s for Cd, Zn and Hg for bacterial consortia from B6MCdM and P6MCdM found greater MIC and EC50 values for HMs from bacterial consortia from Cd-spiked microcosms compared to those for bacteria from control microcosms ($p < 0.05$). Lower Cd MIC and EC50 values were determined for bacterial consortia from B6MCdM and P6MCdM at Time 0 compared to those for bacteria from same microcosms after 2, 4 and 6 months incubation (Figures 5.20-5.25). Similar trends were determined for MIC and EC50 values of Cd, Zn and Hg for bacterial consortia from B6MZnM, B6MHgM, P6MZnM and P6MHgM (Figures A3.25-A3.36, Appendix 3).

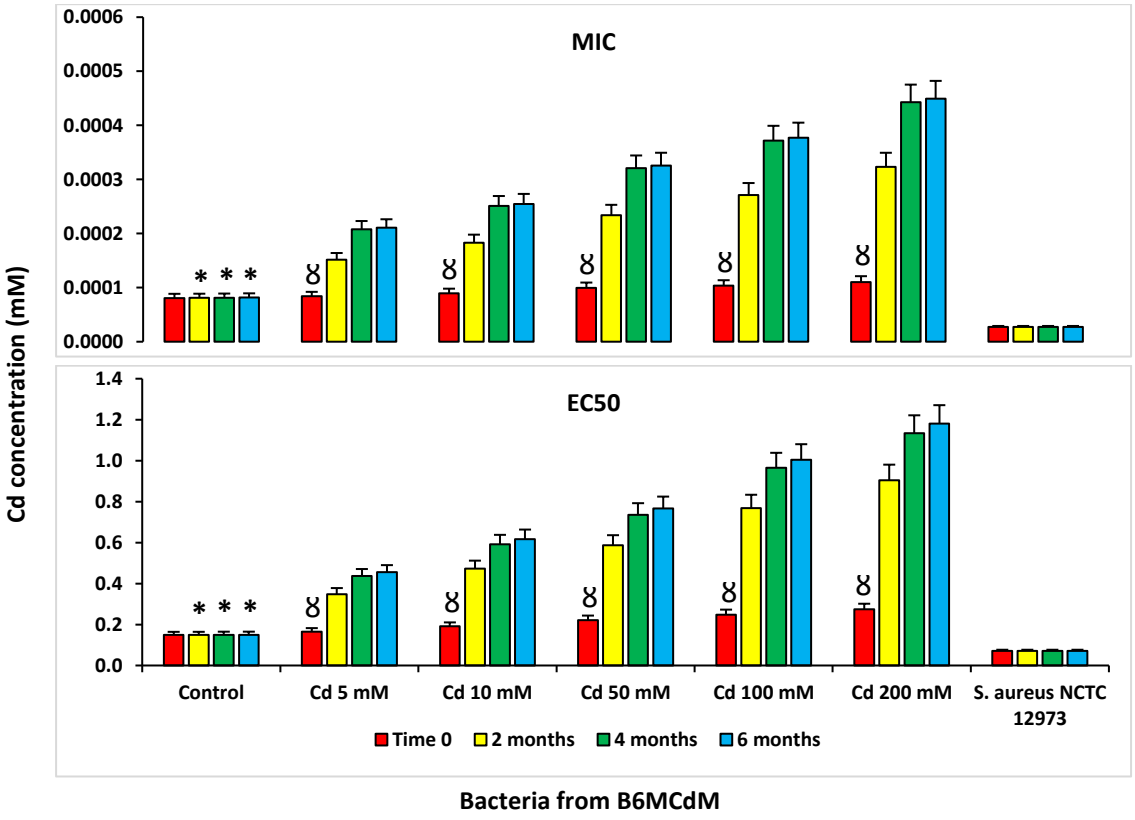


Fig. 5. 20. Mean MIC and EC50 values (mM) of PICT analysis with Cd for bacteria from B6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

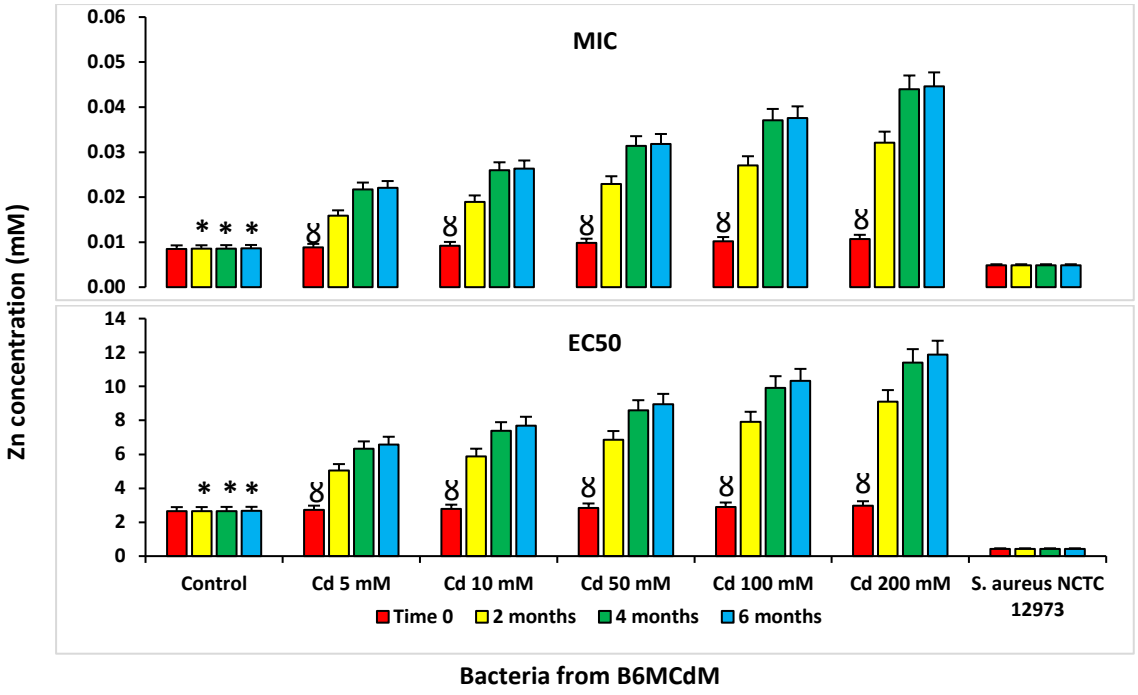


Fig. 5. 21. Mean MIC and EC50 values (mM) of PICT analysis with Zn for bacteria from B6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

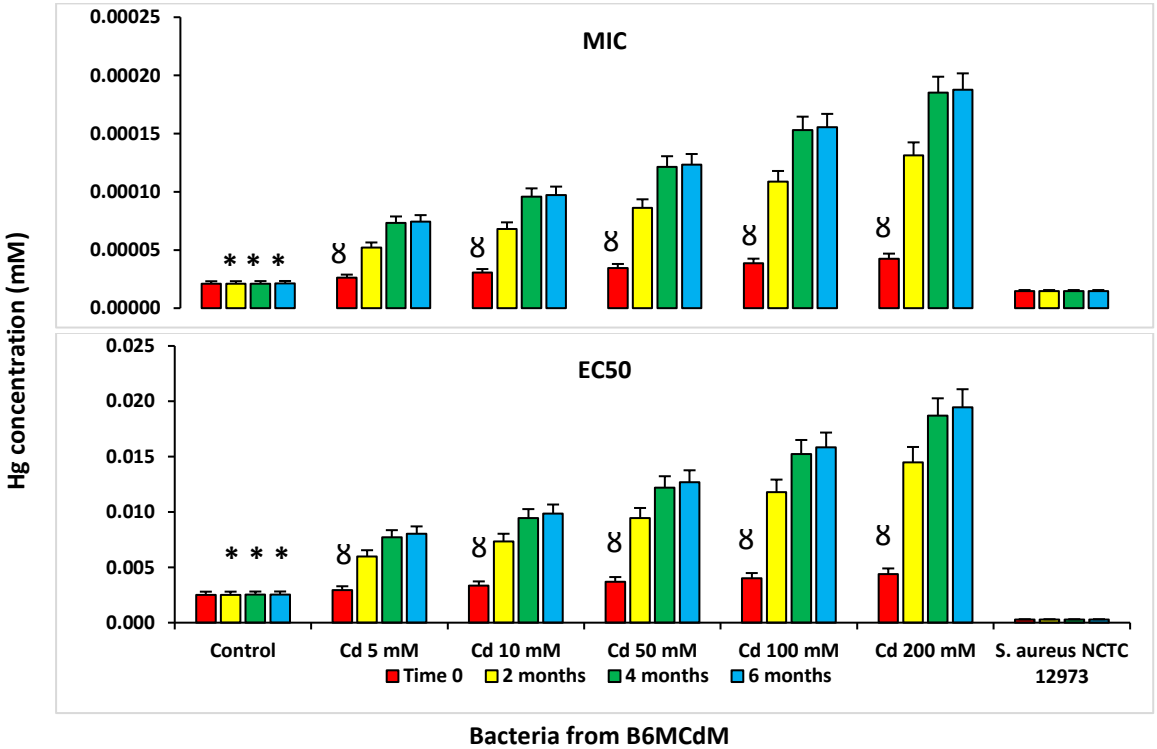


Fig. 5. 22. Mean MIC and EC50 values (mM) of PICT analysis with Hg for bacteria from B6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

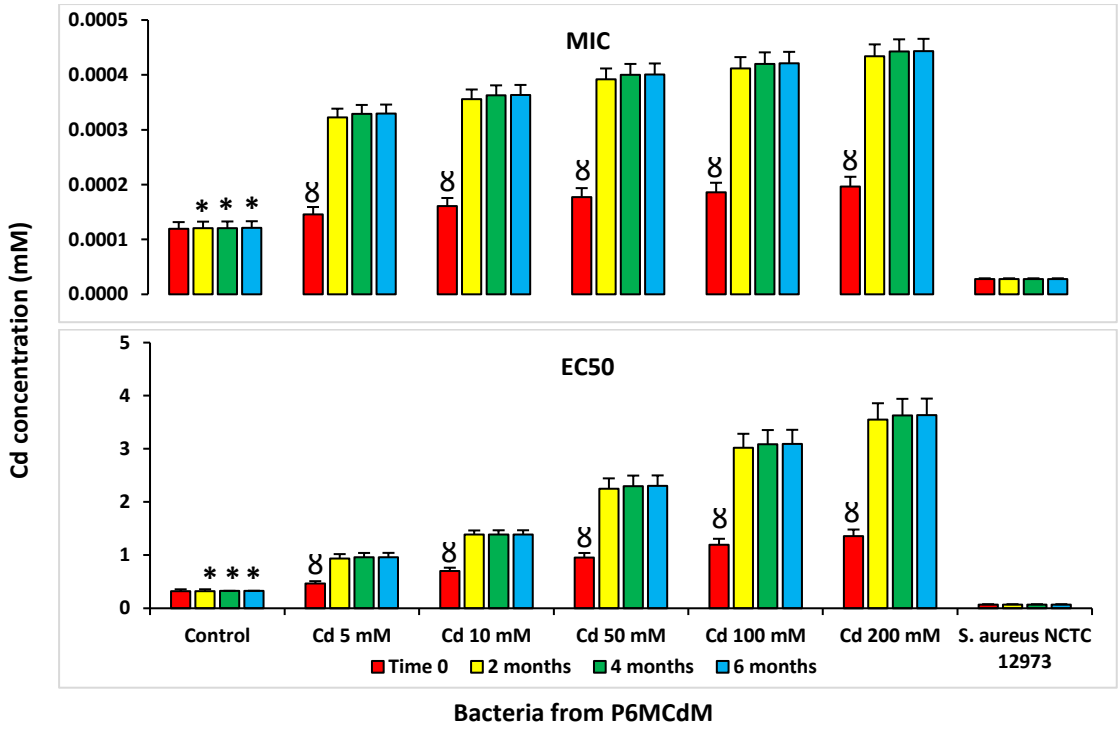


Fig. 5. 23. Mean MIC and EC50 values (mM) of PICT analysis with Cd for bacteria from P6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

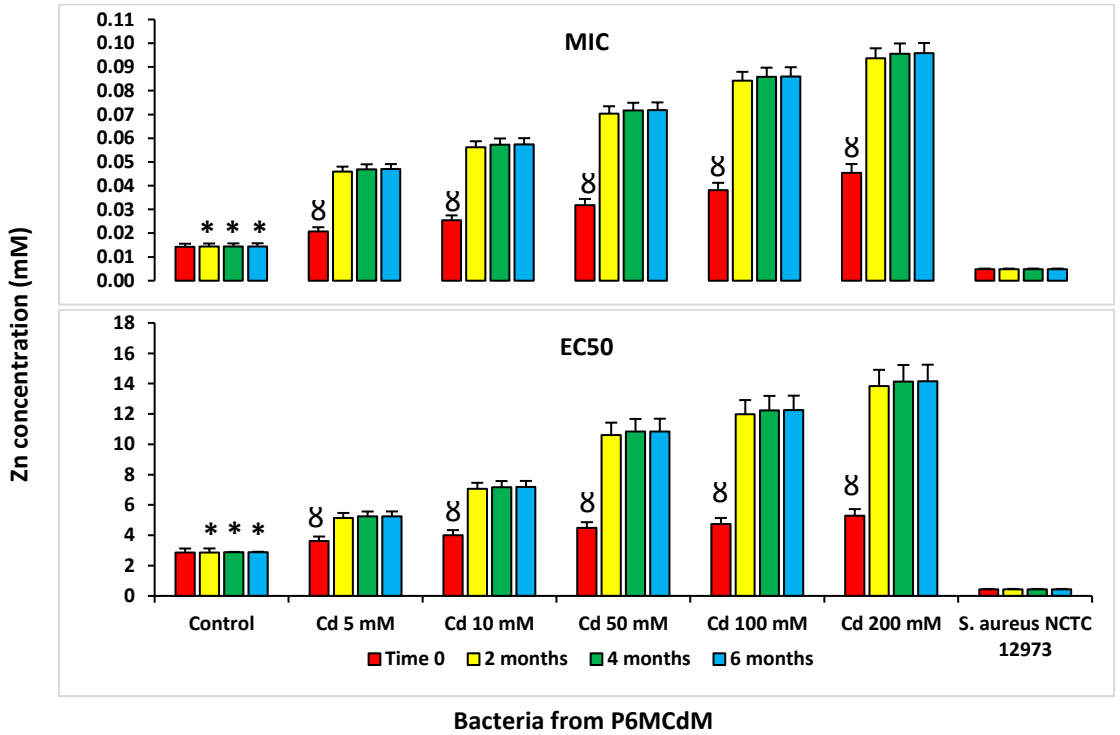


Fig. 5. 24. Mean MIC and EC50 values (mM) of PICT analysis with Zn for bacteria from P6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

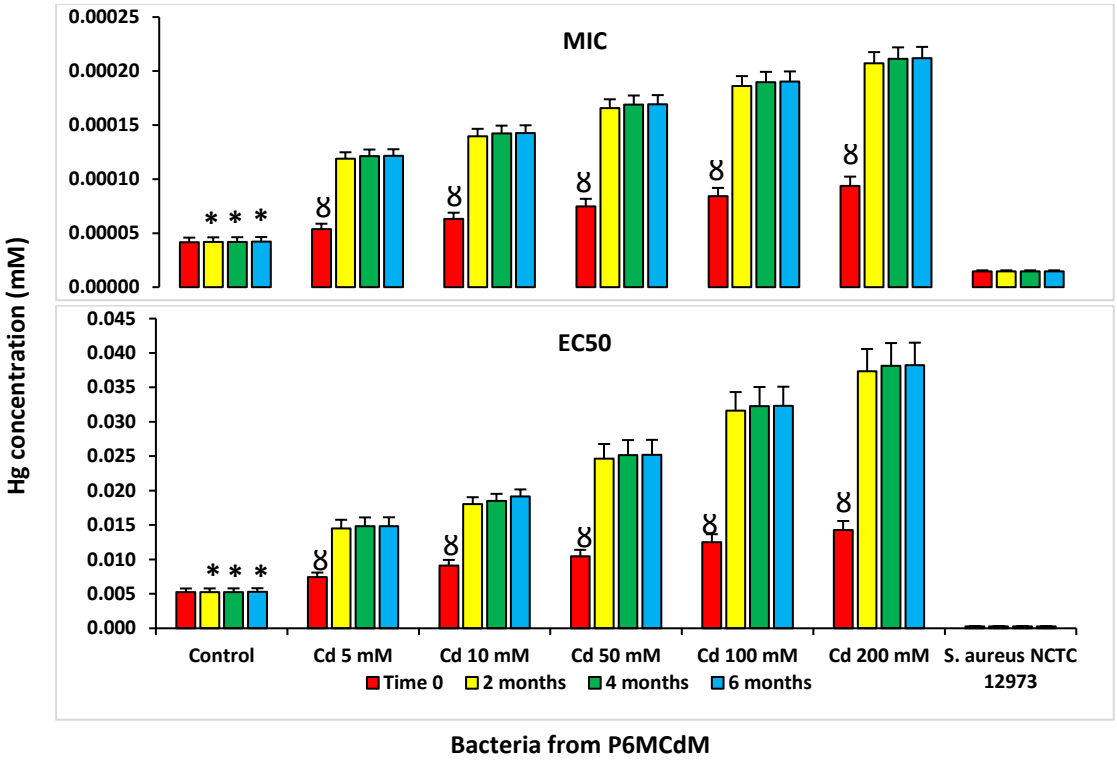


Fig. 5. 25. Mean MIC and EC50 values (mM) of PICT analysis with Hg for bacteria from P6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month.

5.1.5.2.2. Analysis Using Abs

The PICT analysis of MIC and EC50 for Tc, Cm, Ery, Cb and Amp for bacterial consortia isolated from B6MCdM and P6MCdM showed there were significantly higher MIC and EC50 values for Abs from bacterial consortia from Cd-spiked microcosms compared to those from control microcosms ($p < 0.05$). There were lower Abs MIC and EC50 values for bacteria from B6MCdM and P6MCdM at Time 0 compared to those isolated from same microcosms after 2, 4 and 6 months (Figure 5.26-5.27). According to the recommendations [220, 221], the MIC values determined for Tc for bacterial consortia isolates from B6MCdM and P6MCdM were greater than 20 µg mL^{-1} at timepoints 2, 4 and 6 months of incubation. However, they were greater than 20 µg mL^{-1} at for pastoral soil-containing microcosms at Time 0 too. Similar trends were determined for MIC and EC50 values of Tc for bacterial consortia from B6MZnM, P6MZnM, B6MHgM and P6MHgM (Figures 5.28-5.31). The MIC and EC50 values of Cm, Ery, Cb and Amp

for bacterial consortia from B6MZnM, P6MZnM, B6MHgM and P6MHgM are shown in Appendix 3 (Figures A3.37-A3.60, Appendix 3). The MIC values determined for bacterial consortia (according to EUCAST ECOFF recommendations [218]) from B6MM and P6MM soils were higher than 20 µg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs at 2, 4 and 6-month time-points [217, 220], while these were lower than this threshold for Tc and Cm at Time 0.

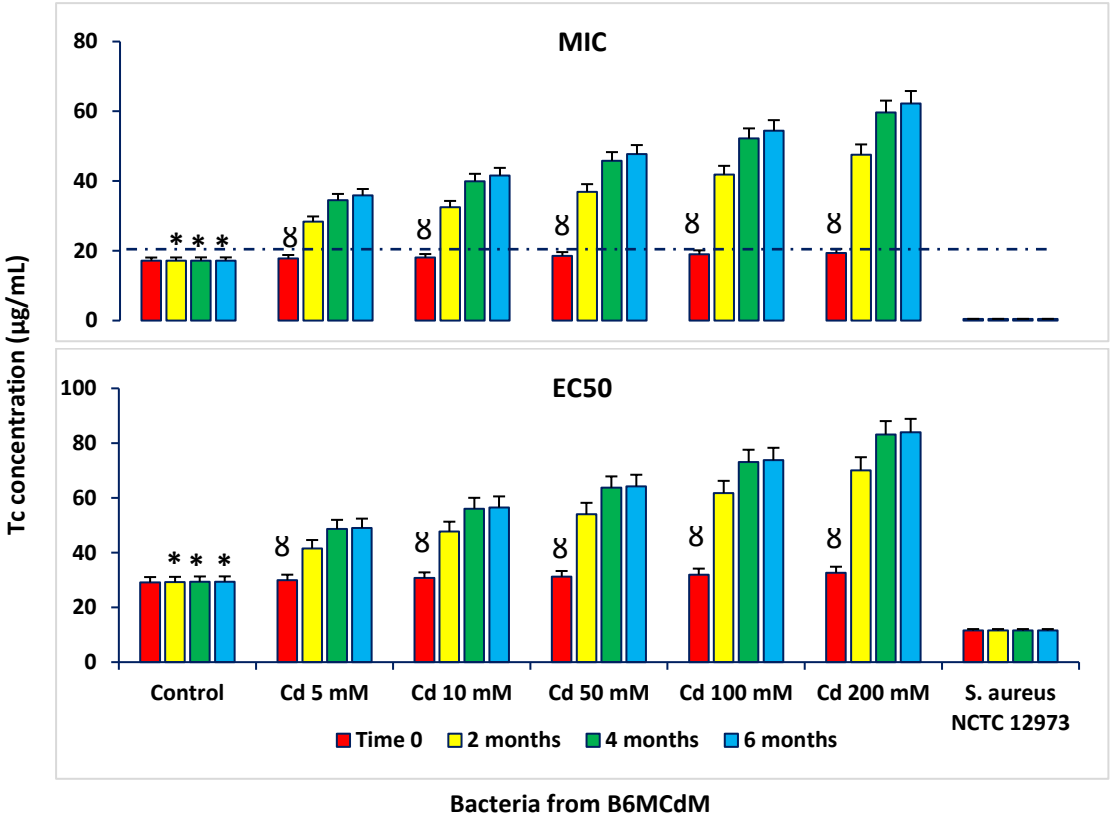


Fig. 5. 26. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from B6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

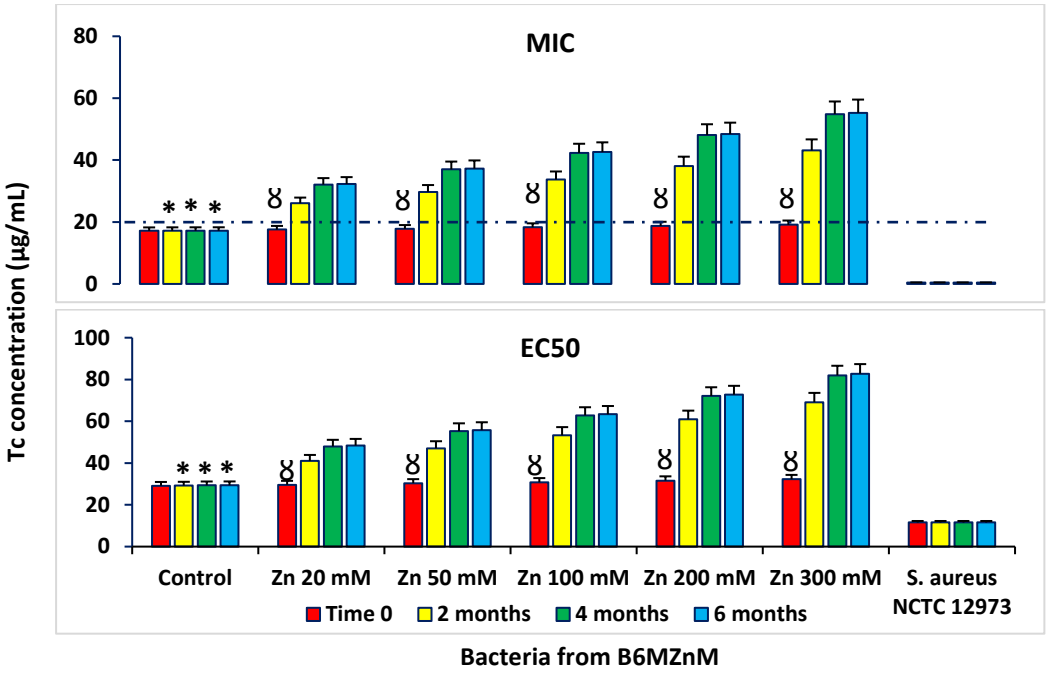


Fig. 5. 27. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from B6MZnM. Zn concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

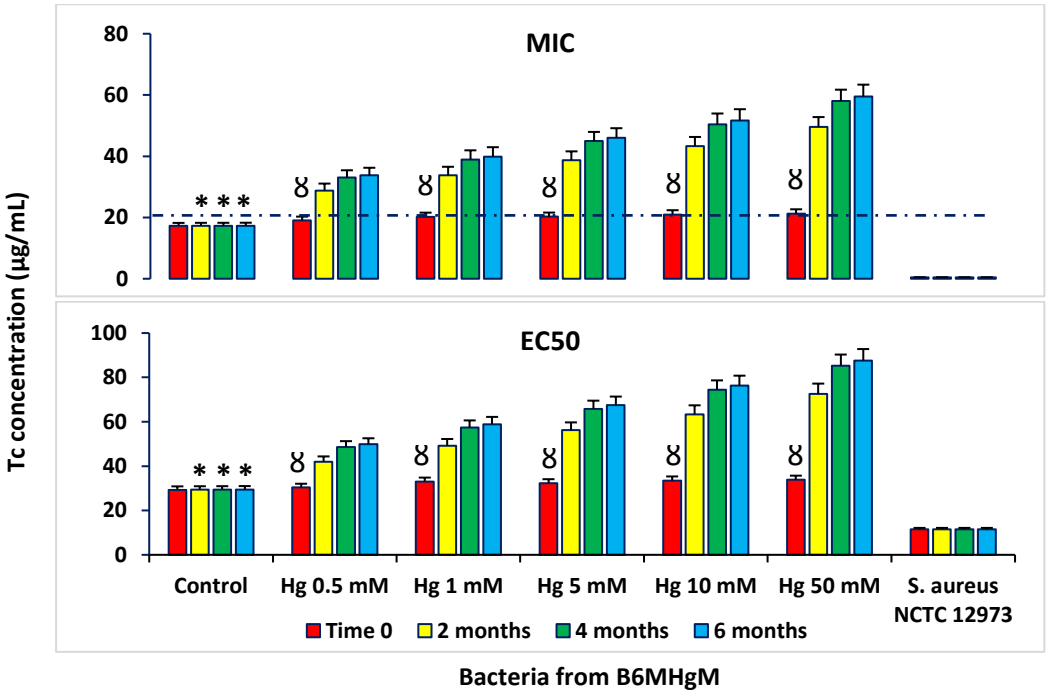


Fig. 5. 28. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from B6MHgM. Hg concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

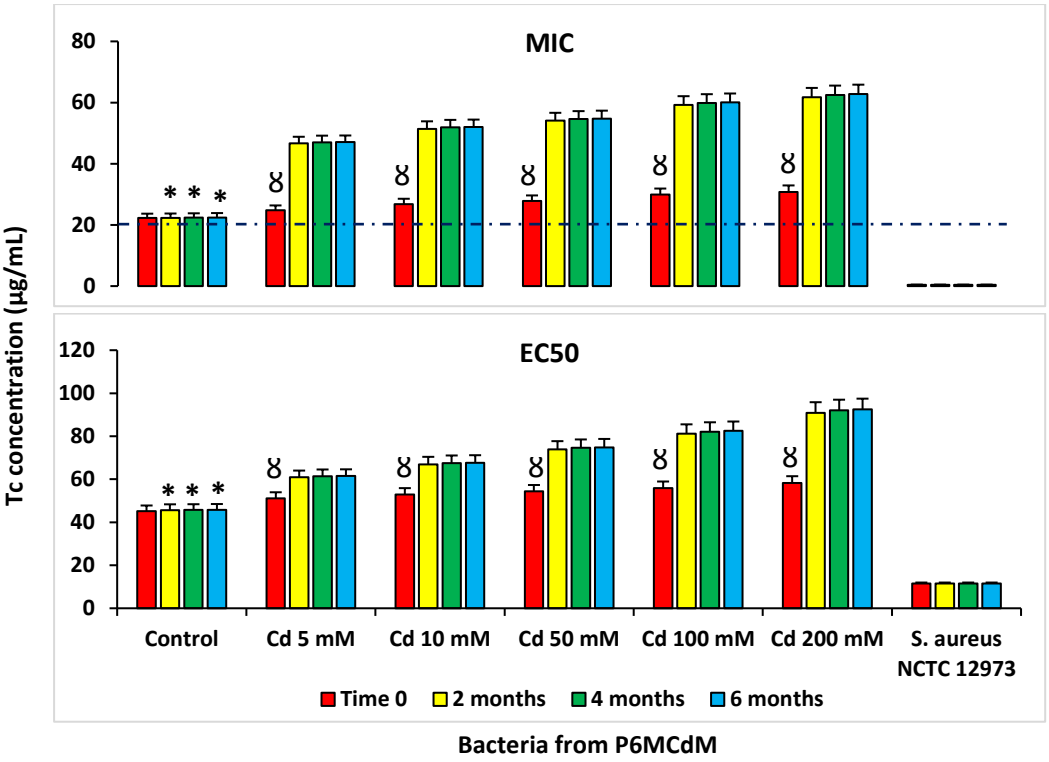


Fig. 5. 29. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from P6MCdM. Cd concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

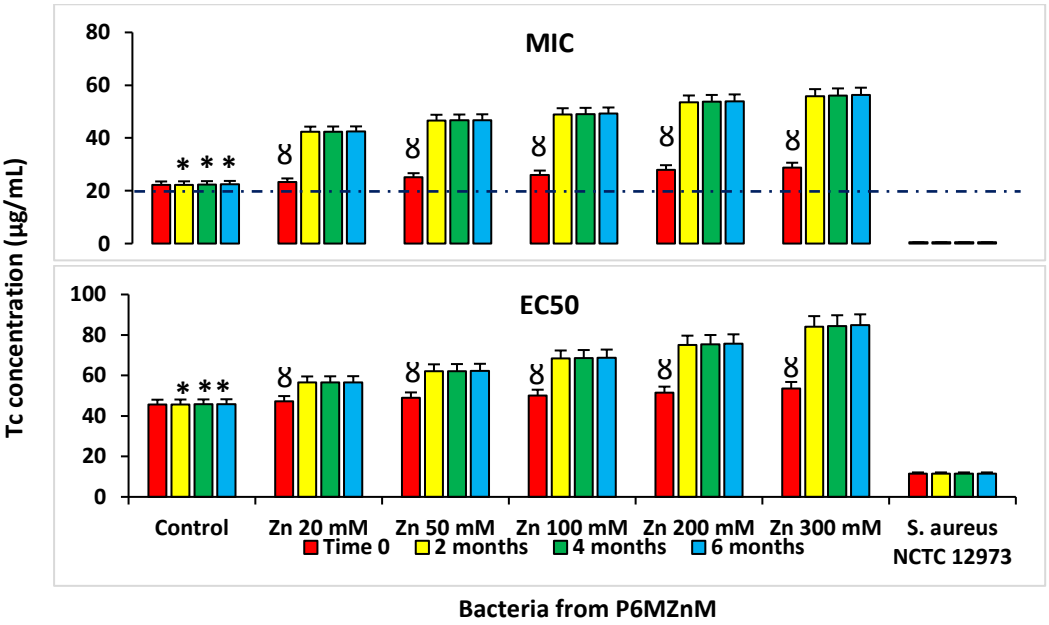


Fig. 5. 30. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from P6MZnM. Zn concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at the sam etimepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

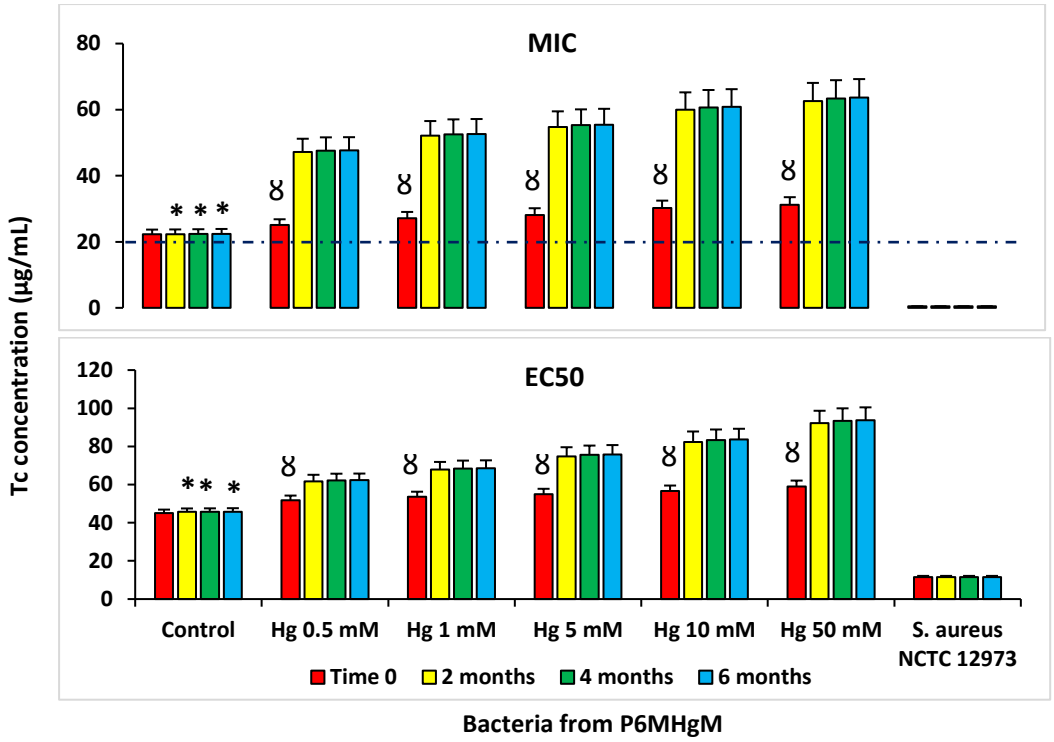


Fig. 5. 31. Mean MIC and EC50 values (µg mL^{-1}) of PICT analysis with Tc for bacteria from P6MHgM. Hg concentrations are mM. *$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Tc MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

5.1.6. Broth Microdilution (BM) Analysis of HMR Bacterial Isolates to Determine MIC and EC50 Values for Heavy Metals and Antibiotics

The determination of susceptibility to the antimicrobials (Cd, Zn, Hg, Tc, Cm, Ery, Cb and Amp) by BM for the HMR bacterial isolates (see Section 3.9) from 6WM and B6MM and P6MM soil samples was performed. A selection of 600 HMR isolates for each microcosm set from R2A agar plates containing a range of concentrations of Cd, Zn and Hg (described in Section 3.4) were compared to 50 HM-sensitive bacterial isolates for each soil sample. Three-Way ANOVA test investigated associations between the MICs and EC50s values for bacterial isolates from different soils samples. Soil sampling from microcosms at appropriate intervals (2 weeks or 2 months), concentration of HM additives to the microcosms and HM or Ab concentrations in

microtitre plates were introduced to the analysis as independent variables. The dependent variable was MIC or EC50 value.

5.1.6.1. Microcosms Incubated for 6-weeks (6WM)

5.1.6.1.1. BM Analysis with HMs

Lower Cd MIC and EC50 values were determined for HMR bacterial isolates from control microcosms (with no added HM) compared to the isolates from HMs-spiked microcosms. In addition, there were lower Cd MIC and EC50 values for the HMR bacterial isolates from HMs-spiked microcosms at Time 0 compared to those from the same microcosms after 6 weeks of incubation (Figure 5.32). Lower Cd MIC and EC50 values found for HM-sensitive bacterial isolates from 6WM spiked with HMs compared to the HMR isolates from the same microcosms ($p < 0.05$). Similar trends were determined for MIC and EC50 values for Zn and Hg in the bacterial isolates from HMs-spiked 6WM (Figures 5.33 and 5.34).

5.1.6.1.2. BM Analysis with Abs

Tetracycline (Tc)

Similar to what was determined for the HM resistance profile of isolates from the 6-week microcosms, there were significantly higher MIC and EC50 values for Tc for the HMR isolates from HMs-spiked 6WM at 6 weeks compared to those from isolated at Time 0 ($p < 0.05$). There were lower MIC and EC50 values for Tc for HMR bacterial isolates from control microcosms compared to those for isolates from HMs-spiked microcosms. Moreover, lower MIC and EC50 values for Tc were determined for the HMR bacterial isolates from HMs-spiked microcosms at Time 0 compared to those from the same microcosms after 6 weeks incubation (Figure 5.35). Similar trends were determined for MIC and EC50 values for the other Abs (Cm, Ery, Cb and Amp) for these isolates (Figures A3.61-A3.64, Appendix 3). The MIC values determined for bacterial isolates (according to EUCAST ECOFF recommendations [218]) from 6WM soil were

higher than 20 μg mL^{-1} threshold defined for Ab resistance in soil bacterial for all of the five Abs at 2, 4 and 6-week time-points [217, 220], while these were lower than this threshold for Tc at Time 0.

5.1.6.2. Background and Pasture soil-contained 6-month microcosms (B6MM and P6MM)

5.1.6.2.1. BM Analysis with HMs

There were significantly lower Cd MIC and EC50 values for the HM-sensitive bacterial isolates from B6MM and P6MM compare to those for the HMR isolates from the same microcosms ($p < 0.05$). Higher Cd MIC and EC50 values were determined for the HMR bacterial isolates from the HM-spiked microcosms compared to those for the HMR bacteria from control microcosm. Furthermore, lower Cd MIC and EC50 values were determined for the HMR bacterial isolates from HMs-spiked microcosms at Time 0 compared to those for HMR bacteria isolated from the same microcosms after 6 months of incubation (Figures 5.36 and 5.37). Similar trends were determined for MIC and EC50 values of Zn and Hg for the bacterial isolates from B6MM and P6MM (Figures A3.65-A3.68, Appendix 3).

5.1.6.2.2. BM Analysis with Abs

There were significantly lower Tc MIC and EC50 values for the HM-sensitive isolates from HMs-spiked B6MM and P6MM compared those for the HMR isolates from the same microcosms ($p < 0.05$). Lower Tc MIC and EC50 values were determined for the HMR isolates from control microcosms compared to those for the HMR isolates from the HMs-spiked microcosms. There were lower Tc MIC and EC50 values for the HMR isolates from HMs-spiked microcosms at Time 0 compared to those determined for the HMR isolates from the same microcosms and after 6 months of incubation (Figures 5.38 and 5.39). Similar trends were determined for MIC and EC50 values of Cm, Ery, Cb and Amp for the bacterial isolates from B6MM and P6MM (Figures A3.69-A3.76, Appendix 3). The MIC values determined for bacterial isolates (according to EUCAST ECOFF recommendations [218]) from B6MM and P6MM soil were higher than 20 μg mL^{-1}

threshold defined for Ab resistance in soil bacterial for all of the five Abs at 2, 4 and 6-week timepoints [217, 220], while these were lower than this threshold for isolates HMR isolates from B6MM for Tc at Time 0.

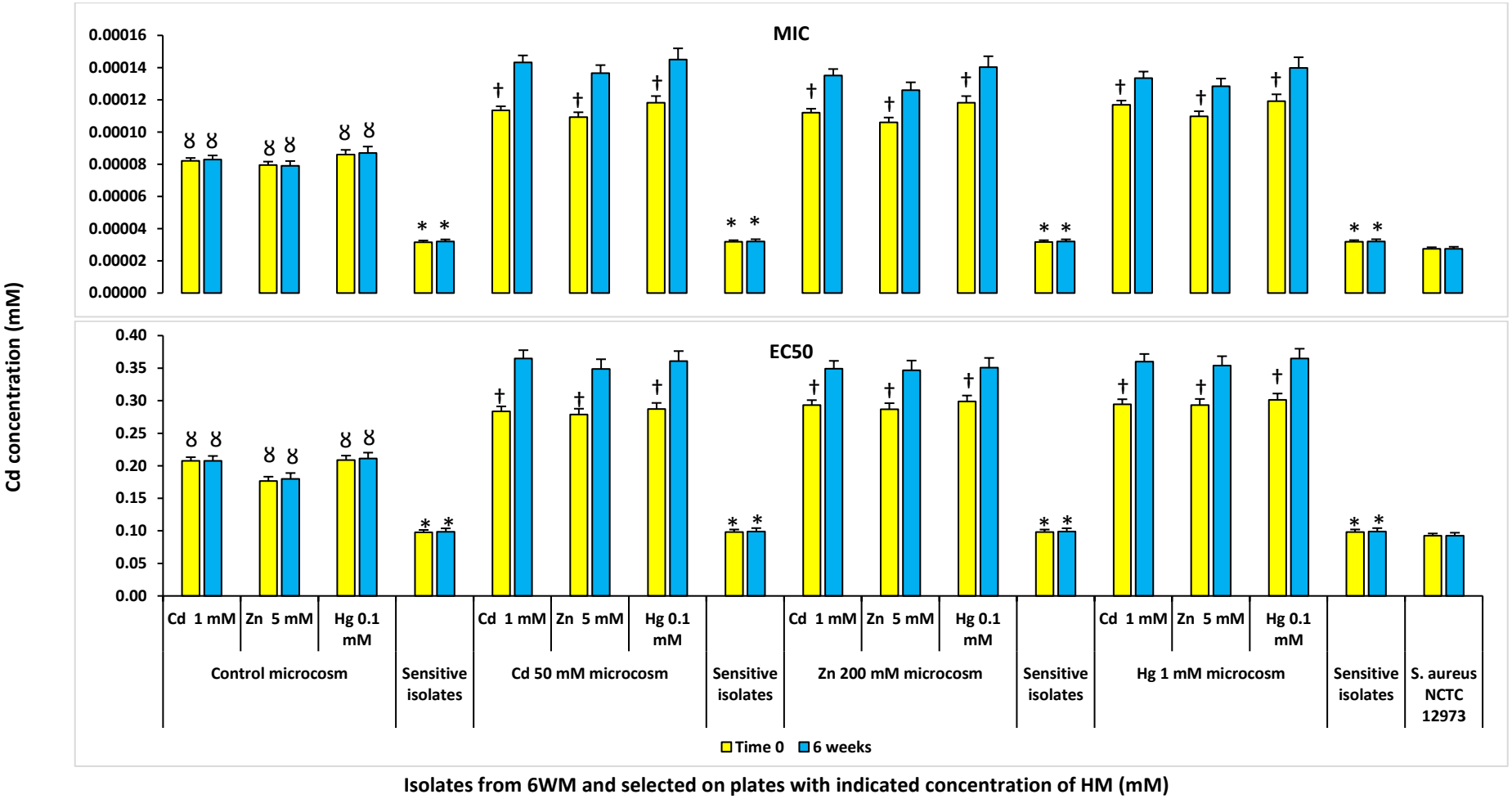


Fig. 5. 32. Mean MIC and EC50 values (mM) of BM analysis with Cd for HMR isolates from 6WM. *$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation.

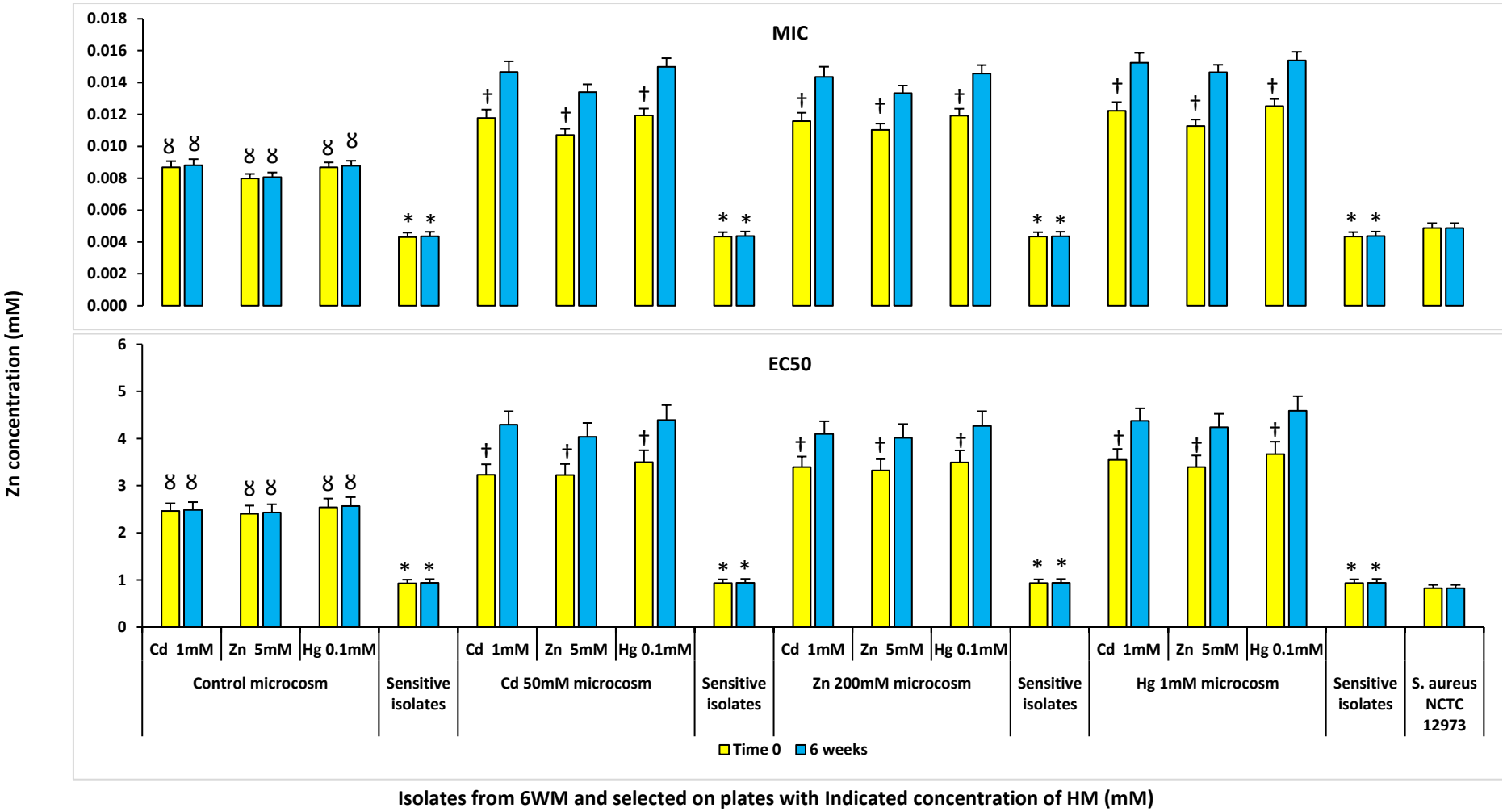


Fig. 5. 33. Mean MIC and EC50 values (mM) of BM analysis with Zn for HMR isolates from 6WM. *$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Zn MIC AND EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation.

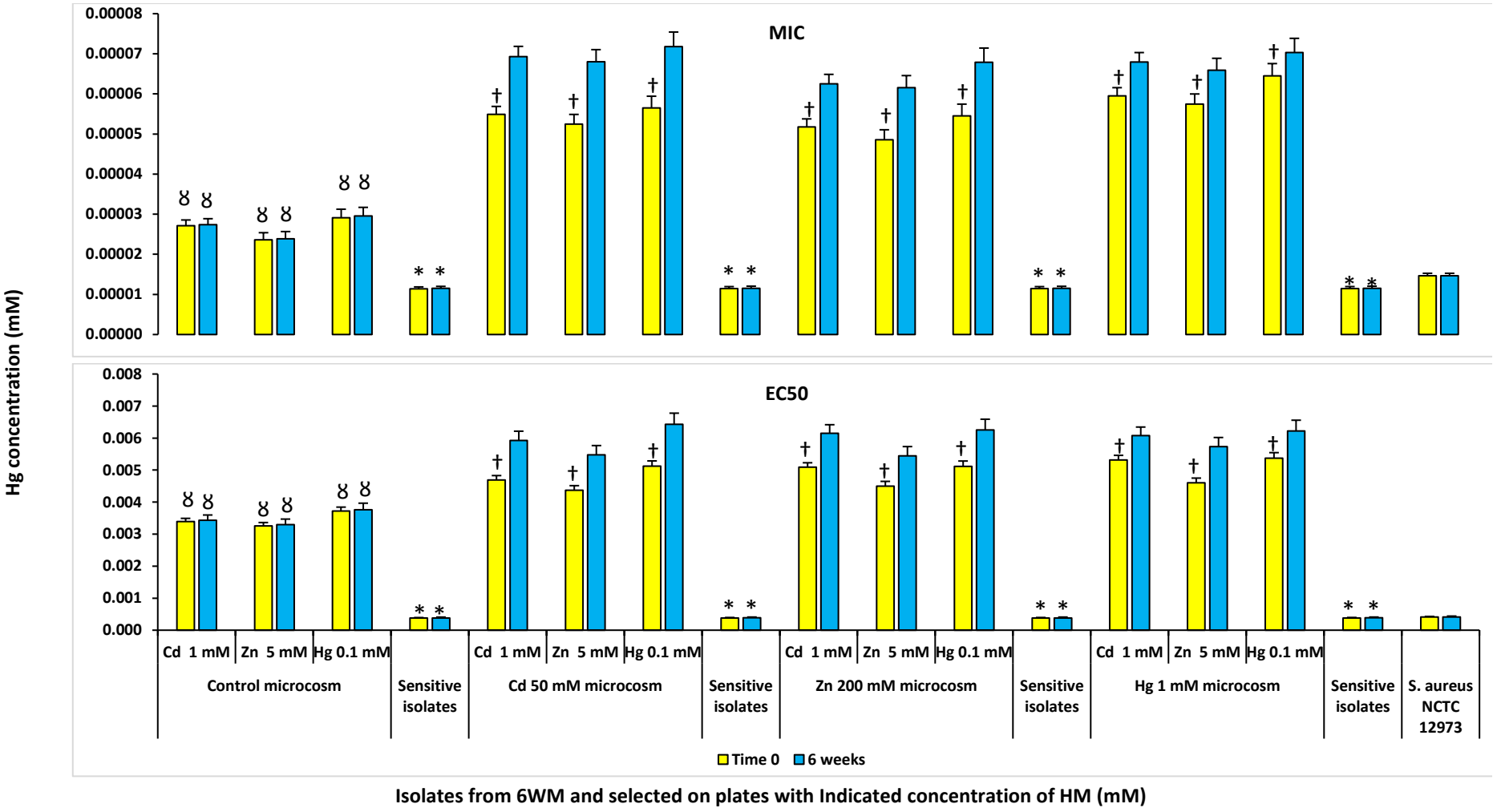


Fig. 5. 34. Mean MIC and EC50 values (mM) of BM analysis with Hg for HMR isolates from 6WM. *$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation.

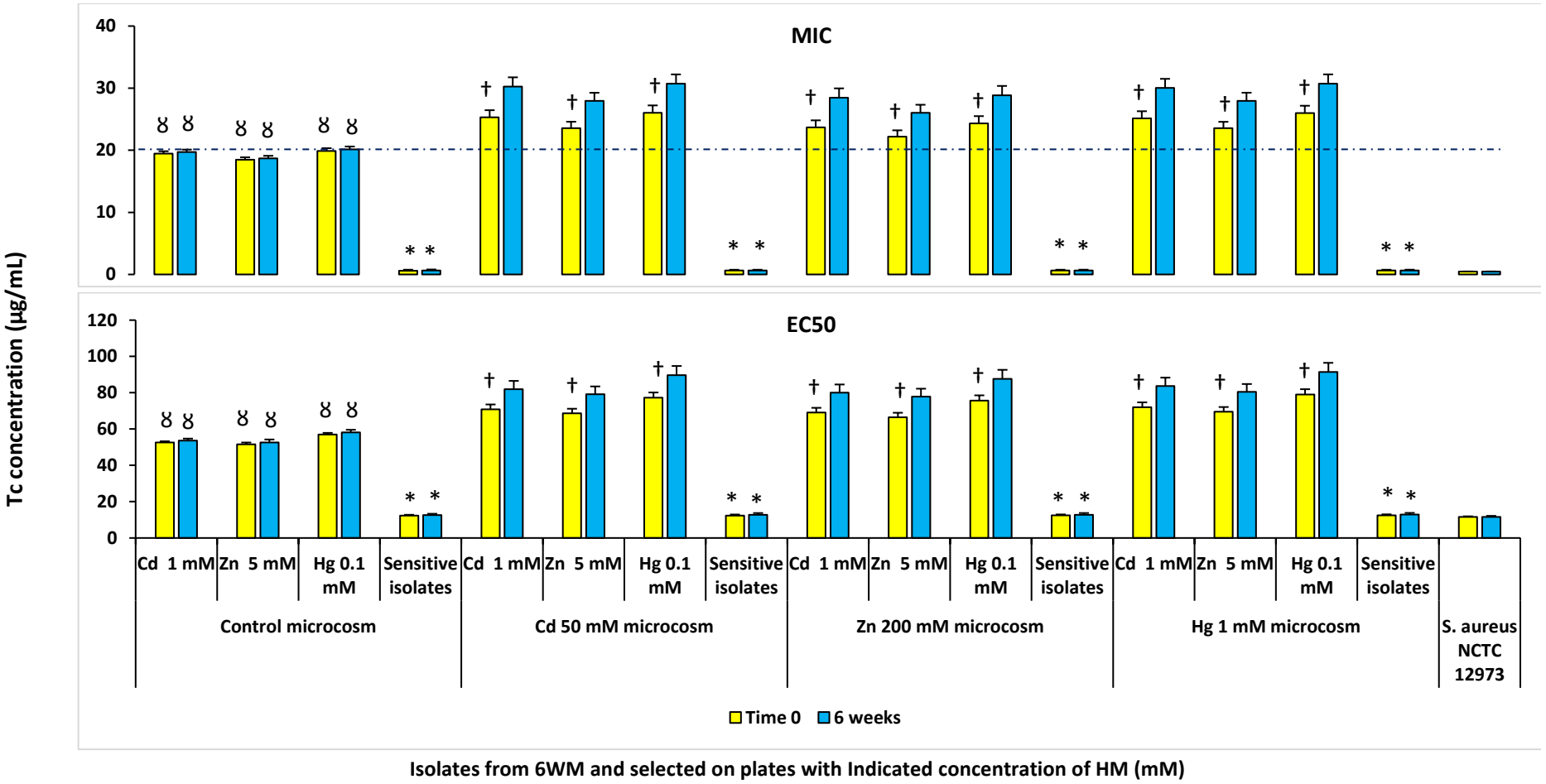


Fig. 5. 35. Mean MIC and EC50 values (µg mL^{-1}) of BM analysis with Tc for HMR isolates from 6WM. *$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation. The dash line defines AbR level of soil bacteria.

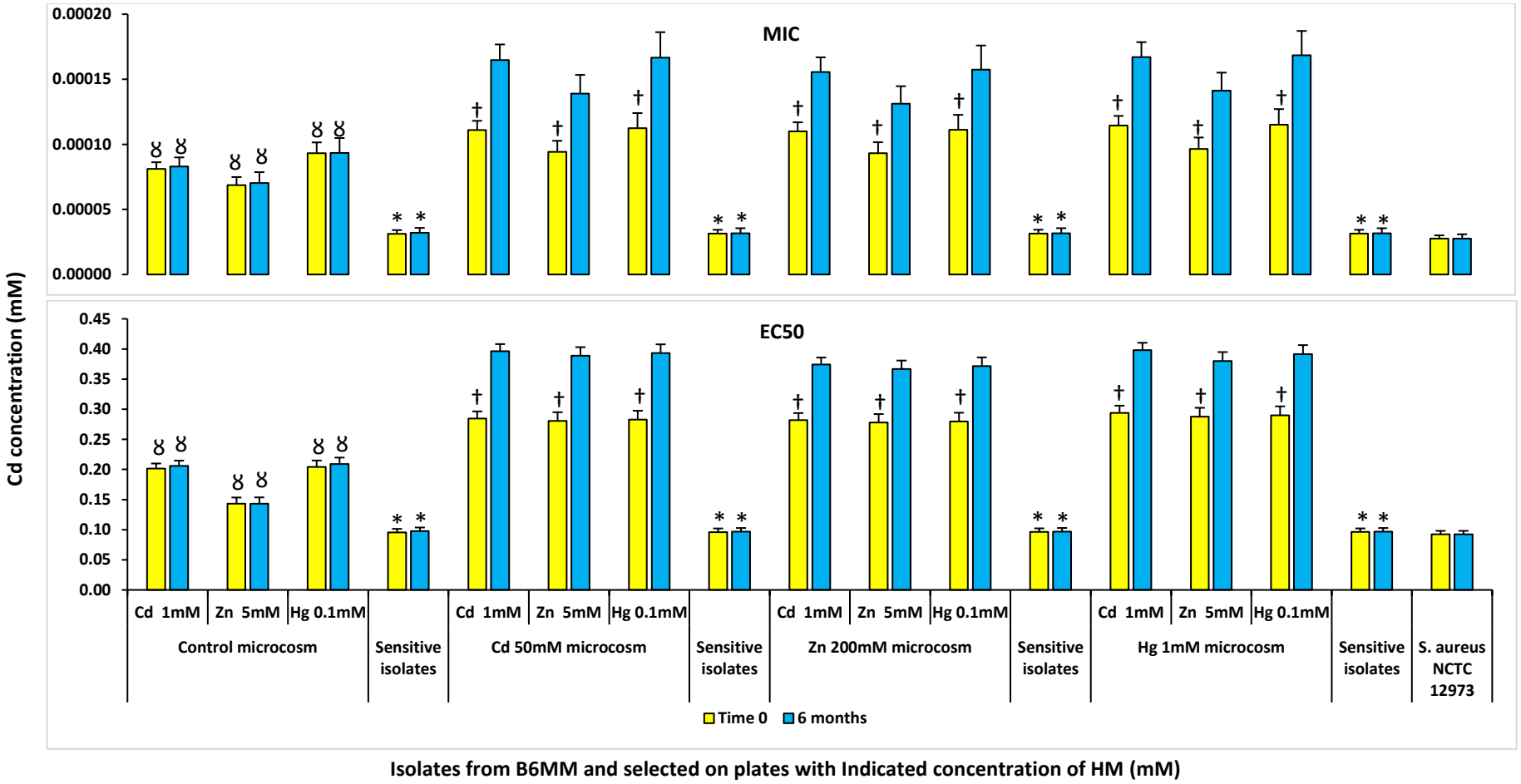


Fig. 5. 36. Mean MIC and EC50 values (mM) of BM analysis with Cd for HMR isolates from B6MM. *$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

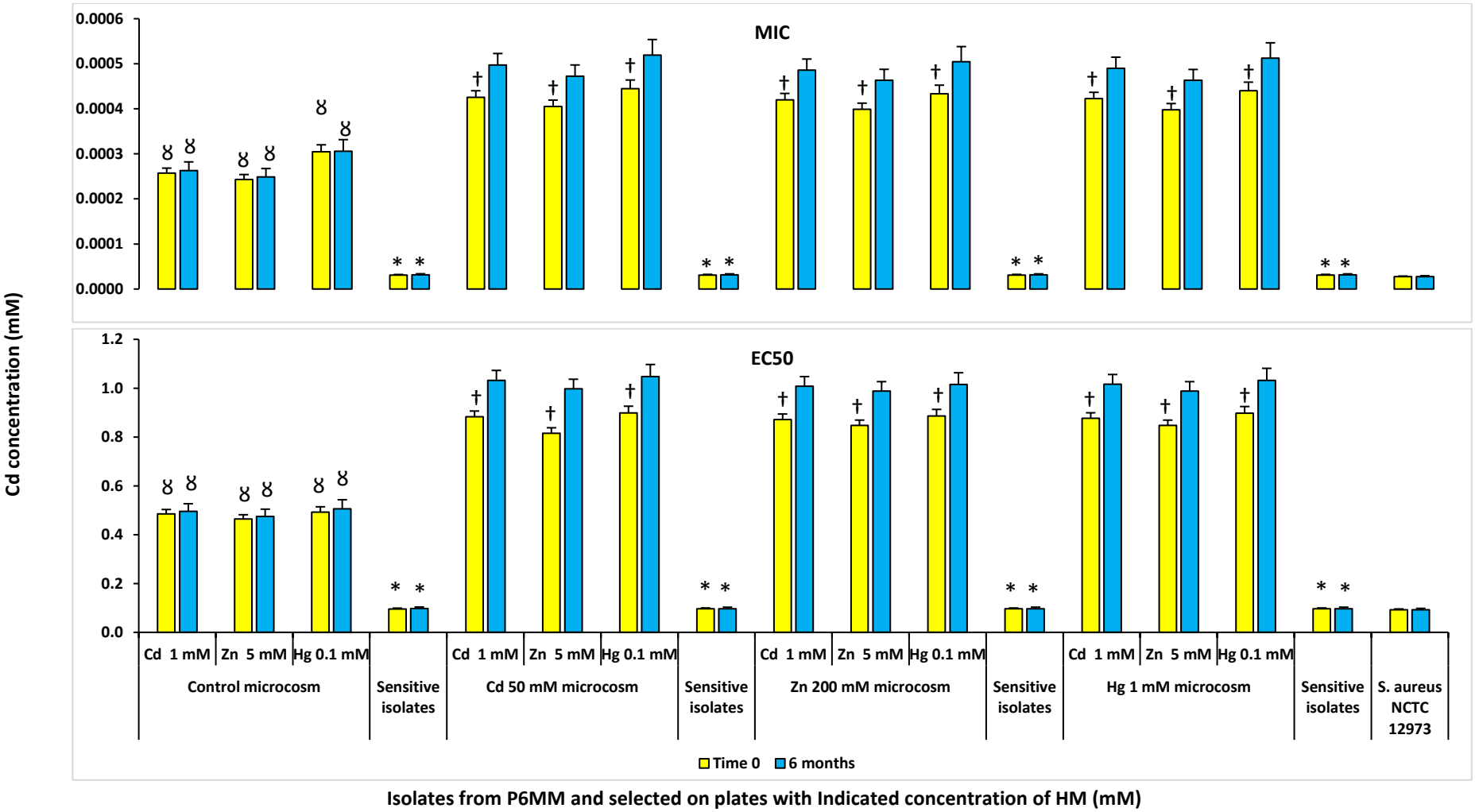


Fig. 5. 37. Mean MIC and EC50 values (mM) of BM analysis with Cd for HMR isolates from P6MM. *$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cd MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

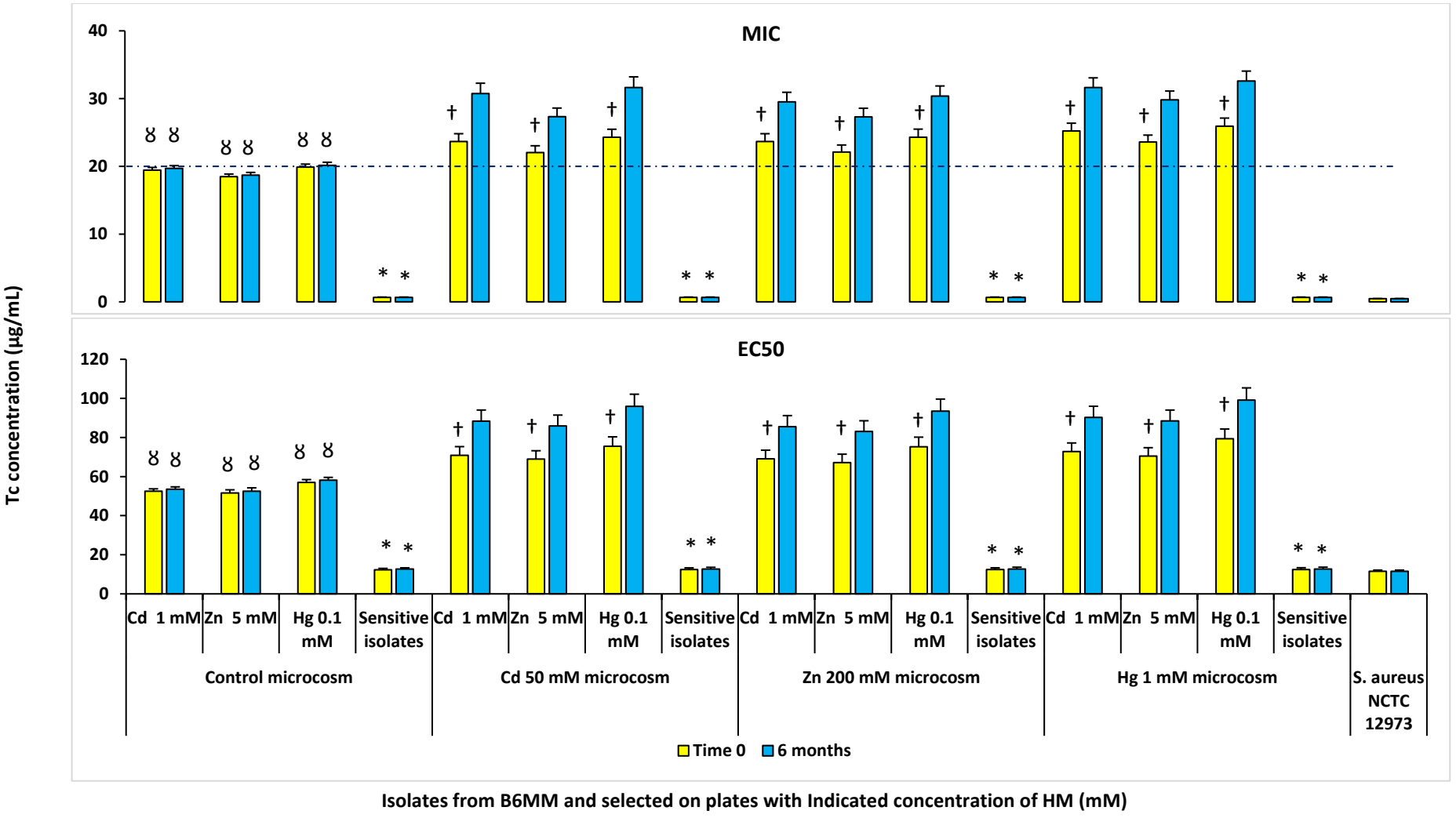


Fig. 5. 38. Mean MIC and EC50 values (µg mL^{-1}) of BM analysis with Tc for HMR isolates from B6MM. *$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

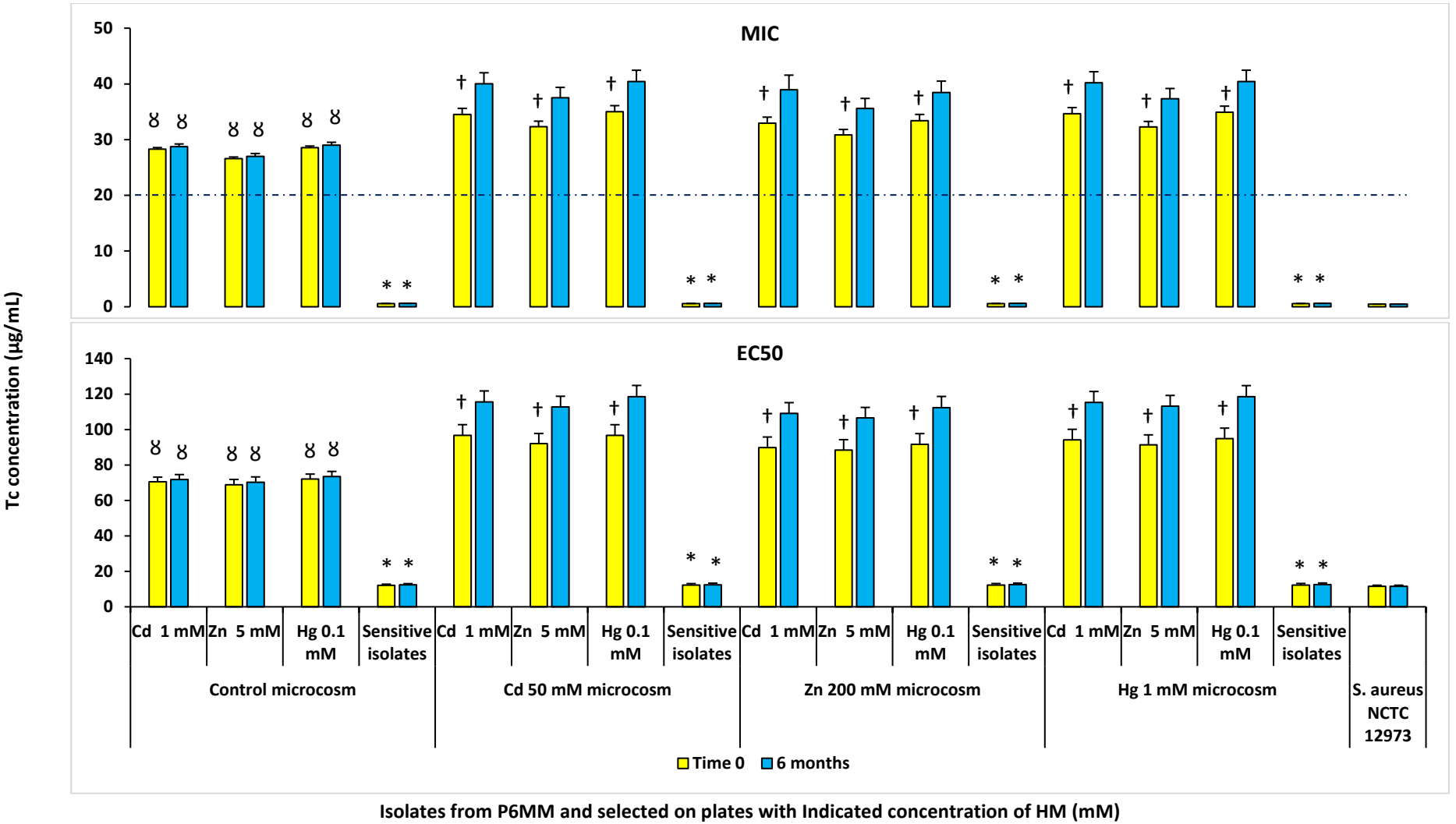


Fig. 5. 39. Mean MIC and EC50 values (µg mL^{-1}) of BM analysis with Tc for HMR isolates from P6MM. *$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Tc MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

5.1.7. Microcosms leachate metal concentration analysis

The concentration of HMs in drainage leachate samples collected from 6WM, B6MM and P6MM trays after 6 hours of incubation were analysed by Atomic Absorption Spectrophotometry (AAS).

The result showed Cd, Zn and Hg were adsorbed strongly to the background soil particles in 6WM after 6 hours of incubation. In general, less than 1% of the added HMs were detected in the microcosms' drainage after 6 hours of incubation (Table 5.2).

Leachate HMs analysis for B6MM and P6MM implied that HMs concentrations were increased in soils from both B6MM and P6MM by addition of a range concentrations of HMs to microcosms. The amount of adsorption of HMs to the soil particles was higher in microcosms with pastoral soil microcosms. It shows the substantial adsorption of HMs to the soils particles. (Tables 5.3).

Table 5. 2. Leachate HM concentration analysis of 6WM after 6 hours of incubation.

Microcosm	Microcosm HM conc. (mg L^{-1})	Original volume of added HM stock (L)	Total HM originally added (mg)	Total HM left in drainage after adsorption (mg)	Total HM lost by adsorption (mg)	Increase of HM conc. in soil (mg kg^{-1})	HM total adsorption (%)
Cd-spiked	Control	0.3	0	0.0026	-0.0026	0	0
	1	0.3	0.3	0.0087	0.29	1	97.09
	5	0.3	1.5	0.011	1.48	5	99.26
	10	0.3	3	0.019	2.98	10	99.36
	50	0.3	15	0.084	14.91	50	99.43
	100	0.3	30	0.19	29.80	99	99.36
Zn-spiked	Control	0.3	0	0.011	-0.011	0.0	0
	20	0.3	6	0.043	5.95	19.9	99.26
	50	0.3	15	0.057	14.94	49.8	99.61
	100	0.3	30	0.065	29.93	99.8	99.78
	200	0.3	60	0.22	59.77	199.3	99.62
	300	0.3	90	0.40	89.59	298.6	99.54
Hg-spiked	Control	0.3	0	0	0	0.0	0
	0.5	0.3	0.15	0.0038	0.14	0.5	98.93
	1	0.3	0.3	0.0077	0.29	1.0	98.75
	5	0.3	1.5	0.038	1.46	4.9	98.55
	10	0.3	3	0.077	2.92	9.7	98.52
	50	0.3	15	0.38	14.61	48.7	98.50

Table 5. 3. Leachate HM concentration analysis of B6MM and P6MM after 6 hours of incubation.

Microcosm	Microcosm HM	Microcosm HM conc. (mg L^{-1})	Original volume of added HM stock (L)	Total HM originally added (mg)	Total HM left in drainage after adsorption (mg)	Total HM lost by adsorption (mg)	Increase of HM conc. in soil (mg kg^{-1})	HM total adsorption (%)
B6MM	Cd-spiked	Control	0.3	0	0	0	0	0
		5	0.3	1.5	0.28	1.28	4.27	85.47
		10	0.3	3	0.61	2.34	7.83	78.30
		50	0.3	15	3.16	11.81	39.38	78.76
		100	0.3	30	6.09	23.93	79.77	79.77
		200	0.3	60	14.20	45.74	152.46	76.23
	Zn-spiked	Control	0.3	0	0	0	0	0
		20	0.3	20	0.54	5.47	18.25	91.26
		50	0.3	50	1.56	13.41	44.71	89.42
		100	0.3	100	3.10	26.83	89.43	89.43
		200	0.3	200	5.56	54.47	181.58	90.79
		300	0.3	300	8.10	81.87	272.90	90.97
	Hg-spiked	Control	0.3	0	0.001	0	0	0
		0.5	0.3	0.5	0.031	0.11	0.39	79.11
		1	0.3	1	0.058	0.24	0.80	80.56
		5	0.3	5	0.26	1.23	4.11	82.24
		10	0.3	10	0.56	2.43	8.11	81.13
		50	0.3	50	3.92	11.07	36.93	73.86
P6MM	Cd-spiked	Control	0.3	0	0	0	0	0
		5	0.3	1.5	0.19	1.30	4.35	87.06
		10	0.3	3	0.44	2.55	8.51	85.13
		50	0.3	15	2.51	12.49	41.63	83.27
		100	0.3	30	6.00	23.99	79.98	79.98
		200	0.3	60	11.45	48.55	161.83	80.92
	Zn-spiked	Control	0.3	0	0	0	0	0
		20	0.3	20	0.53	5.46	18.21	91.07
		50	0.3	50	1.25	13.74	45.82	91.65
		100	0.3	100	1.80	28.19	93.98	93.98
		200	0.3	200	4.02	55.97	186.59	93.30
		300	0.3	300	6.23	83.76	279.22	93.07
	Hg-spiked	Control	0.3	0	0.001	0	0	0
		0.5	0.3	0.5	0.01	0.13	0.44	89.20
		1	0.3	1	0.043	0.25	0.85	85.51
		5	0.3	5	0.14	1.35	4.51	90.28
		10	0.3	10	0.32	2.67	8.91	89.10
		50	0.3	50	1.60	13.39	44.63	89.28

5.1.8. Sequential extraction of HMs

Sequential extraction of HMs from B6MM and P6MM soils samples after 6 months of incubation using $MgCl_2$ to detect the exchangeable ions by AAS was performed. Mercury was more strongly bound onto the soil compared to Cd and Zn, so the 1 M $MgCl_2$ exchangeable fraction is always ≤3% of the total. Cadmium is at the other end of the scale, with a range of ˜63-98% exchangeable fraction, and zinc was about 14% on average. These results indicated that in terms of potential for release of HM ions into soil pore-water, the order is Cd >> Zn >> Hg (Table 5.4). Toxicity is influenced by that order, but microbes may often be able to access the metals that are bound to the soil phases less affected by 1 M $MgCl_2$, as well as this fraction, that is the 'most' readily available. Mercury for example is strongly bound to soil organic matter. Microbes could well have access to that Hg, to the extent they consume soil organic matter, and are part of the organic matter pool themselves.

Table 5. 4. HMs exchanable ions extracted from B6MM and P6MM after 6 months of incubation.

Microcosm	Microcosm HM	Microcosm HM conc. (mg L^{-1})	Measured HM conc. (mg L^{-1})	HM adsorped to soil (mg kg^{-1})	1 *M* $MgCl_2$ exchangeable fraction
B6MM	Cd-spiked	Control	0.021		
		5	0.13	4.27	76.89
		10	0.21	7.83	65.32
		50	1.13	39.4	69.38
		100	2.17	79.8	65.42
		200	4.03	153	63.26
	Zn-spiked	Control	0.027		
		20	0.063	18.3	8.38
		50	0.13	44.7	7.37
		100	0.20	89.4	5.50
		200	0.41	182	5.46
		300	0.66	273	5.85
	Hg-spiked	Control	<0.05		
		0.5	<0.05	0.39	< 3
		1	<0.05	0.80	< 3
		5	<0.05	4.11	< 3
		10	<0.05	8.11	< 3
		50	<0.05	33.6	< 3
P6MM	Cd-spiked	Control	0.05		
		5	0.17	4.35	97.75
		10	0.29	8.51	82.00
		50	1.34	41.6	77.87
		100	2.74	80.0	82.46
		200	5.75	162	85.27
	Zn-spiked	Control	0.46		
		20	0.42	18.2	56.67
		50	0.46	45.8	24.59
		100	0.37	93.9	9.57
		200	0.72	187.0	9.29
		300	1.10	279.0	9.46
	Hg-spiked	Control	<0.05		
		0.5	<0.05	0.44	< 3
		1	<0.05	0.85	< 3
		5	<0.05	4.51	< 3
		10	<0.05	8.91	< 3
		50	<0.05	44.6	< 3

5.1.9. Discussion

The physicochemical properties of the soil samples from microcosms with Waikato region pastoral and background soils are listed in the Tables 5.1 and A3.1 (Appendix 3). The soil types and major variables of the two sites are different and have had different land uses. Levels of organic matter, total P and trace elements reflect the histories and land management practices that have occurred on the properties since land clearance. One property stayed as an undisturbed 'background' site (still covered with native bush), and the second one has been used as a pastoral farm. The soil sample from native bush site was a sandy loam soil, classified as an Immature Orthic Pumice soil. Sample from pastoral site was a clay loam soil classified as a Typic

Orthic Brown Soil. These two sites were the sampling sites available at the time of performing the investigation, however, they have had different soil types. It was found that the history of land usage and levels of HMs concentrations were more substantial factors, influencing HM and Ab resistance levels in soil bacteria, rather than soil types. The background and pastoral soils with different levels of HMs concentrations were included in the 6-month microcosms to be able to contrast the levels of HMR and AbR bacteria in bacterial consortia and also individual isolates.

The concentration of heavy metals including Cd and Zn increased from native bush soils to pastoral soils. For pastoral soils the two main sources of elemental change were superphosphate fertilisers, which contain P, S, Ca, Cd, F and U; and facial eczema remedies, which contain elevated Zn [251, 252].

The levels of P (Table 4.33) (as a factor which increase the bioavailability of Cd and Zn in soil thorough an increase in soil fertility) in pasture soils (site EW-24) is higher compared to background soil (site EW-13) [262]. The levels of organic material in Waikato region pastoral soils are higher than the background soils, reflecting organic matter depletion in the latter [251]. Impacts on availability are harder to determine for several reasons. Some soil-related factors could work in opposition to each other. Cd and Zn bind to organic matter, usually reducing their bioavailability. Pastoral soils have more organic matter, but also have higher total Cd and Zn. Higher levels of Cd and Zn in pastoral soil could compensate for the higher levels of organic material [251].

However, more importantly, bioavailability is partly determined by various interactions between living organisms and the soil environment. Bioavailability can also be defined in different ways. The bioavailability measured as the relative proportion taken up within an organism can be significantly different from the bioavailability determined by a weak chemical extraction [294].

Overall, no specific assumptions could be made about the potential influence of bioavailability at the outset in this work. However, the results consistently suggest that effects of HMs levels

on microbial populations follow the order pastoral soils > background soils. This order is the same as for the total contaminant concentrations in these soils. This suggests that any differences in actual bioavailability of contaminants between the two different land uses to their microbial populations were not significant enough to noticeably influence the results.

Plate culturing of bacteria isolated from 6WM showed the ratios of HMR/total bacterial CFUs in control microcosms were significantly lower than those from HM-spiked microcosms. Gremion et. al., 2004 [295], reported the presence of HMs in microcosm soil as the most drastic factor by which bacterial population and communities are changed. There are reports indicating the presence of high levels of HMs in soil for a short period of time can induce resistance reactions in the bacterial communities, although adaptation to HMs stresses can also happen over longer periods of time, especially with levels much higher than bacterial tolerance thresholds as exists in the microcosms used here [264, 270]. Caliz et. al., 2011 [202], reported that the bioavailability of HMs is a substantial factor in effectively inducing bacterial resistance, along with the potential toxicity of the HMs, which is Cd and Hg > Zn in this study. Soil moisture content is likely the important factor affecting bacterial community induced HM resistance which was kept consistent in the current investigation [269]. High levels of exchangeable HMs can lead to the higher levels of bacterial resistance to these HMs, as well as other HMs, and a range of antibiotics owing to various resistance mechanisms, e.g. same cellular efflux pumps and presence of resistance genes on the same genetic elements [16, 36, 253, 277, 278].

The levels of Ab resistance in bacteria isolated from 6WM were significantly higher in HM-spiked microcosms' soil compared to those from control microcosms. This suggests that inducing different levels of HM resistance in bacterial consortia in the presence of these HMs in soil can lead to co-resistance of various Abs [36, 253, 277, 278, 296].

Resistant bacterial counts from B6MM and P6MM showed the HMR/total bacterial CFU ratios were significantly higher in HM-spiked microcosms compared to those from control microcosms,

and these ratios were greater in P6MM compared to those from B6MM. It suggests that the long term usage of HM-contained compounds, e.g. facial eczema remedies, and the higher levels of Cd and Zn in pastoral soil used in P6MM has resulted in higher levels of HM resistance in bacteria isolated from pastoral soil [16]. In addition, different levels of carbon, nitrogen, and contaminants in this pastoral soil compared to the background soil, affects the levels of HM resistance in bacteria. It is suggested that bacterial resistance to Cd and Zn would result in greater levels of resistance to Hg too, due to various mechanisms, e.g. cellular efflux pumps [23, 271-275]. It means resistance to Hg happens in bacterial isolates which have Cd and Zn resistance mechanisms as efflux pumps. The levels of Hg in the soil samples used in this study were not higher in pastoral soils compared to background soil, but this feature can be interpreted as the main factor responsible for higher level of bacterial resistance to Hg for bacterial isolates from pastoral soils (with the higher levels of Cd and Zn concentrations) compared to background soil [16].

Similar to 6WM, the levels of HMR/total bacterial CFU ratios in both B6MM and P6MM were increased from Time 0 to the 2-month interval. The increase in this ratio was slower from 2 months onward in B6MM, while this ratio was almost same at 2, 4 and 6 months in P6MM (Figures 5.5-5.10). It suggests the adaptation of soil bacteria in both soil-type microcosms to the different levels of HMs present in soil after a period of time [264, 270]. While bacteria in background soil continue to develop resistance with a slower trend after 2 months of incubation compared to earlier incubation periods, the HM and Ab resistance of the bacterial consortia is mostly stable after 2 months of incubation compared to shorter incubation periods. It can be interpreted as the presence of higher levels HM and Ab's resistance mechanisms in bacterial consortia in pastoral soils which results in faster adaptation to high levels of HMs in soils, while these mechanisms continue to develop also after 2 month of incubation, although with a slower trend compared to the earlier period of incubation. Several reports mention the levels of exchangeable HMs in soil decrease over time, as we observed here from 2 months' incubation

onward [295, 297]. However, Acosta-Martínez et. al. (2010), suggested the bacterial adaptation to HMs happens in similar timeframes [264].

The MIC and EC50 values of HMs determined by the BM method for HMR isolates from 6WM soils differed between those for isolates from HM-spiked microcosms compared to those for isolates from control microcosm soils at Time 0 and after 6 weeks of incubation (Figures 4.49-4.51; and Figures A2.7-A2.11, Appendix 2). Isolates from HM-spiked microcosms soils had higher MIC and EC50 values for HMs with p-values as low as < 0.001 [282, 287, 288].

Greater MIC and EC50 values of Abs for 6WM soils' HMR isolates compared to those for HM-sensitive bacteria from the same microcosms soil were determined. Higher levels of HM resistance in bacteria can lead to higher levels of Ab resistance in bacteria [283, 287]. Ab resistance threshold in soil bacterial isolates is defined as growth at 20 μg mL^{-1} for all classes of Abs [217, 220]. According to this, the percentage of isolates categorised as resistant amongst HMR isolates were significantly higher than the HM sensitive bacteria, verifying the HM and Ab co-resistance phenomenon.

The results of BM analysis for bacteria from B6MM and P6MM soils showed there were significantly higher HM and Ab's MIC and EC50 values for bacteria from the HM-spiked microcosms at Time 0 and after 6 months of incubation compared to those from control microcosms. There were initial higher levels of HM and Ab's MIC and EC50 values for bacteria from P6MM microcosms compared to those from B6MM at Time 0, due to initial lower levels of HMs in background soil used for B6MM. In addition, according to the recommendations [217], there were significantly higher proportions of HMR and AbR bacteria in HM-spiked microcosms soils compared to those from soils of control microcosms. Similar to 6WM soils' bacteria, higher levels of HMs in 6-month microcosms' soils lead to the higher bacterial HM resistance, thereby, it may lead to higher levels of Ab resistance in bacteria according to the previous surveys [255, 283, 286, 287].

The results of PICT analysis of bacterial consortia for samples from 6WM, B6MM and P6MM soils revealed that there were greater HMs and Abs' MIC and EC50 values for HM-spiked microcosms compared to those from control microcosm soil. The presence of higher levels of HMs in HM-spiked microcosms soils and also based on previous reports [281-283], it is interpreted that added different concentrations of HMs into microcosms soils leads to higher levels of HM resistance in soil's bacterial isolates. This project's results and previous reports [281-283], can be interpreted that similar processes occur in the wider environment. Co-resistance for Ab can occur in bacterial isolates with different levels of HM resistance [284].

The analysis of HMs concentrations in the microcosms leachate at the time of 6 hours of incubation showed strong adsorption of Cd, Zn and Hg to the soil particles (Tables 5.2 and 5.3). It means the levels of HMs in microcosms soils were raised significantly and only a small amount of HM ions were detected in the microcosms leachate. The levels of organic matter in soil could be one of the main factors involved in HMs adsorption to the soil particles and subsequently may reduce the levels of bioavailability of HMs in soils [251].

The levels of exchangeable HMs ions in 6 months microcosms were determined. The aim of this analysis was to determine the levels of bioavailable HMs in microcosm soils after 6 months of incubation (Table 5.4), although bioavailability is also determined by interactions between the organisms and soil environment [251]. It can be interpreted that bacteria could be able to access the HM ions bound to soil particles more than those detected as exchangeable ions. However, this analysis used here is one of the methods to determine the available HM ions affecting soil bacteria and development of resistance [193].

In conclusion, this project showed that the ratios of HMR and AbR/total CFU bacteria are higher in soil samples with higher levels of HMs compared to those with lower concentrations. The bacteria subjected to the selective pressure of HMs in soil show higher resistance to different levels HMs and Abs. These findings support the hypothesis of this study that the levels of HM

and Ab resistance in soil bacteria is increased by elevated concentrations of HMs in the environment.

Chapter 6, Results Part 3

6.1. Study 3: Molecular and DNA Sequencing Investigations

6.1.1. Introduction

In response to accumulation of heavy metals in soils, some microorganisms including bacteria develop a range of resistance mechanisms, which work to reduce the impact of metal toxicity, as well as co-occurrence of antibiotics resistance. Several lines of evidence suggest that resistance to different kinds of HMs and Abs can occur in the presence of resistance genes. These genes induce resistance to HMs and Abs, posing a threat to both human and animal health. The focus of Study 3 was to investigate bacterial communities' structures in soil samples from WR, BRP, 6WM and 6MM. This was investigated using a Terminal Restriction Fragment Length Polymorphism (TRFLP) protocol. Further, selected BRP soil samples and 6WM soil samples, spiked with different levels of HMs were used as representative samples for investigations of bacterial communities' structure and metagenomic differences using Next Generation Sequencing (NGS) analysis. Genes profile differences which may have occurred due to presence of HMs in soil and involvement of these genes in the occurrence of HM and Ab resistance were investigated as described in this chapter. TRFLP and NGS are the established tools for these explorations [298-301]. Bacterial community structures in soil are changed in the presence of elevated levels of HMs and this can change lots of phenotypic characteristics of bacteria in soil. For example, levels of bacterial reproduction, introduction of extracellular compounds involved in biofilm production, introduction of tolerance features in the soil bacteria to a given HM or other HMs, and Metabolism changes of bacterial cells. Bacterial community structure changes can affect the quality of farmland and affect the quality and quantity of plant and animal production. Expanding knowledge to know which bacterial groups are affected more by HM levels in soil and its environmental and financial consequences are of importance, and justify conducting investigations on bacterial community structure in soils with elevated levels of HMs.

6.1.2. Methodology

The methodology used for this chapter is described in Section 3.12 of the Methodology Chapter. In short, soil DNA extraction, TRFLP and 16s rDNA were employed. In this part of the molecular investigation of soil bacterial communities' structure, selected BRP and 6WM soil samples with various levels of HMs, were used. The representative samples from BRP were B10 (70 m from the start of the airstrip), B14 (30 m from the start of the airstrip) and B17 (at the start of the airstrip) for 16s rDNA sequencing. The samples for 6WM were from 100 mM Cd-spiked, 200 mM Zn-spiked, 50 mM Hg-spiked and Control microcosms for 16s rDNA sequencing. The microcosms samples were selected according to the toxicity of the HMs, Hg > Cd > Zn to bacteria and their probable effect on bacterial community changes in soil. The samples from 200 mM Zn-spiked microcosm was chosen as Zn-microcosm representative; it was the same concentration which showed highest ratios of ZnR/total bacterial CFU compared to other microcosms spiked with a range of concentrations of Zn. The sample from 100 mM Cd-spiked microcosm was chosen as Cd-microcosm representative; it was one concentration higher than the concentration which showed highest ratios of CdR/total bacterial CFU compared to other microcosms spiked with Cd. The sample from 50 mM Hg-spiked microcosms was chosen as Hg-microcosm representative; it was two concentrations higher than the concentration which showed highest ratios of HgR/total bacterial CFU compared to other microcosms spiked with a range of Hg concentrations.

Figure 6.1 illustrates different steps and experiments used for Chapter 6.

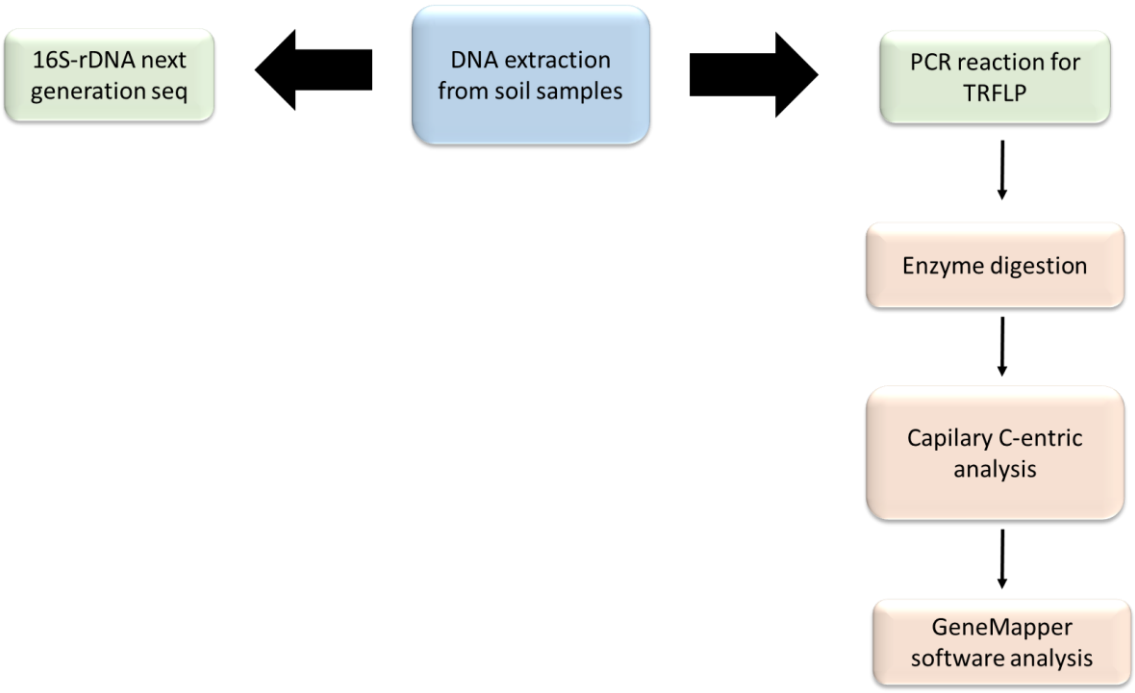


Fig. 6. 1. Flow chart illustrating different steps and experiments of Chapter 6.

6.1.3. Terminal Restriction Fragment Length Polymorphism (TRFLP)

DNA extraction from the soil samples and PCR reactions targeting the bacterial 16s rDNA gene and restriction endonuclease digestion of these amplimers using *Msp*I was performed (Section 3.12.3). The TRFLP samples were applied to an ABI3730 Capillary Genetic Analyser. The raw data were processed on GeneMapper software and non-metric Multi-Dimensional Scaling (NMS) analysis was performed with PRIMER v. 7 software using the Bray-Curtis similarity index [233]. The data for bacterial community structures were analysed by three-way ANOVA. Soil samples, history of soils usage (for samples from Waikato Region) and distance of sub-sets from the fertiliser storage (for Belmont Park soil samples), and soils HM concentrations were introduced to the analysis as independent variables. The dependent variable was the number of T-RFs reads. Three-way ANOVA was conducted for Terminal Restriction Fragment Length Polymorphism (TRFLP) analysis of soil samples from microcosms. Soil samplings at appropriate intervals (2 weeks or 2 months), initial soils HM concentrations and concentration of HM additives to the microcosms were introduced to the analysis as independent variables. The dependent variable was the number of T-RFs reads.

6.1.3.1. Waikato Region soil samples

Soil samples collected from WR, including pastoral, arable and background soils of WRSS1, WRSS2 and WRSS3 were analysed in this part of the study. The result of NMS analysis of WR soil communities' structure, analysed according to the abundance and size of bacterial T-RFs, showed there were >80% similarity between the bacterial communities from background soils on one hand and those from pastoral and arable soil on the other hand. There were >40% similarity between bacterial communities from background, pastoral and arable soils. Three-Way ANOVA analysis determined that there were significant difference between the relative abundance of T-RFs in background soils' bacterial communities compared to those for pastoral and arable soils ($p < 0.05$) (Figure 6.2).

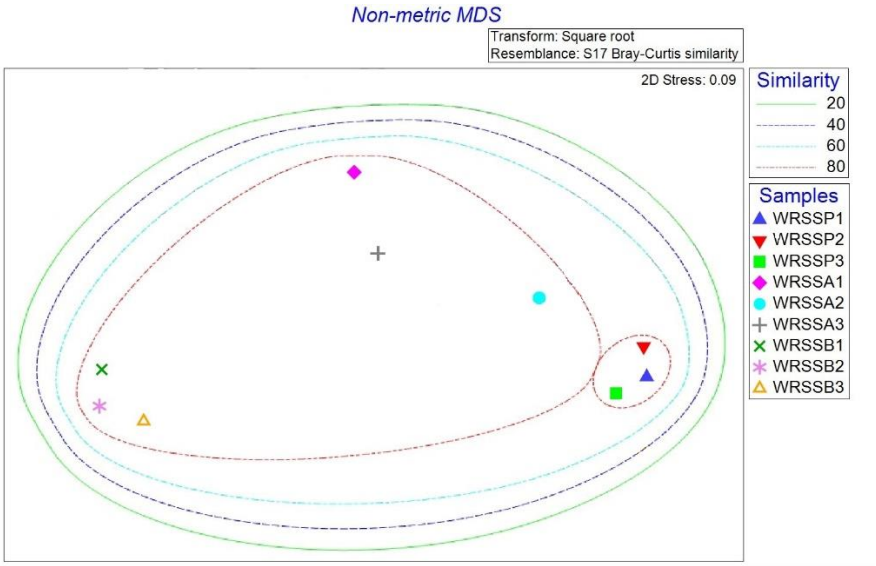


Fig. 6. 2. NMS analysis plot of TRFLP relative peak height for WR soils' bacterial communities' data, using the Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the two clusters characterised with >80% of similarity.

6.1.3.2. Belmont Regional Park soil samples

A total number of 8 pastoral soil samples collected from the airstrip at BRP were analysed in this part of the investigation. The result of NMS of data from BRP soil samples' bacterial communities showed there was >80% similarity between the bacterial communities from B17 and B16 soils.

In addition, there were >80% similarity between the bacterial communities from B10-B15 soil samples. The data determined that there were >40% similarity between all of bacterial communities from BRP soils. Three-way ANOVA showed there were significant differences between the relative abundance of T-RFs in B17 and B16 soils' bacterial communities compared to those form other BRP soil samples ($p < 0.05$) (Figure 6.3).

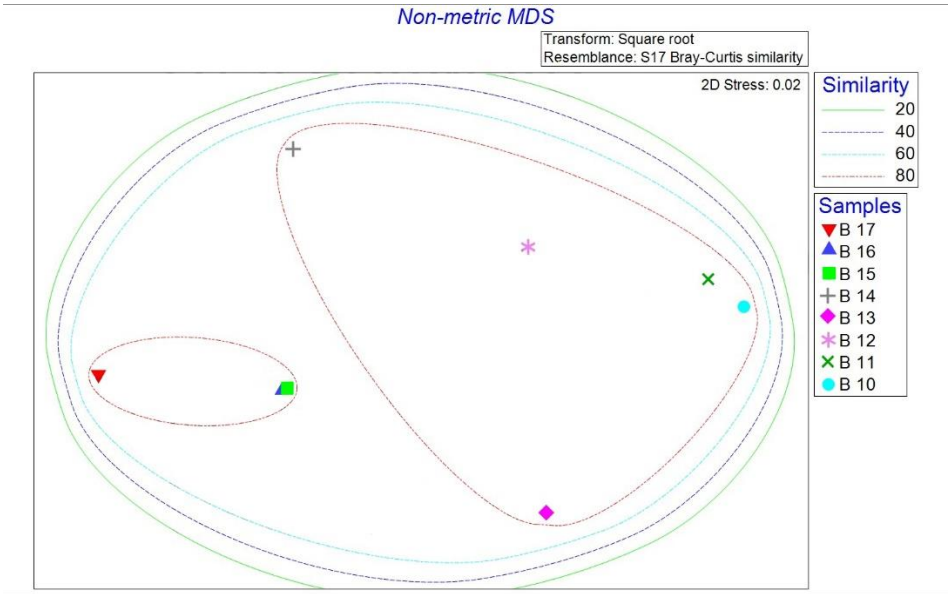


Fig. 6. 3. NMS analysis plot of TRFLP relative peak height for BRP soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the two clusters specified with >80% of similarity.

6.1.3.3. TRFLP Analysis of Soil DNA from 6-week Microcosms

6.1.3.3.1. Cd-spiked microcosms (6WCdM)

The result of NMS of data from 6WM bacterial communities showed at Time 0 there were 60-80% similarity between the bacterial communities from control, 5 and 10 mM Cd-spiked microcosms. In addition, bacterial communities from Cd 50, 100 and 200 mM microcosms were > 80% similar at Time 0. At week 2, the Cd spiked communities appear to be transitioning towards greater similarity with each other. After 4 and 6 weeks the bacterial communities from most of Cd-spiked communities were >80% similar and only 60% similar to the control microcosm's bacterial communities. Three-Way ANOVA showed that there were significant differences between the relative abundance of bacterial T-RFs in the control microcosm

compared to those from 50, 100 and 200 mM Cd-spiked microcosms' bacterial communities at Time 0, and 2, 4 and 6-week timepoints; and also those from all of 6WCdM at 6-week time-point ($p < 0.05$) (Figure 6.4).

6.1.3.3.2. Zn-spiked microcosms (6WZnM)

The NMS result on data from 6WM soil samples revealed that bacterial communities from control, 20 and 50 mM Zn microcosms were 60-80% similar at Time 0 and 2 weeks. The bacterial communities from 100, 200 and 300 mM Zn microcosms were >80% similar at Time 0 and 2-week time-points. After 4 or 6 weeks the bacterial communities from 20 and 50 mM Zn and 100, 200 and 300 mM Zn microcosms became more similar and less similar to the control. Three-Way ANOVA revealed that there were significant differences between the relative abundance of bacterial T-RFs in the control microcosms compared to those from 100, 200 and 300 mM Zn at Time 0 and 2-week timepoints and also all of the 6WZnM at Time 0 and after 2, 4 and 6 weeks of incubation ($p < 0.05$) (Figure 6.5).

Hg-spiked microcosms (6WHgM)

The NMS analysis of 6WHgM soil samples' bacterial communities revealed that there were >80% similarity between bacterial communities from control, 0.5 and 1 mM Hg microcosms, and also between communities from 5, 10 and 50 mM Hg microcosms at Time 0. The similarity of the communities from control microcosms to other Hg-spiked microcosms changed to >60% after 2 weeks. The bacterial communities from 0.5, 1, 5 and 10 mM Hg microcosms showed >80% similarity after 2 weeks. In addition, the communities from 1, 5, 10 and 50 mM Hg were >80% similar at 2-week timepoint. After 6 wees, the similarity of control microcosm's communities to other microcosms reduced by >40%. The communities from 0.5 and 1 mM Hg microcosms were >80% similar after 4 and 6 weeks. Moreover, communities from 5, 10 and 50 mM Hg microcosms showed >80% similarity. Three-Way ANOVA showed there were significant differences between

the relative abundance of bacterial T-RFs in the control microcosms compared to those from 6WHgM at 2, 4 and 6-weeks timepoints ($p < 0.05$) (Figure 6.6).

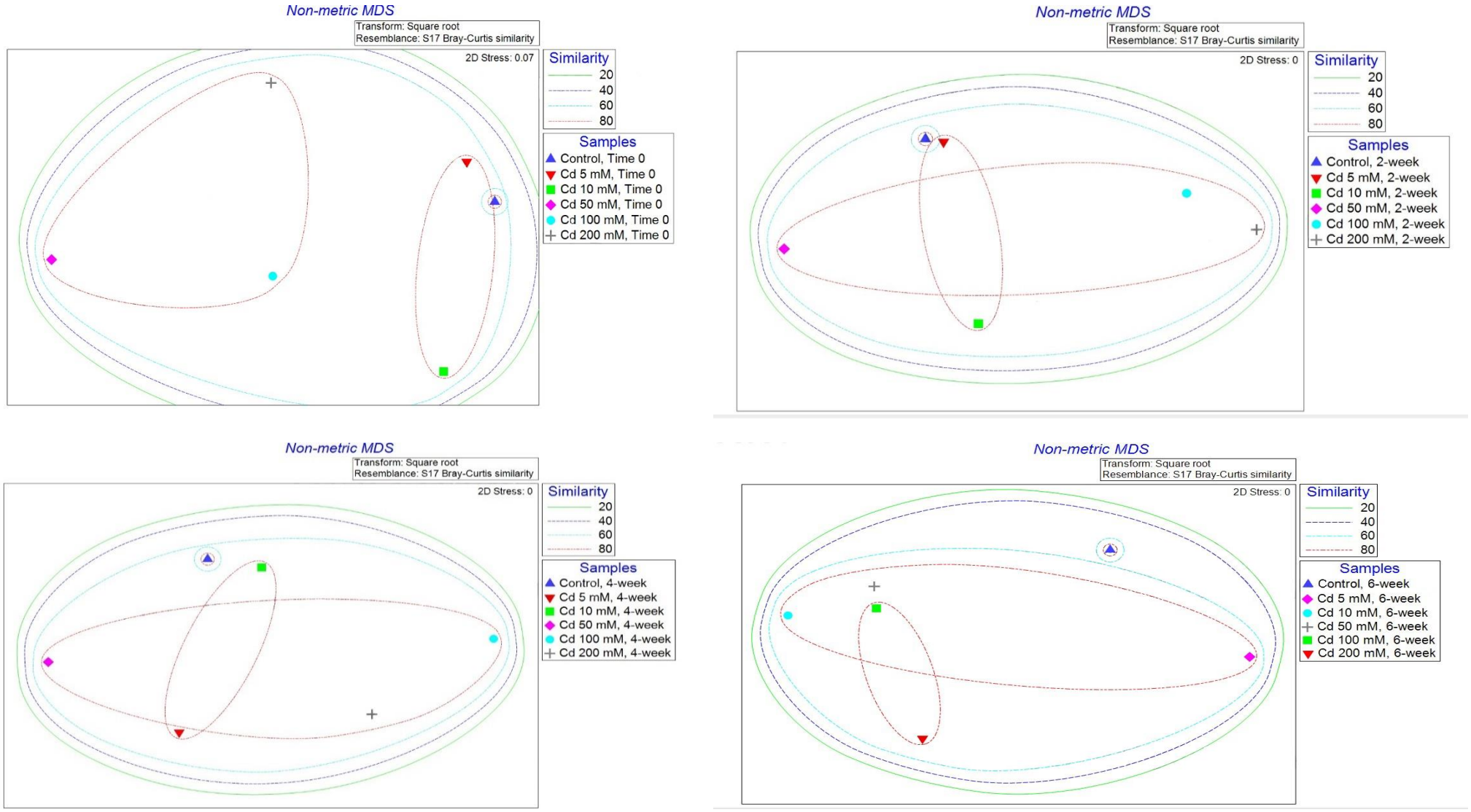


Fig. 6. 4. NMS analysis plot of TRFLP relative peak height for 6WCdM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the clusters specified with >60% of similarity compared to the control microcosms'.

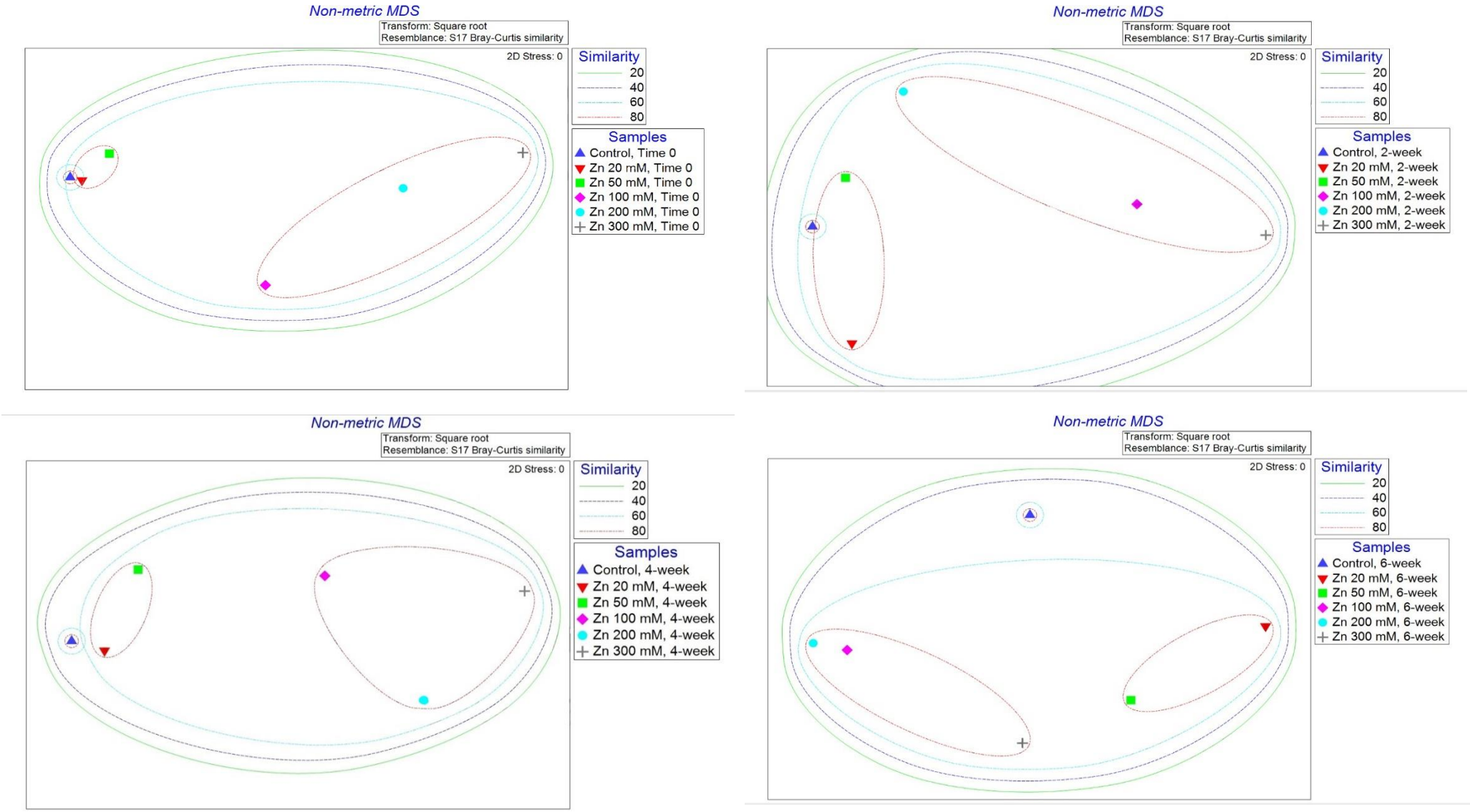


Fig. 6. 5. NMS analysis plot of TRFLP relative peak height for 6WZnM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the clusters specified with >60% of similarity compared to control microcosms'.

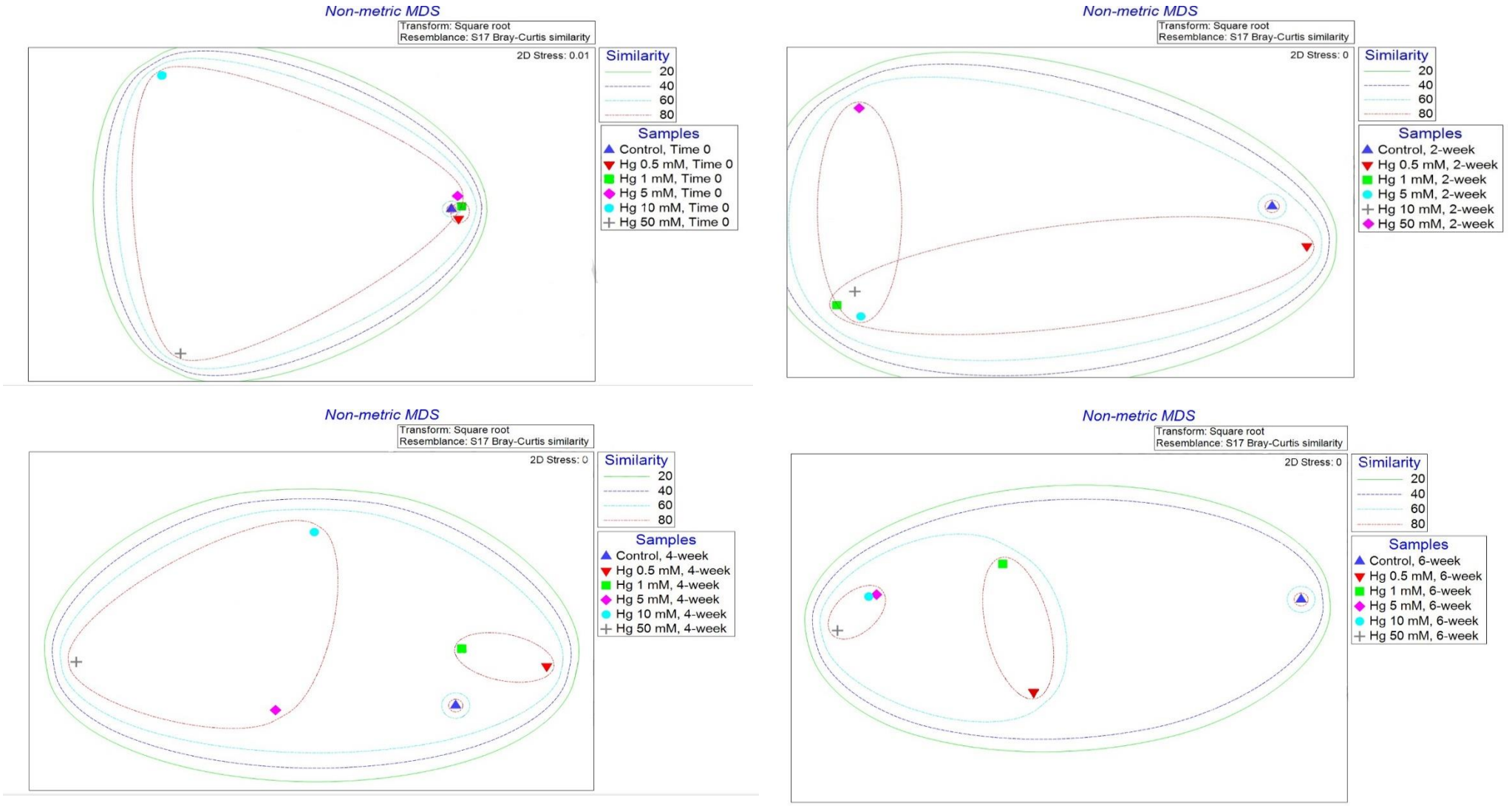


Fig. 6. 6. NMS analysis plot of TRFLP relative peak height for 6WHgM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the clusters specified with >60% similarity compared to control microcosms'.

4.3.3.4. 6-month microcosms soil samples

Cd-spiked background soil microcosms (B6MCdM)

NMS of data from B6MCdM soil samples showed there was >40% similarity between the bacterial communities from control microcosm and those from Cd-spiked microcosms. There was >60% similarity between the bacterial communities from Cd-spiked microcosms soils at Time 0 and after 2, 4 and 6-months of incubation. There was >80% similarity between bacterial communities from Cd 5 and 10 mM-spiked microcosms at Time 0 and after 2, 4 and 6-months of incubation. Similarly, there was >80% similarity between Cd 50, 100 and 200 mM-spiked microcosms at the mentioned time intervals. Three-Way ANOVA analysis showed there were significant differences between the relative abundance of bacterial T-RFs in the control microcosms compared to B6MCdM soils' bacterial communities at all of the time intervals ($p < 0.05$) (Figure 6.7).

Zn-spiked background soil microcosms (B6MZnM)

The NMS of data from B6MZnM soil samples revealed that there was >40% similarity among all of the bacterial communities from Zn-spiked background soil microcosms at Time 0 and after 2, 4 and 6 months of incubation. There was >60% similarity between the bacterial communities from B6MZnM at Time 0 and after 2, 4 and 6-months of incubation. In addition, there was >80% similarity between the bacterial communities from B6MZnM spiked with 20 and 50 mM concentrations of Zn at Time 0 and after 2, 4 and 6-months of incubation. Likewise, there was >80% similarity between B6MZnM spiked with 100, 200 and 300 mM of Zn at all of the mentioned time intervals. Three-Way ANOVA analysis showed there were significant differences between the relative abundance of bacterial T-RFs in the control microcosms compared to those from Zn-spiked B6MM at Time 0 and after 2, 4 and 6 months of incubation ($p < 0.05$) (Figure 6.8).

Hg-spiked background soil microcosms (B6MHgM)

The NMS of data from B6MHgM soil samples showed that there was >40% percent similarity among the bacterial communities from control microcosms compared to those from the B6MHgM. Similarity of >60% was determined between Hg-spiked microcosms at Time 0 and after 2, 4 and 6 months of incubation. In addition, there was >80% similarity between bacterial communities from Hg 0.5 and 1 mM-spiked microcosms on one hand and those from Hg 5, 10 and 50 mM-spiked microcosms at Time 0. Similarity of >80% was detected between bacterial communities from B6MHgM spiked with 1, 5, 0 and 50 mM concentrations of Hg after 2 months of incubation, however, there was >80% similarity between Hg 0.5 and 1 mM-spiked microcosms at the 2-months time interval. In addition, there was >80% similarity between bacterial communities from Hg 0.5, 1, 10 and 50 mM-spiked B6MHgM after 4 months of incubation, however, similarity of >80% was detected between bacterial communities from Hg 5, 10 and 50 mM-spiked B6MHgM. At the time interval of 6 months of incubation similarity of >80% was determined between all of the B6MHgM, although, the same similarity level was detected between those from Hg 5, 10 and 50 mM-spiked B6MHgM. Statistical analysis using three-Way ANOVA showed there were significant differences between the relative abundance of bacterial T-RFs from control microcosms compared to those from B6MHgM at Time 0 and after 2, 4 and 6 months of incubation ($p < 0.05$) (Figure 6.9).

Cd-spiked pastoral soil microcosms (P6MCdM)

The NMS of data from P6MCdM soil samples showed there was >40% similarity among the bacterial communities from control microcosm and those from Cd-spiked microcosms. There was >60% similarity between the bacterial communities from Cd-spiked microcosms soils at Time 0 and after 2, 4 and 6 months of incubation. Similarity of >80% was detected between bacterial communities from Cd 5 and 10 mM-spiked microcosms at Time 0 and after 2, 4 and 6-months of incubation. Similarly, there was >80% similarity between Cd 50, 100 and 200 mM-

spiked microcosms at the mentioned time intervals. Three-Way ANOVA statistical analysis showed there were significant differences between the relative abundance of bacterial T-RFs from control microcosms compared to those from the P6MCdM soils ($p < 0.05$) (Figure 6.10).

Zn-spiked pastoral soil microcosms (P6MZnM)

The NMS of data from P6MZnM soil samples showed there was >40% similarity among the bacterial communities from control microcosm and P6MZnM at Time 0 and after 2, 4 and 6-weeks of incubation. There was >80% similarity between the bacterial communities from P6MZnM soils at all of the time points. Similarity of >80% was detected between bacterial communities from Zn 20 and 50 mM-spiked microcosms on one hand and those from Zn 100, 200 and 200 mM-spiked microcosms on the other hand at all of the time intervals. Three-Way ANOVA showed there were significant differences between the relative abundance of bacterial T-RFs in the control microcosms compared to those from P6MZnM at Time 0, and after 2, 4 and 6 months of incubation ($p < 0.05$) (Figure 6.11).

Hg-spiked pastoral soil microcosms (P6MHgM)

The NMS of data from P6MHgM soil samples' bacterial communities revealed that there was >20% similarity among the bacterial communities from control microcosms and all of the Hg-spiked microcosms. In addition, there was >60% similarity between P6MHgM at all of the time intervals. Similarity of >80% was determined between bacterial communities from Hg 0.5 and 1 mM-spiked microcosms and also between those from Hg 5, 10 and 50 mM-spiked microcosms at all of the time intervals. Three-Way ANOVA determined that there were significant differences between the relative abundance of bacterial T-RFs from the control microcosms compared to those from P6MHgM at Time 0 and after 2, 4 and 6 months of incubation ($p < 0.05$) (Figure 6.12).

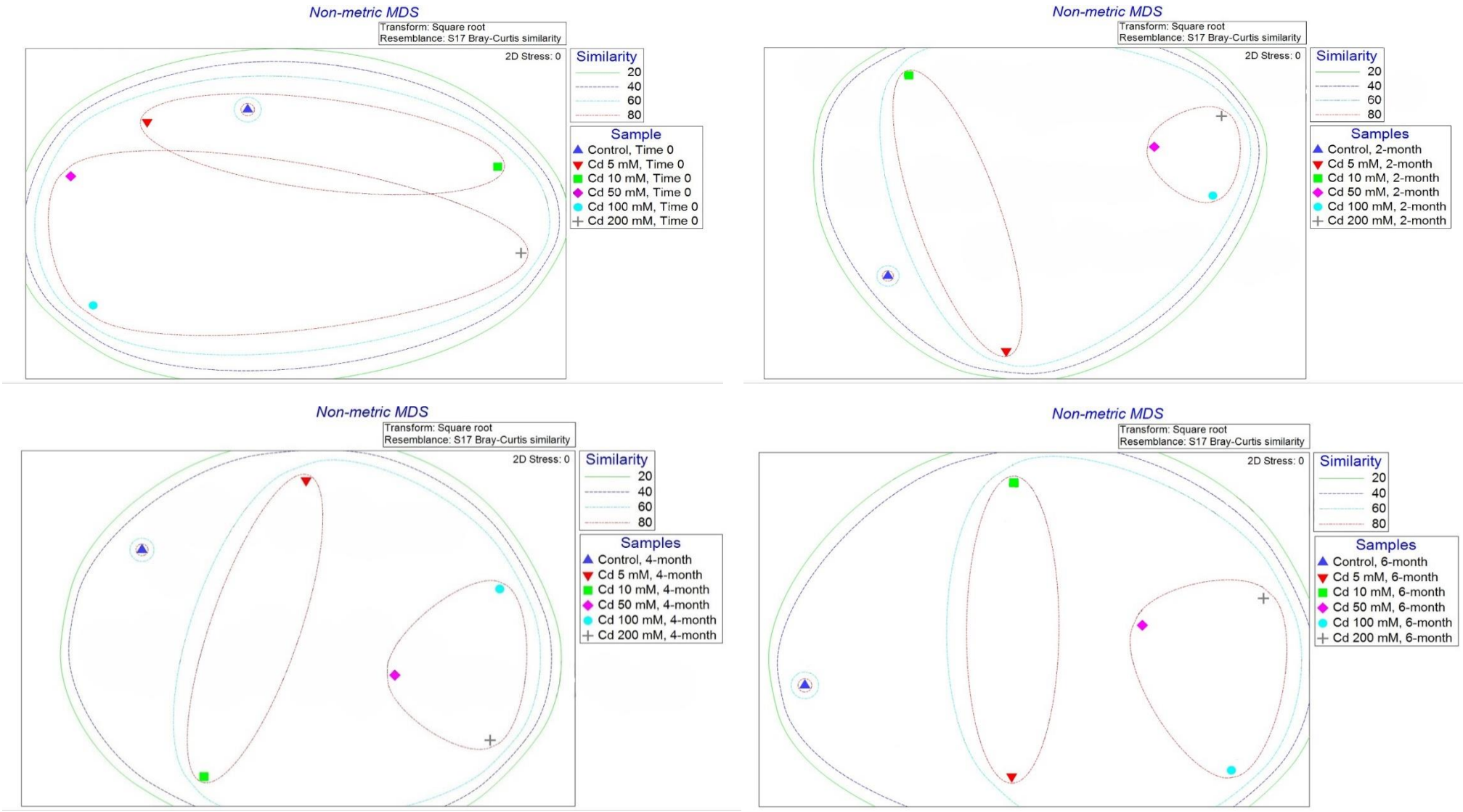


Fig. 6. 7. NMS analysis plot of TRFLP relative peak height for B6MCdM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

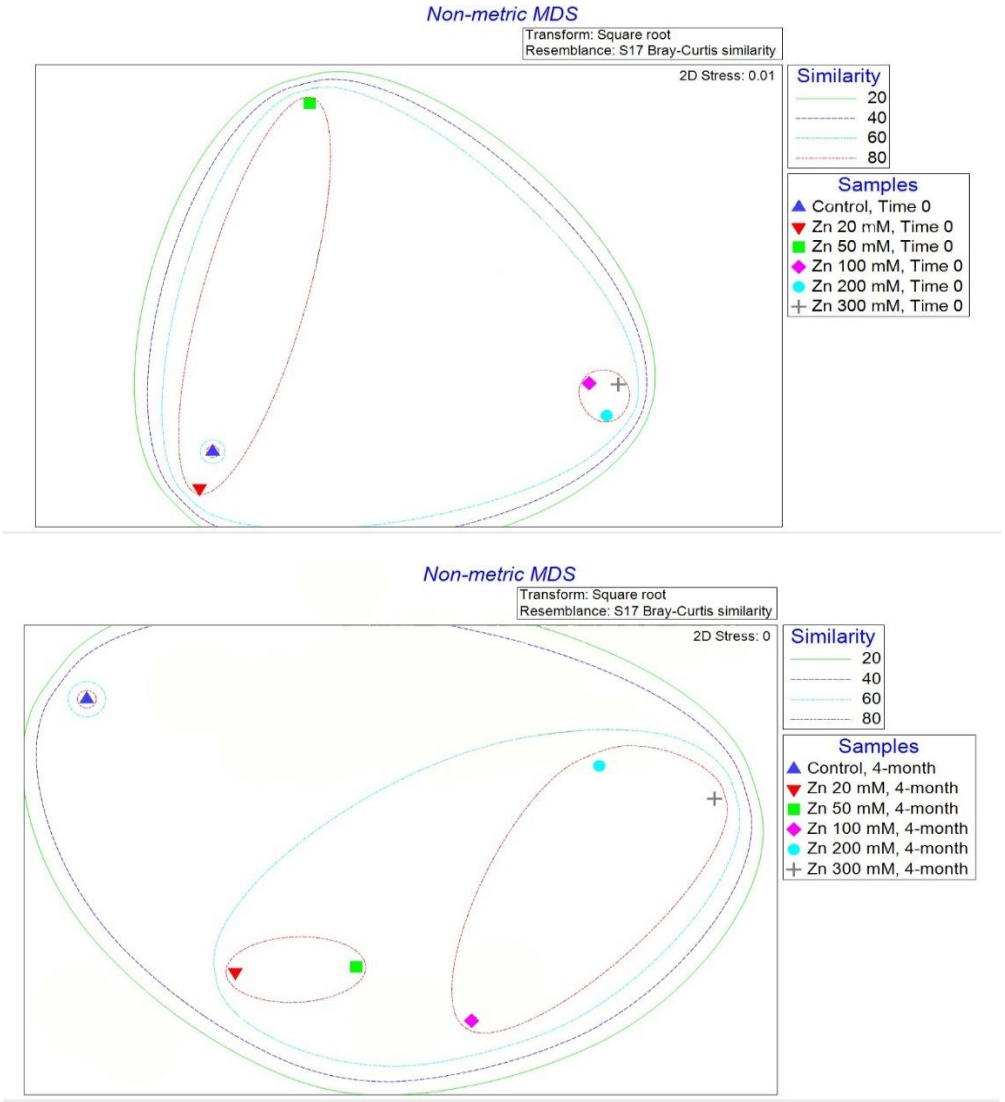


Fig. 6. 8. NMS analysis plot of TRFLP relative peak height for B6MZnM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

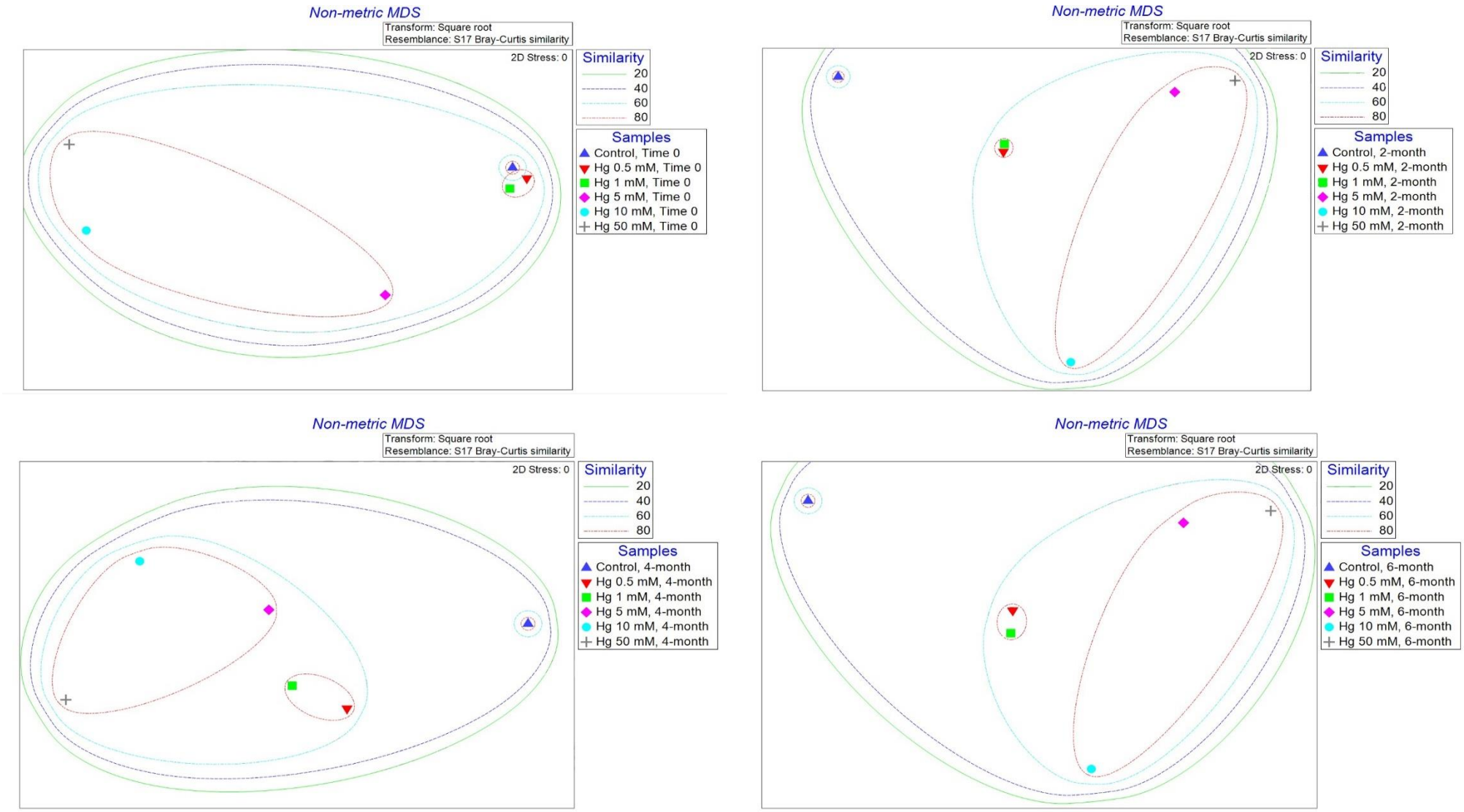


Fig. 6. 9. NMS analysis plot of TRFLP relative peak height for B6MHgM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

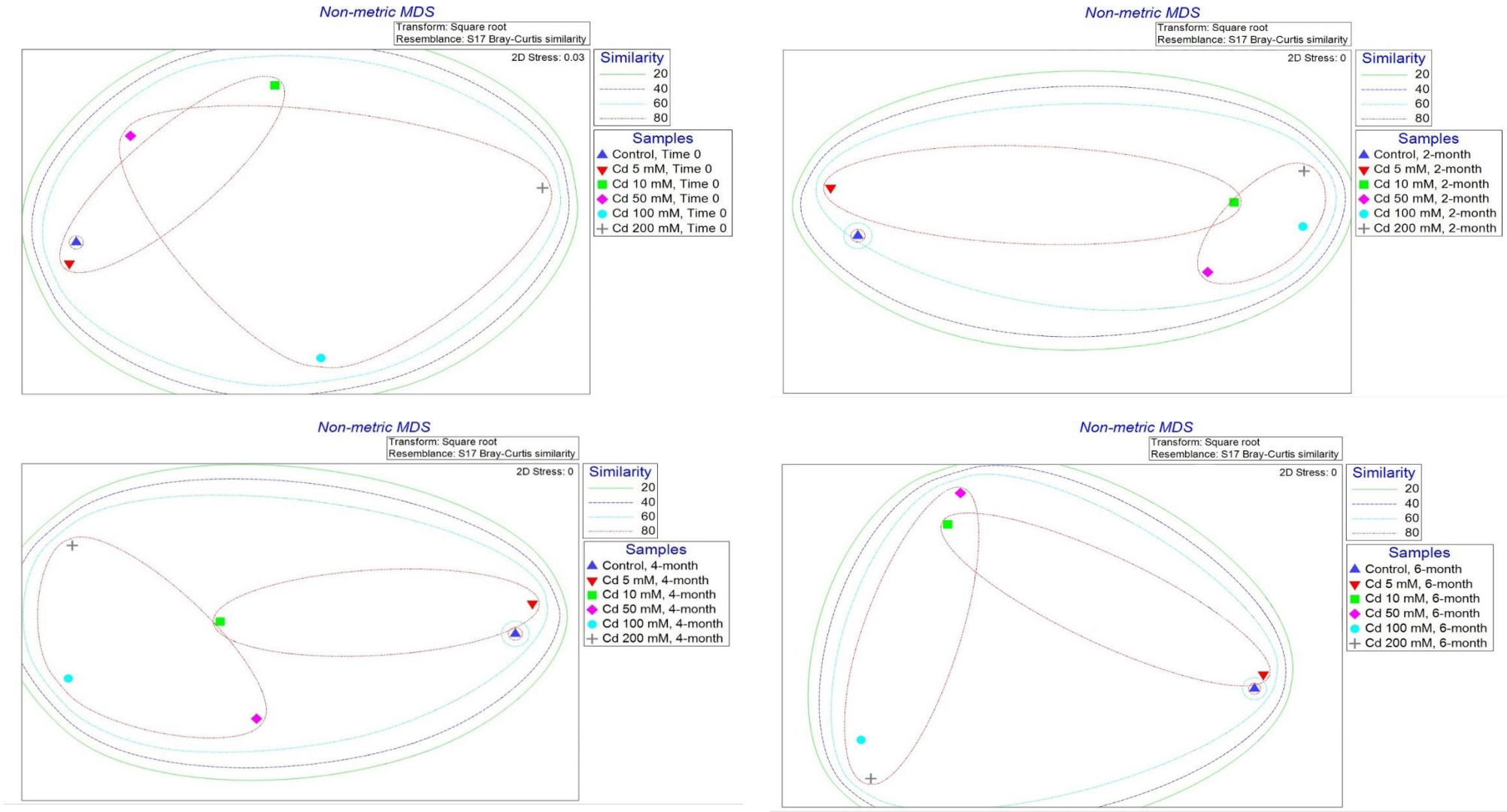


Fig. 6. 10. NMS analysis plot of TRFLP relative peak height for P6MCdM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

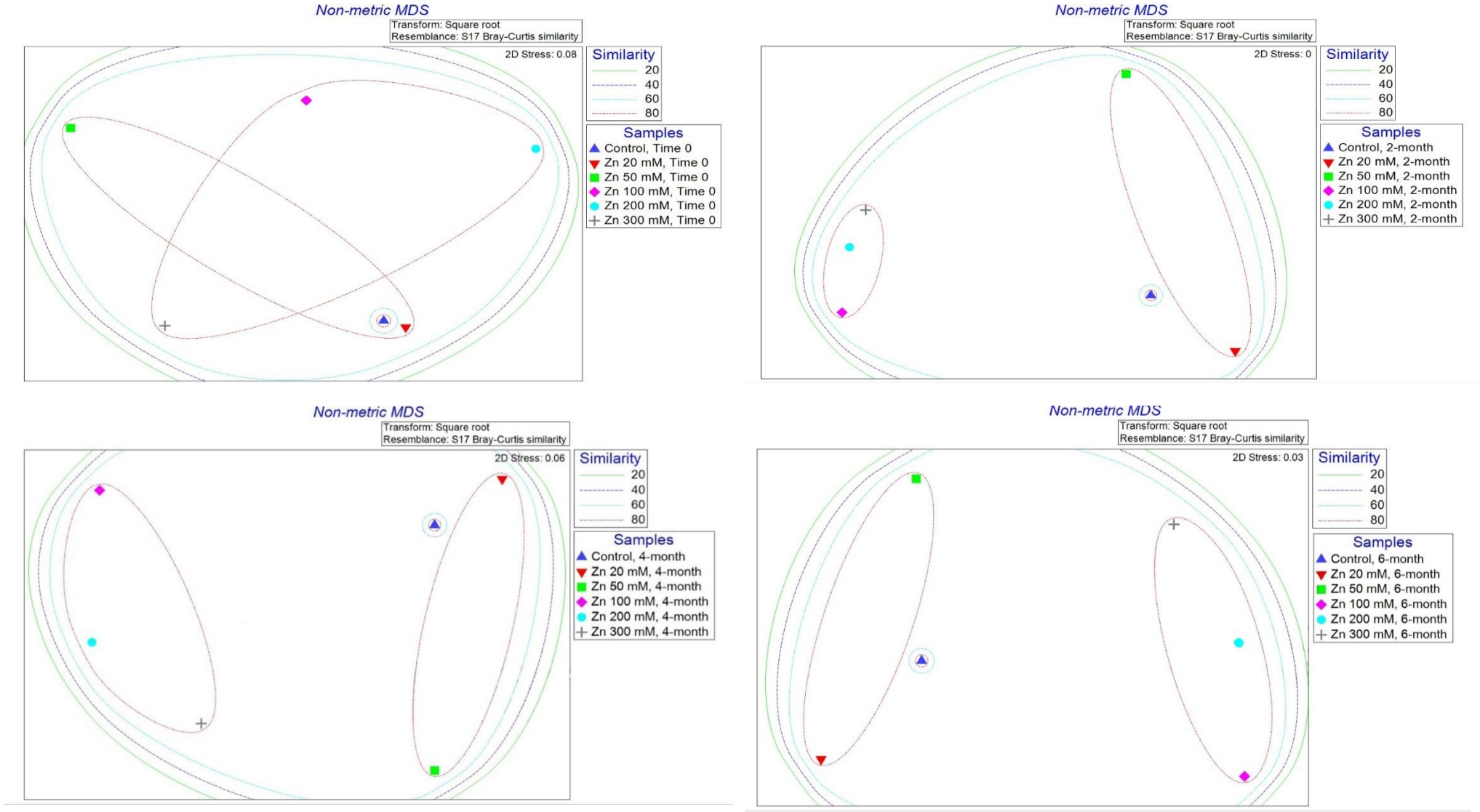


Fig. 6. 11. NMS analysis plot of TRFLP relative peak height for P6MZnM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

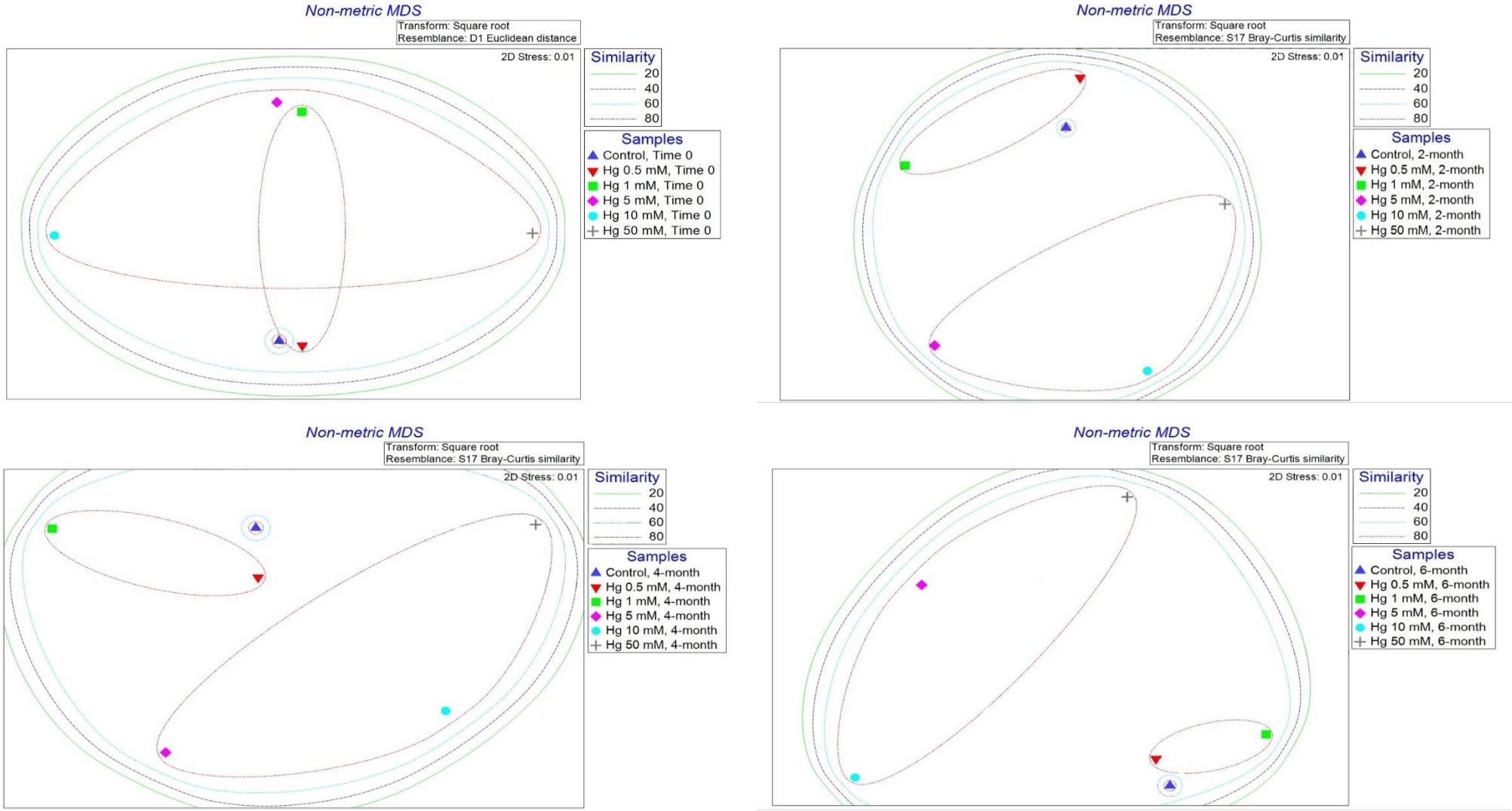


Fig. 6. 12. NMS analysis plot of TRFLP relative peak height for P6MHgM soils' bacterial communities' data, using Bray-Curtis similarity index. Significant difference ($p < 0.05$) between the cluster specified with >60% similarity compared to control microcosms.

6.1.4. 16s rDNA Next Generation Sequencing

6.1.4.1. Belmont Regional Park soil samples

Analysis of NGS of metagenomic 16s rDNA gene of bacterial communities from selected BRP soil samples by QIIME (Quantitative Insights Into Microbial Ecology) software, v.2, showed there were significant differences between the number of reads of this gene, related to the relative abundance of each bacterial phylum, in B10 (70 m from the start of the airstrip) and B14 (30 m from the start of the airstrip) soil samples compared to those in B17 (at the start of the airstrip) soil sample ($p < 0.05$). Figure 6.13 illustrates the diversity of bacterial communities in the selected BRP soils at the level of phylum. The most abundant phyla detected belonged to Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi, Acidobacteria and Chloroflexi were the phyla that differed most among the samples ($p < 0.05$). Table 6.1 lists the p-values for comparisons of the number of 16s rDNA gene reads in B10 and B14 compared to B17 for the taxonomical levels, including phyla, class, order, family, genus and species. The data are only shown to the level of phylum (Figure 6.13) due to the huge number of groups detected in lower taxonomic levels, e.g. class, order, etc. The highest differences amongst BRP samples were detected for Acidobacteria Verrucomicrobia, Bacteroidetes and Chloroflexi.

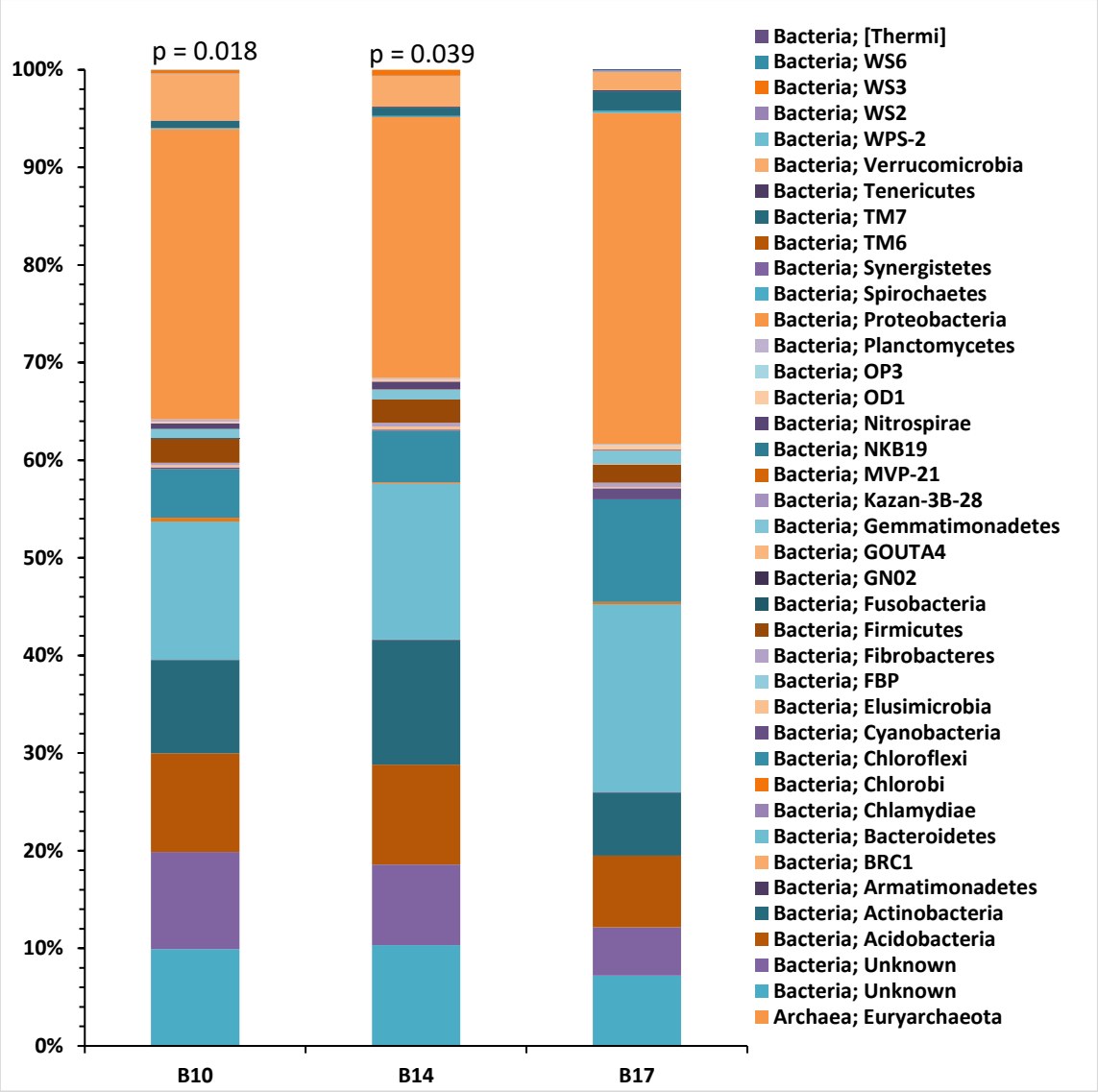


Fig. 6. 13. Chart depicting assignment of sequenced metagenomic Next Generation 16s rDNA gene to various bacterial phyla for selected Belmont Regional Park soil samples. B10 (70 meters from start of the airstrip), B14 (30 meters from start of the airstrip), and B17 (0 meters from start of the airstrip). P values compared to B17 sample.

Table 6. 1. Comparison of NGS of bacterial 16s rDNA gene reads in various taxonomical levels from B10 and B14 soil samples compared to B17 soil from BRP.

Taxonomy levels	B10	B14
Phylum	p=0.018	p=0.039
Class	p = 0.0015	p = 0.031
Order	p < 0.001	p = 0.0221
Family	p < 0.001	p = 0.0217
Genus	p < 0.001	p = 0.0149
Species	p < 0.001	p = 0.0146

4.3.4.2. 6-week microcosms soil samples

Analysis of the metagenome by NGS of 16s rDNA genes for bacterial communities from selected 6WM soil samples by QIIME software was performed. The samples were control, Cd 100 mM, Zn 200 mM and Hg 50 mM-spiked microcosms taken after 6 weeks of incubation. There were significant differences between the number of reads for 16s rDNA gene, related to each bacterial phylum, for the HM-spiked soil samples compared to those for control sample ($p < 0.05$). Figure 6.14 illustrates the diversity of the bacterial communities in the selected 6WM soils at the level of phylum. Similar to the most abundant detected phyla in the BRP soil samples, the most abundant phyla determined in the 6WM soil samples were Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi. Table 6.2 lists the p-values for comparison of the number of 16s rDNA gene reads in Cd, Zn and Hg-spiked 6WM soil samples for the lower taxonomical levels, including phylum, class, order, family, genus and species.

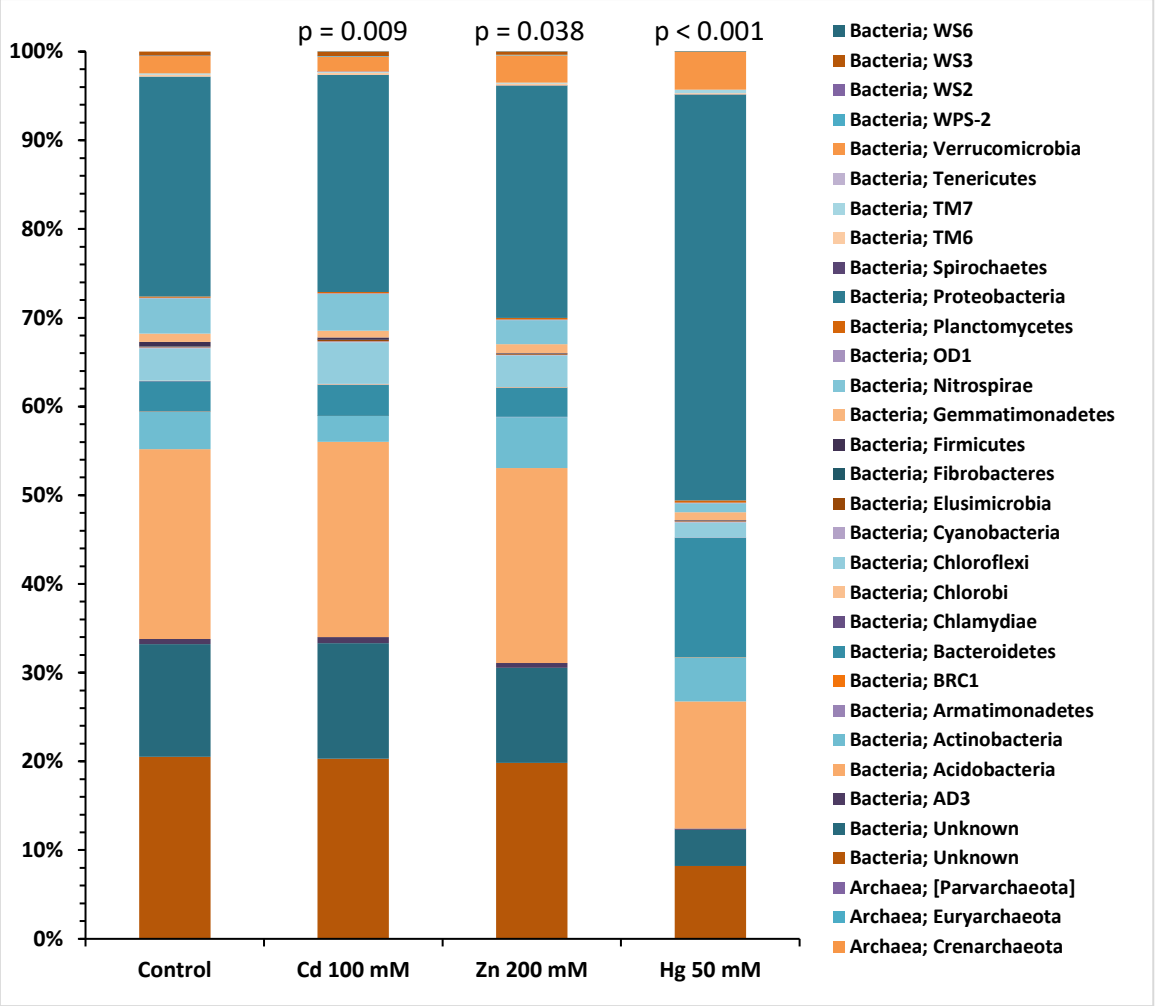


Fig. 6. 14. Chart depicting assignment of sequenced metagenomic Next Generation 16s rDNA gene to various bacterial phyla for 6WM selected HM-spiked microcosms (Cd 100 mM, Zn 200 mM and Hg 1 mM, as well as control) microcosms soil samples. P value compared to Control sample.

Table 6. 2. Comparison of bacterial 16s rDNA gene reads in various taxonomical levels of Cd, Zn and Hg-spiked 6WM soil samples compared to control microcosm soil.

Taxonomy levels	Cd 100 mM-spiked 6WM	Zn 200 mM-spiked 6WM	Hg 50 mM-spiked 6WM
Phylum	p=0.009	p=0.038	p<0.001
Class	p = 0.008	p = 0.030	p < 0.001
Order	p = 0.007	p = 0.020	p < 0.001
Family	p = 0.006	p = 0.015	p < 0.001
Genus	p = 0.006	p = 0.010	p < 0.001
Species	p = 0.006	p = 0.010	p < 0.001

6.1.6. Discussion

TRFLP analysis of bacterial 16s rDNA gene profiles is a trusted technique for comparing soil microbial community structures [302, 303]. The statistical analysis investigated the distribution

of variation in the T-RFs associated with various levels of HMs in different soil samples. There are some reports indicating that HMs present in soil impose changes on bacterial community structures and these changes are for almost all of T-RF abundances of bacterial communities [298-300]. However, Gough and Stahl (2011) [304] contradicts this. The TRFLP analysis on the samples from Waikato region, Belmont Regional Park, 6-week microcosms and 6-month microcosms showed that higher levels of HMs in soil result in greater changes in the bacterial community structures in soil.

TRFLP fingerprinting of WR soil samples revealed that higher levels of HMs in pastoral and arable soils compared to background soil were associated with distinct bacterial community structures suggesting the selection of particular species in the presence of high levels of HM. According to several published studies the high levels of HMs in pastoral and arable soils had been a selective influence on lots of bacterial taxa in these soils [304-307]. Based on the result in the current study, there were higher levels of Cd and Zn in pastoral and arable soils compared to background soils, which resulted in relatively distinct clusters of T-RFs abundance in pastoral and arable samples, though there was >80% similarity between these soils (Figure 4.90). This finding is in concordance with the report of Brodie et. al. (2002) [308].

The soil samples collected from BRP pastoral sub-sites were subjected to TRFLP analysis to explore associations between HM profile and bacterial T-RFs variations. Macdonald et. al. (2011) [306], suggested TRFLP analysis as a robust tool to perform the analysis of microbial diversity in soils with similar land use. TRFLP analysis of the BRP soil samples showed that higher levels of Cd and Zn at B17 (at the start of the airstrip) and B16 (10 m from the start of the airstrip) (See Section 4.1.3) had more variation in bacterial T-RFs abundance compared to other BRP sub-sites soil samples (Figure 4.91). This result indicates the direct relationship between the effect of higher levels of HMs in soil and greater changes in bacterial community structures [306].

Bacterial 16s rDNA gene TRFLP analysis was performed on the 6WM soil samples spiked with a range of Cd, Zn and Hg. Lazzaro et. al. (2006) [309], reported 16s rDNA gene TRFLP analysis was an effective tool to investigate the effects of HMs causing changes in soil bacteria community structures in microcosms during short and long term incubation.

There were significant differences between the control microcosms' soil bacterial profiles compared to those from Cd, Zn and Hg-spiked microcosms, especially for those with higher levels of HMs and after longer periods of incubation (Figures 4.92-4.94). Various studies have reported the effects of HMs on soil bacteria communities' structures was effectively investigated by TRFLP fingerprinting [309, 310]. Li et. al. (2014) [310], reported that 30 days of incubation of soil spiked with HMs had major influence on bacterial community structures in microcosms.

ANOVA analysis of 6MM soil bacteria TRFLP data showed there were significant differences between T-RF abundances in control microcosms compared to HM-spiked ones, especially in the microcosms spiked with the higher levels of HMs. T-RF variation was greater compared to control in the samples from HM-spiked microcosms with longer incubation periods (4 and 6 months).

Metagenomics is considered a sophisticated tool to analyse DNA extracted from environmental samples e.g. soil. This method is used to investigate the bacterial community structures in soil without the requirement of *in vitro* cultures [311, 312]. Practically, only less than 1% of soil bacteria are able to be cultured *in vitro*, therefore, the importance of metagenomics to provide a clear picture of bacterial communities' structure in soil samples is evident [313, 314]. Metagenomics involves the exploration of gene composition of bacterial communities; therefore, this method provides a much wider view than phylogenetic perspectives alone from the sequencing of 16S rDNA gene in individual bacterial isolates [315-317]. The descriptive phylogenetic relations of uncultured bacteria and evolutionary profiles of bacterial community structures are some examples of the application of metagenomics [301]. In the present study,

bacterial communities' structures in representative samples from BRP soils, and also selected 6WM soil samples spiked with Cd, Zn and Hg were subjected to metagenomic 16s rDNA sequencing.

High-throughput NGS of metagenomic 16s rDNA is an established method to describe the diversity of bacterial communities in soil samples [267] and was employed for this purpose in the current study. Comparatively, there are a huge number of reports of bacterial community structures utilising DNA-fingerprinting methods [268, 312], however the number of studies employing DNA sequencing are still rare [267, 318-322]. The 16s rDNA sequencing data presented in this thesis demonstrate that sequencing of bacterial metagenomic 16S rDNA fragment using NGS is an effective tool to investigate bacterial communities structures in soil samples containing various levels of HMs. Vierheilig et. al. (2015) [323], suggested that NGS remains a strong molecular research method which can change the fundamentals of the current knowledge about bacterial community structures in different environments.

There are some reports suggesting of NGS 16s rDNA analysis has higher power compared to TRFLP fingerprinting tools to provide a clearer insight of bacterial community structures in soil. For example, Suzuki et. al. (2019) [324], cited that Lopes et. al. (2011) [325] compared the microbial diversity of organic and conventional paddy soils using PCR-DGGE analysis, and suggested that although these analyses provided useful information, but the techniques utilised for the bacterial communities structures investigations remained inadequate to provide a full perspective of bacterial diversity in soil samples compared to high-throughput NGS 16s rDNA sequencing. This report is in concordance with the report of Lynch et. al. (2012) [326] on bacterial phylogenetic investigation using NGS of 16s rDNA gene.

Hermans et.al. (2017) [267] reported that there are fundamental relationships between the levels of HMs and evolution of bacterial community structures in soil. In the current study, employing 16s rDNA sequencing analysis revealed that bacterial community compositions in the

BRP soil samples had sustained some levels of evolutionary changes under the selection pressure of HM contaminants. There were significant differences between the relative abundance of the major phyla in B10 (lowest concentrations of HMs, 1.14 and 49 mg kg^{-1} of dry soil for Cd and Zn, respectively) and B14 soil samples in contrast with B17 soil sample (highest concentrations of HMs, 7.2 and 95 mg kg^{-1} of dry soil for Cd and Zn, respectively). The most prevalent bacterial phyla in BRP soil samples were Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi, though a wide number of other bacterial phyla were determined. Aislabie and Deslippe (2013) [35], and Fierer at. al. (2007) [327], reported Proteobacteria, Actinobacteria and Acidobacteria as the most numerous bacterial phyla in soil samples. There were some bacterial groups which were not able to be classified to a particular phylum (˜ 10% of determined phyla in B17 and microcosms soils, and ˜20% in B10 and B14 soils). There are reports in concordance with this finding (Janssen (2006) [328] and Nacke et. al. (2011) [329]). Also, Li et. al. (2019) [330] reported that the most abundant Cd-resistant bacteria determined by 16s rDNA sequencing were the members of Proteobacteria, Bacteroidetes and Actinobacteria phyla and the genera *Chryseobacterium*, *Cupriavidus*, *Curtobacterium*, and *Sphingomonas*.

The presence of elevated levels of HMs in soil can change many phenotypic characteristics of bacterial reproduction in soil, for example, introduction of extracellular compounds involved in biofilm production, introduction of tolerance features in the soil bacteria to the added HM or other HMs, and metabolism of bacterial cells.

In the present study, 16s rDNA sequencing analysis showed that bacterial community structures in the selected 6WM soil samples had changed due to presence of elevated HMs concentrations in soil. ANOVA showed that there were significant differences between the relative abundance of the most abundant phyla in 6WCdM (spiked with 100 mM Cd), 6WZnM (spiked with 200 mM Zn) and 6WHgM (spiked with 50 mM Hg). The most prevalent bacterial phyla in BRP soil samples were Proteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria and Chloroflexi, as well as a

large number of other less numerous bacterial phyla. The detected major phyla as well as the unknown groups for microcosms and BRP soils were in concordance with previous reports [35, 327-330].

In conclusion, the results of this investigation showed that structure of bacterial communities in soil is changed under selective pressure due to the presence of HMs. The bacterial communities with less developed HM resistance strategies (e.g. bacteria in background soil), sustain greater structural changes when exposed to HMs, compared to those bacterial communities that have already sustained a long term of exposure to HMs (e.g. bacteria in pastoral soil).

Chapter 7, Results Part 4

7.1. Study 4, Investigation of Mobility of Heavy Metal and Antibiotic Resistance Genes.

7.1.1. Introduction

Horizontal Gene Transfer (HGT) in bacteria is an important mechanism for gene transfer in the environment. This phenomenon demonstrates gene transfer without vertical transmission through generations and has an important role in the evolution and the spread of resistance to antimicrobial compounds. The most common mechanisms of HGT in prokaryotes are conjugation, transformation and transduction [119]. Physical contact of the donor and recipient cells is important for HGT by conjugation. The focus of this chapter was to investigate HGT of Cd resistance genes, *czcA* and *cadA*, in bacterial isolates carrying these genes to the recipient bacterial strain, *Pseudomonas aeruginosa* ICMP 6286. The taxonomy of bacteria carrying the mobilizable Cd resistant genes were identified by 16s rDNA sequencing.

7.1.2. Methodology

The methodology used for this chapter is described in Sections 3.12.5, 3.12.6 and 3.13. In short, PCR reactions using specific primers to amplify Cd resistance genes in individual bacterial isolates, Replica Plate Mating (RPM), and identification of bacterial isolates using 16s rDNA Sequencing were employed. In this part of the study some of the archived individual CdR bacterial isolates in the HM BM experiments (Sections 3.11) were used. The bacterial isolates used for this investigation were chosen as representatives of Cd resistant isolates selected from WR, BRP and microcosms soil samples. Figure 7.1 illustrates different steps and experiments used in this study.

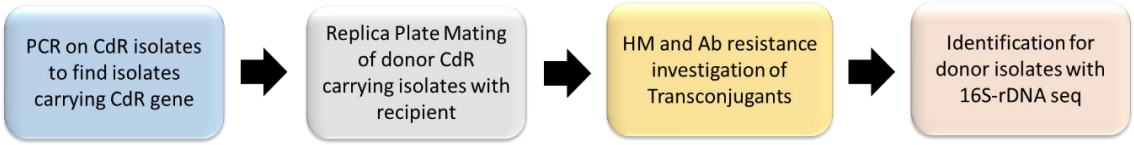


Fig. 7. 1. Flow chart illustrating different steps and experiments used in Chapter 7.

7.1.3. PCR Reaction Amplifying Cd-Resistance genes Using Specific Primers

Following DNA extraction from fresh overnight culture of a total number of 906 individual purified bacterial isolates by the boiling method, PCR reactions using specific primers, amplifying the *czcA* (252 bp) and *cadA* (1052 bp) Cd resistance genes, was performed. A total number of 378 bacterial isolates (42 %) carried *czcA* and 22 isolates (2.3 %) carried *cadA* (Figures 7.2 and 7.3). Table 7.1 lists the number of Cd resistant bacterial isolates from WR, BRP and microcosms soil samples and the abundance of isolates carrying *czcA* and *cadA* genes.

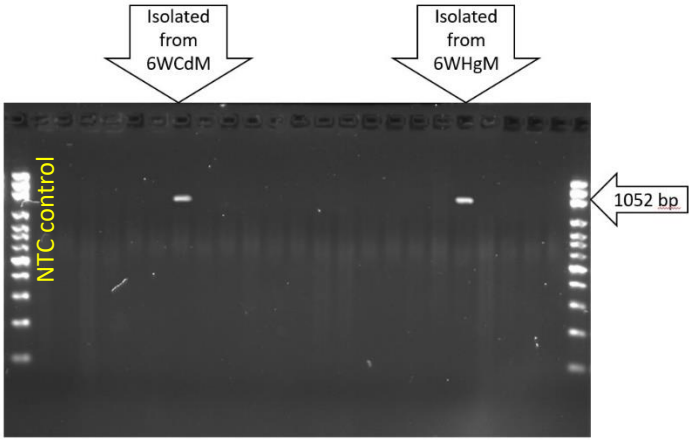


Fig. 7. 2. Amplified 1052 bp *cadA* gene on agarose gel. (NTC: No Template Control).

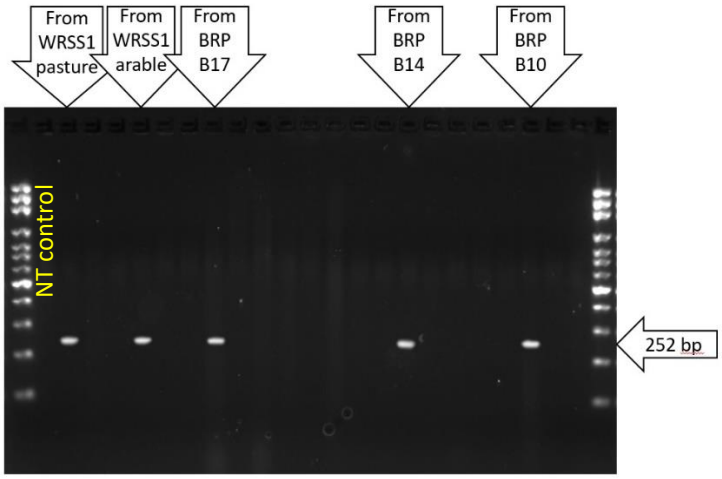


Fig. 7. 3. Amplified 252 bp *czcA* gene on agarose gel. (NT control: No Template Control).

Table 7. 1. Number of Cd resistant bacterial isolates from WR, BRP and microcosms soil samples and the number of isolates carrying *czcA* and *cadA* genes.

Soil samples	**Number of Cd resistant isolates**	**Cd resistance genes**	
		cadA	***czcA***
WRSS1 pasture	15	1 (6.6%)	4 (28.5%)
WRSS2 pasture	15	0	3 (20%)
WRSS3 pasture	15	0	3 (20%)
WRSS1 arable	15	0	3 (20%)
WRSS2 arable	15	0	2 (13.3%)
WRSS3 arable	15	0	2 (13.3%)
WRSS1 background	15	1 (6.6%)	1 (6.6%)
WRSS2 background	15	0	0
WRSS3 background	15	0	1 (6.6%)
BRP B17	40	1 (2.5%)	37 (92.5%)
BRP B16	40	2 (5%)	35 (87.5%)
BRP B15	40	1 (2.5%)	34 (85%)
BRP B14	40	0	25 (62.5%)
BRP b13	40	0	17 (42.5%)
BRP B12	40	0	11 (27.5%)
BRP B11	40	0	8 (20%)
BRP B10	40	1 (2.5%)	8 (20%)
6WCdM	40	2 (5%)	28 (70%)
6WZnM	40	1 (2.5%)	23 (57.5%)
6WHgM	40	0	5 (12.5%)
6WM, control	30	0	2 (6.6%)
B6MCdM	40	2 (5%)	24 (60%)
B6MZnM	40	2 (5%)	23 (57.5%)
B6MHgM	40	0	5 (12.5%)
B6MM, control	30	0	2 (6.6%)
P6MCdM	40	4 (10%)	31 (77.5%)
P6MZnM	40	2 (5%)	29 (72.5%)
P6MHgM	40	2 (5%)	5 (12.5%)
P6MM, control	30	0	7 (23.3%)

7.1.4. Horizontal Gene Transfer (HGT) by Replica Plate Mating (RPM)

HGT by conjugation using an SmR derivative of *P. aeruginosa* ICMP 2686 as recipient cells, and the bacterial isolates carrying *cadA* or *czcA* Cd resistance genes as donor cells on Brain Heart infusion plates (containing 100 µg mL^{-1} of streptomycin and 1 mM of $CdCl_2$) was investigated using the RPM method. A total of 400 bacterial isolates were tested for their ability to transfer CdR to the recipient, including 378 isolates carrying *czcA* and 22 isolates carrying the *cadA* gene. A total number of 104 isolates out of these CdR 400 isolates, were found to transfer these genes to the recipient cells (Figure 7.4). Table 7.2 lists the number of bacterial isolates carrying Cd

resistance genes from WR, BRP and microcosms soil samples and the number of isolates able to transfer their Cd resistance genes to the recipient while conjugated.

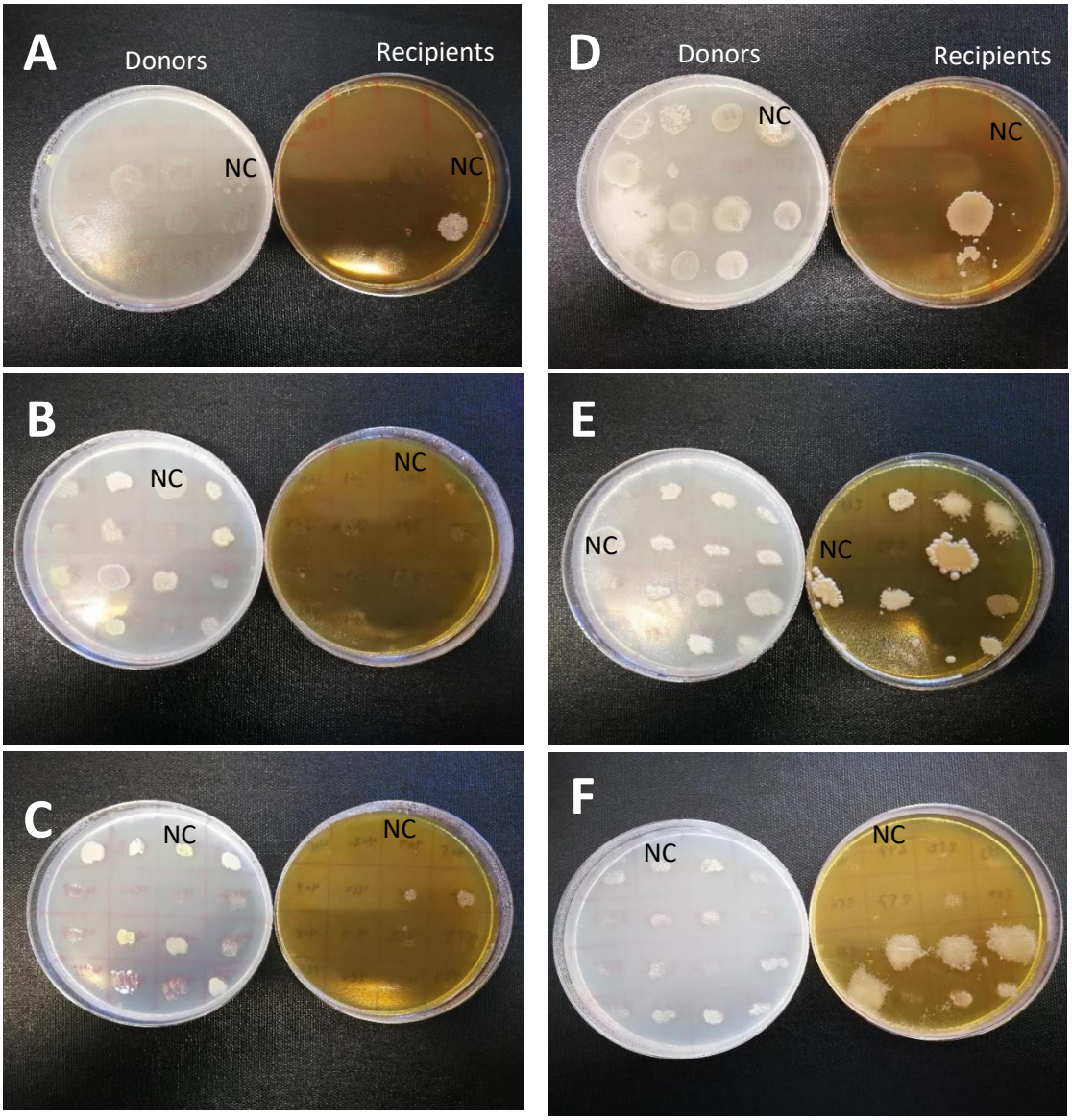


Fig. 7. 4. HGT of *cadA* (A, B and C images) and *czcA* (D, E and F images) Cd resistant genes from donor cells to the transconjugant SmR *P. aeruginosa* ICMP 2686 strain. Donor isolates are on 2% Nutrient Agar (left plates) and 2% Brain Heart Agar containing 100 µg mL^{-1} streptomycin and 1 mM of $CdCl_2$. NC: Negative Control.

Table 7. 2. Number of bacterial isolates carrying *cadA* and *czcA* Cd resistance genes transferring these genes to the transconjugants (SmR *P. aeruginosa* ICMP 2686) from WR, BRP and microcosms soil samples.

Soil samples	**Number of isolates carrying Cd resistance genes**	
	cadA	***czcA***
WRSS1 pasture	1	2
WRSS2 pasture	0	1
WRSS3 pasture	0	2
WRSS1 arable	0	2
WRSS2 arable	0	1
WRSS3 arable	0	0
WRSS1 background	1	0
WRSS2 background	0	0
WRSS3 background	0	0
BRP B17	0	10
BRP B16	0	11
BRP B15	1	8
BRP B14	0	7
BRP b13	0	6
BRP B12	0	3
BRP B11	0	2
BRP B10	1	3
6WCdM	0	7
6WZnM	1	5
6WHgM	0	1
6WM, control	0	0
B6MCdM	1	7
B6MZnM	0	5
B6MHgM	0	0
B6MM, control	0	0
P6MCdM	1	7
P6MZnM	1	5
P6MHgM	0	0
P6MM, control	0	1

7.1.5. Examination of *cadA* and *czcA* Genes in Recipient Cells by PCR

The total number of 104 transconjugants that received *cadA* and *czcA* genes were subjected to PCR investigations. The result showed that *cadA* gene was amplified from all of the recipients that putatively received the *cadA* gene (total number of 8 isolates), and *czcA* gene was amplified from all of the recipients that received the *czcA* gene (n=96 isolates) (Figures 7.5 and 7.6).

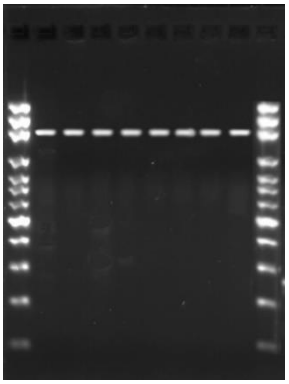


Fig. 7. 5. Amplified *cadA* (1052 bp) gene from transconjugants after conjugation with donor isolates carrying *cadA* gene.

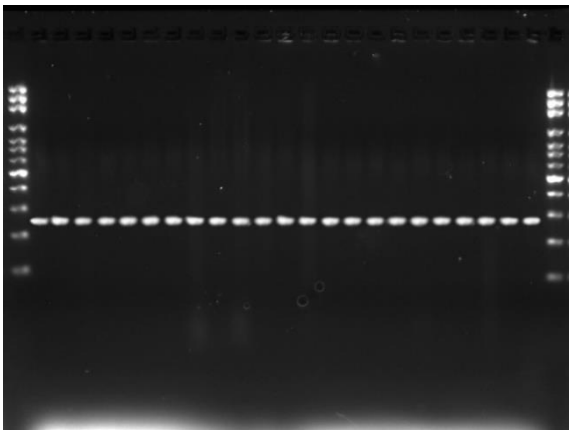


Fig. 7. 6. Amplified *czcA* (252 bp) gene from transconjugants after conjugation with donor isolates carrying *czcA* gene.

7.1.6. Heavy Metal and Antibiotic Resistance Examination of the Recipient Strains

To investigate the HMR and AbR phenotypes of transconjugants isolated in Section 7.1.4, the isolates were cultured on Nutrient Agar plates containing HMs concentrations including 1 and 0.1 mM of Cd, 5 and 1 mM of Zn and 0.1 and 0.01 mM of Hg. BM analysis was performed to examine the resistance of these isolates to Abs.

This showed 100% of transconjugants that had received *czcA* or *cadA* genes, were resistant to 0.1 and 1 mM Cd. In addition, 100% of bacterial *czcA*$^{+}$ transconjugants showed resistance to 1 and 5 mM Zn. For the transconjugants which received *cadA* gene, there was 100% resistance to 1 mM and 97% to 5 mM of Zn. The result for Hg resistance showed 78% of transconjugants

carrying *czcA* gene could grow on 0.1 mM Hg, while 96% of them grew on 0.01 mM of Hg. Further, 31% of *cadA*$^{+}$ transconjugants were resistant to 0.1 mM Hg, and 73% of them grew on 0.01 mM of Hg (Figure 7.7).

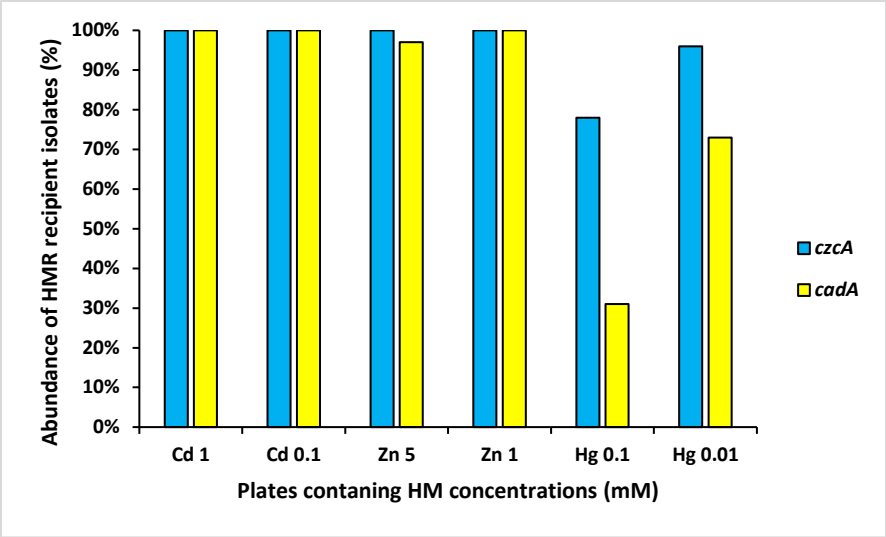


Fig. 7. 7. Percentage of HMR for *czcA*$^{+}$ and *cadA*$^{+}$ tranconjugants *P. aeruginosa* ICMP 2686.

The results of BM analysis to determine MIC values (according to EUCAST ECOFF recommendations [218]) for the genetically modified *P. aeruginosa* ICMP 2686 obtained *czcA* and *cadA* genes with Tc, Cm, Ery, Cb and Amp showed 75% of isolates which obtained *czcA* and 59% of isolates with *cadA* gene were resistant to Tc. In addition, 74% of *czcA*-obtained isolates and 61% of those carrying *cadA* gene were resistant to Cm. Result showed 82% of *czcA* gene-obtained isolates and 85% of those carrying *cadA* gene were resistant to Ery. Resistance to Cb was determined for 88% of isolates with *czcA* gene and 83% of those with *cadA* gene. Finally, 88% of isolates with *czcA* gene and 86% of those carrying *cadA* gene showed resistance to Amp (Figure 7.8).

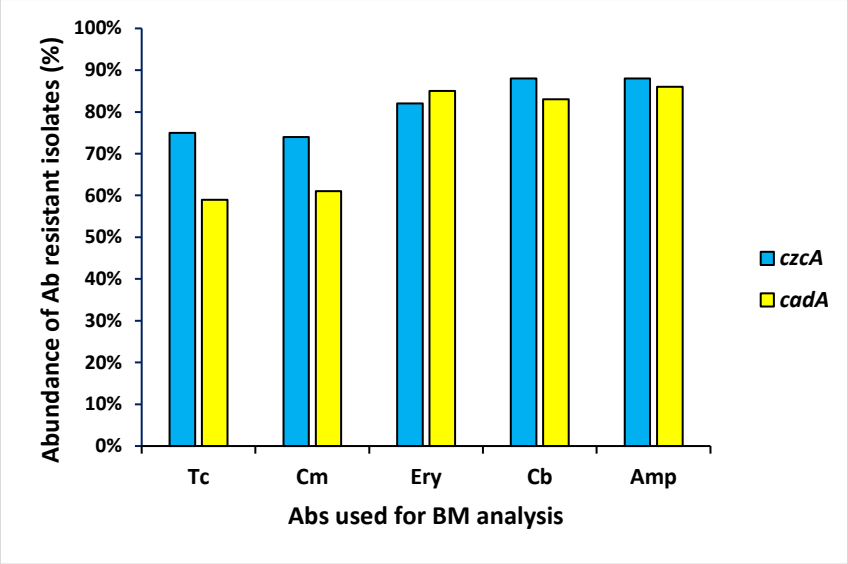


Fig. 7. 8. Percentage of tranconjugant *P. aeruginosa* ICMP 2686 carrying *czcA* and *cadA* genes resistant to Abs in BM analysis.

7.1.7. Bacterial Isolates Identification Using 16s rDNA Sequencing

DNA was extracted from n=60 individual bacterial isolates used as donors in HGT investigation followed by PCR amplification of 16s rDNA amplimers using the unlabelled primers 63F and 1087R (Section 3.12.3). The samples were subjected to 16S rDNA sequencing with an ABI3730 DNA Analyzer. The sequencing data were compared to NCBI (National Centre for Biotechnology Information) BLAST using blastn suite to find the identity from the sequences. Table 7.3 lists the identifies of these isolates, including their soil sample origins, description from NCBI and the percentage of identity to the candidates on the NCBI database. Most of the identified bacteria which had successfully transferred CdR genes to new recipients were from the genera *Pseudomonas, Achromobacter, Stenotrophomonas* (Proteobacteria phylum) and *Chryseobacterium* (Bacteroidetes phylum) (Figure 7.9 and Table 7.3).

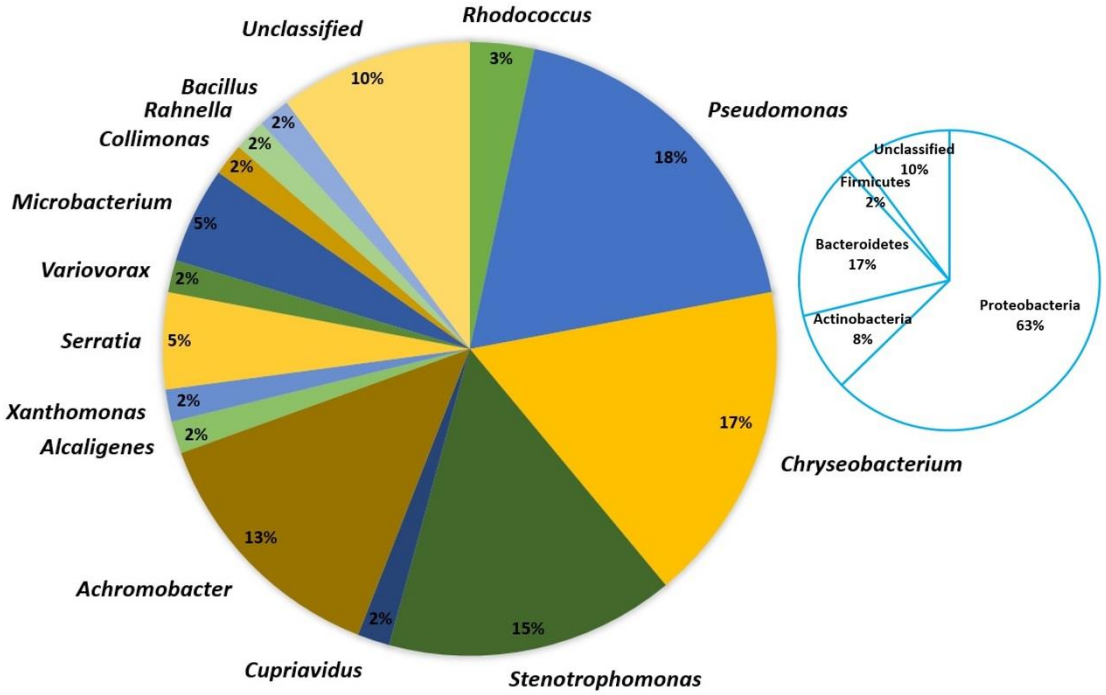


Fig. 7. 9. Abundance (%) of genera (left) and phyla (right) of isolates identified by 16s rDNA sequencing. These isolates were able to transfer their *cadA* and *czcA* Cd resistance genes to new recipients via conjugation.

Table 7. 3. Individual bacterial isolates involved in HGT test as donor cells identified by 16s rDNA sequencing.

Bacterial isolate ID	Carrying Cd resistant gene	Description	Accession number	Percent identity
No. 4, WRSS1, Pasture soil	*cadA*	*Rhodococcus erythropolis* partial 16S rRNA gene, strain SBUG 107.	FR745420.1	99.10%
No. 31, WRSS1, Pasture soil	*czcA*	*Pseudomonas azotoformans* strain P45A chromosome, complete genome.	CP041236.1	99.80%
No. 180, WRSS1, Pasture soil	*czcA*	*Chryseobacterium rhizosphaerae* strain WTB5 16S ribosomal RNA gene, partial sequence.	MK240433.1	98.90%
No. 184, WRSS2, Pasture soil	*czcA*	*Stenotrophomonas maltophilia* strain Tj 16S ribosomal RNA gene, partial sequence.	MF280131.1	99.40%
No. 204, WRSS3, Pasture soil	*czcA*	Bacterium strain BS1294 16S ribosomal RNA gene, partial sequence.	MK824482.1	97.73%
No. 212, WRSS1, arable soil	*czcA*	*Chryseobacterium lactis* partial 16S rRNA gene, strain R-52618.	LN995695.1	99.20%

No. 217, WRSS1, arable soil	*czcA*	Bacterium strain BS1294 16S ribosomal RNA gene, partial sequence.	MK824482.1	99.32%
No. 239, BRP, B17	*czcA*	*Cupriavidus sp.* strain JS3054 16S ribosomal RNA gene, partial sequence.	MH588163.1	99.40%
No. 244, BRP, B17	*czcA*	*Achromobacter xylosoxidans* strain E2 16S ribosomal RNA gene, partial sequence.	MK849863.1	99.20%
No. 252, BRP, B17	*czcA*	*Alcaligenes sp.* 242 16S ribosomal RNA gene, partial sequence.	KT461862.1	99.10%
No. 257, BRP, B17	*czcA*	*Stenotrophomonas sp.* strain D1 16S ribosomal RNA gene, partial sequence.	MH814356.1	99.12%
No. 260, BRP, B17	*czcA*	*Pseudomonas protegens* strain Exi5-13 16S ribosomal RNA gene, partial sequence.	MK235212.1	99.10%
No. 268, BRP, B17	*czcA*	*Xanthomonas sp.* PG15 16S ribosomal RNA gene, partial sequence.	KU350604.1	90.22%
No. 269, BRP, B17	*czcA*	*Stenotrophomonas maltophilia* partial 16S rRNA gene, isolate R5_A9_IIIA.	LR215089.1	98.52%
No. 290, BRP, B17	*czcA*	*Pseudomonas sp.* strain ICMP 22295 16S ribosomal RNA gene, partial sequence.	MH392636.1	99.50%
No. 306, BRP, B17	*czcA*	*Serratia proteamaculans* strain P4_BA1R 16S ribosomal RNA gene, partial sequence.	MK883049.1	99.22%
No. 307, BRP, B16	*czcA*	*Chryseobacterium nakagawai* strain NCTC13529 genome assembly, chromosome: 1.	LR1234386.1	82.50%
No. 345, BRP, B16	*czcA*	*Stenotrophomonas maltophilia* strain DGN5 16S ribosomal RNA gene, partial sequence.	MK764970.1	91.53%
No. 348, BRP, B16	*czcA*	*Pseudomonas geniculata* strain IARI-HHS1-19 16S ribosomal RNA gene, partial sequence.	KF054771.1	95.33%
No. 349, BRP, B16	*czcA*	*Achromobacter pestifer* strain LMG 3431 16S ribosomal RNA, partial sequence.	NR_152016.1	99.21%
No. 350, BRP, B16	*czcA*	*Rhodococcus sp.* strain OB0511_247-1 16S ribosomal RNA gene, partial sequence.	KY020332.1	99.90%

No. 355, BRP, B16	*czcA*	*Achromobacter sp.* JW31.5a partial 16S rRNA gene, strain JW31.5a.	FN556572.1	99.11%
No. 356, BRP, B15	*czcA*	*Pseudomonas palleroniana* strain IHB B 7133 16S ribosomal RNA gene, partial sequence.	KJ767328.1	99.80%
No. 358, BRP, B15	*czcA*	*Serratia sp.* SP19E 16S ribosomal RNA gene, partial sequence.	KP126635.1	99.61%
No. 365, BRP, B15	*czcA*	*Achromobacter sp.* strain FW305-C-28 16S ribosomal RNA gene, partial sequence.	MK402967.2	99.12%
No. 367, BRP, B15	*czcA*	*Serratia sp.* A2 16S ribosomal RNA gene, partial sequence.	EU287454.1	99.71%
No. 376, BRP, B14	*czcA*	*Stenotrophomonas maltophilia* clone B2.18.23 16S ribosomal RNA gene, partial sequence.	AY837730.1	99.80%
No. 377, BRP, B14	*czcA*	Bacterium strain YCR3A-3 16S ribosomal RNA gene, partial sequence.	MF143454.1	99.80%
No. 379, BRP, B14	*czcA*	Bacterium strain BS1294 16S ribosomal RNA gene, partial sequence.	MK824482.1	99.51%
No. 388, BRP, B14	*czcA*	*Chryseobacterium sp.* strain DEM Bc1 16S ribosomal RNA gene, partial sequence.	MG893574.1	99.40%
No. 390, BRP, B13	*czcA*	*Pseudomonas fluorescens* strain B16-231 16S ribosomal RNA gene, partial sequence.	MK072682.1	99.70%
No. 391, BRP, B13	*czcA*	*Pseudomonas palleroniana* strain IHB B 7133 16S ribosomal RNA gene, partial sequence.	KJ767328.1	99.80%
No. 393, BRP, B13	*czcA*	*Pseudomonas palleroniana* strain APC14 16S ribosomal RNA gene, partial sequence.	KX528176.1	97.97%
No. 394, BRP, B12	*czcA*	*Chryseobacterium sp.* strain E2-18 16S ribosomal RNA gene, partial sequence.	KY476499.1	99.70%
No. 395, BRP, B12	*czcA*	*Variovorax boronicumulans* strain E2B5 16S ribosomal RNA gene, partial sequence.	KX881472.1	93.09%
No. 396, BRP, B11	*czcA*	*Achromobacter sp.* strain HBUM200336 16S ribosomal RNA gene, partial sequence.	KY945518.1	99.28%
No. 401, BRP, B10	*cadA*	*Microbacterium sp.* strain PHIL_400ppmZn_ML16 16S	MK652511.1	99.59%

		ribosomal RNA gene, partial sequence.		
No. 402, 6WCdM	*czcA*	*Stenotrophomonas sp.* PA81Nov 16S ribosomal RNA gene, partial sequence.	KJ482787.1	88.73%
No. 407, 6WCdM	*czcA*	*Microbacterium sp.* PMS04 16S ribosomal RNA gene, partial sequence.	KF648897.1	95.63%
No. 411, 6WCdM	*czcA*	*Achromobacter xylosoxidans* strain Y5 16S ribosomal RNA gene, partial sequence.	FJ596145.1	98.91%
No. 418, 6WCdM	*czcA*	*Collimonas pratensis* strain EN14AR2 16S ribosomal RNA gene, partial sequence.	KY486811.1	98.81%
No. 419, 6WZnM	*czcA*	*Microbacterium azadirachtae* strain EIP4 16S ribosomal RNA gene, partial sequence.	MH489019.1	99.70%
No. 423, 6WZnM	*czcA*	*Pseudomonas sp.* 2 RIFA 366 16S ribosomal RNA gene, partial sequence.	KF624750.1	98.42%
No. 425, 6WZnM	*czcA*	*Chryseobacterium sp.* strain DEM Bc1 16S ribosomal RNA gene, partial sequence.	MG893574.1	99.60%
No. 429, 6WZnM	*czcA*	*Chryseobacterium sp.* strain N15122 16S ribosomal RNA gene, partial sequence.	MK389289.1	99.29%
No. 430, B6MCdM	*czcA*	*Pseudomonas sp.* strain A3 16S ribosomal RNA gene, partial sequence.	KX859144.1	100%
No. 431, B6MCdM	*czcA*	*Achromobacter sp.* strain FW305-C-28 16S ribosomal RNA gene, partial sequence.	MK402967.2	99.31%
No. 434, B6MCdM	*czcA*	*Pseudomonas simiae* strain LMTK36 16S ribosomal RNA gene, partial sequence.	KY614184.1	100%
No. 436, B6MZnM	*czcA*	*Chryseobacterium rhizosphaerae* strain WTB5 16S ribosomal RNA gene, partial sequence.	MK240433.1	95%
No. 440, B6MZnM	*czcA*	*Rahnella aquatilis* strain DGE5 16S ribosomal RNA gene, partial sequence.	MK764976.1	99.80%
No. 441, B6MZnM	*czcA*	*Stenotrophomonas sp.* strain D1 16S ribosomal RNA gene, partial sequence.	MH814356.1	99.32%
No. 451, P6MCdM	*czcA*	*Achromobacter sp.* strain B3t90 16S ribosomal RNA gene, partial sequence.	MK737302.1	98.11%

No. 453, P6MCdM	*czcA*	Bacterium E4R16print 16S ribosomal RNA gene, partial sequence.	HQ425942.1	98.17%
No. 456, P6MCdM	*czcA*	*Stenotrophomonas sp.* strain SB544 16S ribosomal RNA gene, partial sequence.	MG491568.1	98.82%
No. 473, P6MCdM	*czcA*	*Pseudomonas sp.* PNP-B 16S ribosomal RNA gene, partial sequence.	FJ556901.1	85.42%
No. 481, P6MCdM	*czcA*	*Chryseobacterium sp.* HWJ 16S ribosomal RNA gene, partial sequence.	KJ959612.1	99.81%
No. 491, P6MZnM	*czcA*	Bacterium strain BS1294 16S ribosomal RNA gene, partial sequence.	MK824482.1	99.31%
No. 492, P6MZnM	*czcA*	*Chryseobacterium rhizosphaerae* strain WTB5 16S ribosomal RNA gene, partial sequence.	MK240433.1	98.59%
No. 493, P6MZnM	*cadA*	*Bacillus sp.* (in: Bacteria) strain M4 16S ribosomal RNA gene, partial sequence.	MK007417.1	99.13%
No. 494, P6MZnM	*czcA*	*Stenotrophomonas maltophilia* strain QAZ26 16S ribosomal RNA gene, partial sequence.	MN099392.1	98.16%

7.1.8. Discussion

In study 4, the occurrence of Cd resistant genes in individual HMR bacterial isolates was interrogated. The *cadA* gene is harboured either on a plasmid [331-333] or chromosome [334, 335] and encodes Cd^{2+}-ATPase protein transporter [237, 238], although it can confer resistant to Zn in bacteria too [19, 336]. Oger et. al. (2001) [238] reported that there was a significant increase in the occurrence of the *cadA* gene in bacterial communities in soils with elevated levels of HMs concentrations [238]. Several groups reported that the *cadA* gene was not detected in Gram negative bacteria [237, 240, 241, 337, 338]. These reports are in concordance with the finding in the current study which amplified *cadA* only in Gram positive bacteria including *Bacillus* and *Rhodococcus* species and also members of the Gram-variable [339] *Micobacterium* genus. However, Alonso et. al. (2000) reported the presence of *cadA* in the chromosome of Gram negative *Stenotrophomonas maltophilia* [340]

The *czcA* gene encodes a transmembrane helicale domain (TMH IV) of efflux-RND proteins engaged in Zn2+, Co2+, and Cd2+ efflux [23]. There are several reports outlining the most abundant bacterial phyla carrying *czcA* as Proteobacteria (including representatives of the α, β, γ, and δ subgroups) (especially the genera *Burkholderia, Pseudomonas, Ralstonia, Cupriavidus,* and *Shewanella*), Actinobacteria, Verrucomicrobia, and Bacteroidetes (mainly *Chryseobacterium*) [330, 341-343]. These reports are in accordance with the finding in the present study. It has been suggested that the occurrence of *czcA* in soil bacteria can be selected by HM contamination pressure [237, 342].

Horizontal gene transfer through mobile genetic elements among bacteria is the most important pathway leading to HMR and AbR gene spread [344, 345].

According to previously published reports [237, 238, 346] about the location of *cadA* and *czcA*, on plasmids and transposons it is suggested that these genes are probably spread among bacterial species by horizontal transfer [343]. The presence of *cadA* is reported in four different staphylococcal isolates, which could transfer this gene to a *Bacillus sp.* isolate. This observation provided evidences of HGT among these bacteria [346].

HMR and AbR profiles of the *czcA*$^{+}$ and *cadA*$^{+}$ transconjugants obtained was examined by plate culturing for HMR and BM analysis for AbR with the resistance status of the isolates to antimicrobials allocated according to the defined Ab resistance breakpoint concentration (20 μg mL^{-1}) [220, 221]. HGT of *cadA* into new bacterial isolates induces resistance to methicillin and tetracycline [347, 348].

Proteobacteria made up 63.3% of the isolates identified using 16s rDNA sequencing (especially genera *Pseudomonas*, *Achromobacter* and *Stenotrophomonas*). Other phyla consisting these isolates were Actinobacteria (*Rhodococcus* and *Michobacterium*) (8.3% of the isolates), Bacteroidetes (*Chryseobacterium*) (16.6% of isolates) and Firmicutes (Bacillus) (1.6% of isolates). About 10% of the isolates were not assigned to any known bacterial phyla. According to previous

reports [35, 327, 328, 349] outlining the most common bacterial phyla in soil, the result of the present study shows accordance with these reports.

In conclusion, the results of the present study indicate that Cd resistance genes, *cadA* and *czcA*, in Cd resistant bacterial isolates, introduce co-resistance to a range of antibiotics in bacterial isolates. According to the literature [331, 332], outlining the location of these resistance genes on mobile genetic elements, and the observations of the current study, these CdR genes can be trasnferred horizontally to new bacterial recipients, which results in induced Cd and Ab resistance in these transconjugants. The results of this chapter support the hypothesis that the presence of HMR genes in soil bacteria results in HM resistance as well as Ab resistance in these bacteria, and these resistance features can be introduced into transconjugants by HGT.

Chapter 8, Overall Discussion

There is a growing problem of HMR in soil bacteria in New Zealand due to using high levels of metal-contained superphosphate fertilizers and also different remedies to manage animal diseases [5, 6, 28-30]. Bacterial AbR is another growing problem in New Zealand [350] which may be due to the co-selection for HM resistance or the direct usage of antibiotics in either human and animal health [351] or agriculture [352]. For example, using kasugamycin to control *Pseudomonas syringae PV. actinidiae* on kiwi fruit and streptomycin to control bacterial diseases on pip fruit and stone fruits [353]. It is only in recent years that research in antibiotic resistance has focused on the environment from which the antibiotics were initially extracted. A lot of research focusses on defining the natural antibiotic resistome and understanding the ecology and evolution of antibiotic resistance in the non-clinical environment [217].

Physicochemical properties of the soil samples used in the current project (including the soil samples from WR and BRP) showed that there were higher levels of HMs (Cd and Zn) in pastoral and arable soils compared to background soil from WR, and also in the B17 and B16 soil samples compared to the soil samples from greater distances from the start of the airstrip (e.g. B11 and B10). These results were in accordance with the results of the resistance bacterial counts determined via plate culturing experiments. These experiments investigated the abundance of HM and Ab-resistant bacterial isolates in WR, BRP and microcosms soil samples. It means when there are higher levels of HMs in soil, the bacterial isolates present in these soil samples sustain higher pressure of HMR selection, therefore show resistance to elevated levels of HMs, and subsequently to Abs [53, 61, 354]. There were higher levels of bacterial resistance to HMs (Cd, Zn and Hg) and Abs (Tc, Cm, Ery, Cb and Amp) in soils with the higher levels of HMs. These observations suggest induced HMR in soil bacteria in the presence of elevated HM levels and the subsequent co-selection for Ab resistance. It is interpreted that the HMR bacterial isolates present in soils with high levels of HM, can reproduce and produce their new generations with

less difficulty compared to the HM sensitive bacteria present in these soils, therefore the population of these resistant bacteria are higher in soil containing higher levels of HMs compared to soil with no or lower levels of HMs [16, 88]. These observations are in concordance with several reports corroborating the hypothesis of co-selection of bacterial HMR and AbR while exposing to elevated levels of HMs [86, 102, 355-363]. The resistance bacterial counts using plate culturing for isolates from 6WM and 6MM soil samples showed significantly higher ratios of HMR and AbR/total bacterial CFUs in the microcosms spiked with HMs compared to the controls. The highest ratios of HMR and AbR/total bacterial CFUs were determined in microcosms soils spiked with the Cd 50 mM, Zn 200 mM and Hg 1 mM compared to the microcosms spiked with lower and higher concentrations of HMs. There are reports suggesting the presence of high levels of HMs in soil for a short period of time can lead to induced resistance in bacterial communities, although adaptation to stresses caused by HMs can also happen over longer periods of time, especially with HM levels much higher than the bacterial tolerance threshold as exists in the microcosms used here [191, 264, 270, 296, 364, 365]. It means, the bacterial isolates exposed to very high levels of HMs in microcosms spiked with, for example, Cd concentrations higher than 50 mM resulted in the killing of the bacteria (comprising HM resistant and HM sensitive bacteria) in soil samples, as these levels of Cd are much higher than the tolerance threshold of the bacteria. However, the bacterial isolates which could tolerate the higher levels of Cd, Zn and Hg in microcosms with higher concentrations of HMs than Cd 50 mM, Zn 200 mM and Hg 1 mM, were more resistant to these HMs and Abs, according to PICT analysis which is discussed in the following. Levels of Cd, below 50 mM, were not strong enough to induce CdR in the bacterial isolates, at least in this period of microcosms incubation. The same interpretation is made for stratification of Zn at 200 mM and Hg treatment at 1 mM.

The result of BM and PICT analyses on CdR, ZnR and HgR isolates from WR and BRP sub-sites soils samples revealed there were significantly higher values of the HMs and Abs' MIC and EC50 determined for the bacteria from the soil samples with the higher levels of HMs compared to

those from background soil. Results of BM and PICT analyses of the HMR bacterial isolates from 6WM and 6MM showed higher values of MIC and EC50 for the HMs and Abs for the soil bacteria from the microcosms spiked with the higher levels of HMs compared to those from control microcosms. There are several studies indicating the effects of initial HM levels in different environments to induce HM resistance and subsequent Ab resistance into bacterial isolates [210, 282, 366-372]. It means, the initial presence of elevated levels of HMs in soil will result in HM resistance in the soil bacteria, and Ab co-resistance occurs following the HMR in bacterial isolates. Therefore, higher values of MIC and EC50 were determined for these HMR and AbR bacterial isolates. In addition, the bacterial isolates resistant to higher levels of HMs and Abs, will show higher resistance while determining their MIC and EC50 values [373, 374].

TRFLP (a DNA fingerprinting technique) analysis of DNA samples for WR and BRP showed the bacterial community structure was significantly different for bacteria from the soil samples with higher levels of HMs (pastoral soil for WR and, B17 and B16 for BRP) compared to background soil for WR soil samples, and B10 and B11 for BRP soil samples. The bacteria which developed resistance strategies against the presence of high levels of HMs in these soils, reproduced more HMR bacteria which has led to different bacterial structure profiles compared to those from soil samples with lower levels of HMs [61, 375, 376]. It was determined that bacterial isolates in microcosms with pastoral soil experienced lower community structural changes compared to the microcosms with background soils in response to HM exposure. There was >60% similarity between the T-RFs abundance of bacterial communities from control microcosms and HM-spiked microcosms after the period of incubation. The bacterial isolates from 6PMM soil samples were initially subjected to community structure changes due to the initial presence of HMs in pastoral soil. Therefore, lower bacterial community structure changes occurred with the addition of high levels of HMs to the microcosms with pastoral soil [377, 378]. The result of the Next Generation 16s rDNA sequencing of selected samples from BRP and 6WM revealed relative abundance of each bacterial phylum were significantly different in bacterial isolates from B10

and B14 soils compared to those from B17 soil, and also in bacterial isolates from 6WM control soil compared to those from Cd 100 mM, Zn 200 mM and Hg 50 mM-spiked microcosms. The higher levels of HMs in these soils have resulted in greater bacterial community changes compared to the soils with lower levels of HMs.

The current study investigated the horizontal transfer of CdR genes (*cadA* and *czcA*) by conjugation. *cadA* and *czcA* were able to be transferred to recipients. The bacterial isolates carrying these genes as donor cells in this experiment were resistant to Cd and a range of Abs. Transconjugants received these genes, showed Cd, Zn and Hg, and also Ab resistance features too. The observation of this investigation supports the hypothesis that co-selection for Ab resistance occurs parallel to HM resistance features development [36, 379].

Conclusion

To conclude, accumulation of HMs in soil and the development of HMR and AbR, as a growing problem must not be overlooked, which introduces a major risk to human and animal health and financial losses. This project elucidated the bacterial resistance to HMs and Abs in selected soil samples from two regions in New Zealand, and in soil bacteria from microcosms spiked with different concentrations of HMs. Resistance plate counts (investigating HMR and AbR/total CFU ratios) for WR, BRP and microcosms soil samples, and also BM and PICT analysis, suggests the presence of elevated levels of HMs in soil leads to the development of HM resistance in bacterial isolates. Subsequent Ab co-resistance occurred due to co-selection in parallel with HMR in bacterial isolates. Bacterial isolates from soil samples with higher levels of HMs showed greater resistance to these HMs as well as a range of Abs. Bacterial community structure changes in soil samples was investigated using TRFLP fingerprinting and 16s rDNA Next Generation Sequencing. As another part of this project, I investigated the presence of Cd resistant genes, *cadA* and *czcA*, in selected CdR strains. The result showed that horizontal transfer of these CdR genes to other bacteria is probable, especially if they are located on mobile genetic elements, e.g. plasmids; the entry of these HM resistance genes to new recipients will introduce HM and possibly Ab resistance features into new hosts. These results emphasize that elevated HMs concentrations in soil leads to bacterial resistance to these metals and subsequent resistance to different classes and types of Abs as a major risk for humans and animals. Together, the results in this thesis contributed to the comprehensive understanding of HM and Ab resistance in soil bacteria under the selection pressure of HMs presence in soils with different history of usage.

Future Research

More investigations on other soil samples from other regions of New Zealand to complete a broad insight into resistance to heavy metals and antibiotics by soil bacteria in New Zealand is recommended for future research projects. Investigation on soil samples from different areas

with different history of usage and farms producing different crops will increase the knowledge on the levels of HMR and AbR in bacterial isolates in different soil samples. Comprehensive investigations on the mechanisms of resistance to HMs and Abs in different bacterial isolates from areas with various HMs levels will produce important information on the major mechanism bacteria from different soils apply against HMs and Abs. Further researches on products with lower concentrations of HMs, e.g. superphosphate fertilisers, is suggested. The products with lower HMs-concentrations could help to conduct researches on the safer levels of HMs accumulated in soil samples.

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Appendices

Appendix 1

Table A1. 1. Methods used by Hill Laboratories Ltd. to determine the physicochemical features of WR and BRP soil samples.

Test	Method Description
Soil Preparation	Air dried at 35-40 °C overnight (residual moisture typically 4%).
pH	1:2 (v/v) soil:water slurry followed by potentiometric determination of pH.
Olsen Phosphorous	Olsen extraction followed by Molybdenum Blue colorimetry.
Metals extensive suite, trace level (33 metals)	Dried sample, <2 mm fraction. Nitric/Hydrochloric acid digestion, ICP-MS, trace level.
Total Recoverable digestion	Nitric/hydrochloric acid digestion. US EPA 200.2.
Total Fluoride in solids alkaline fusion	Alkaline fusion of sample. Methods of Soil Analysis 2nd Edition, Pt2, 26-4.3.3.
Total Fluoride in Solids	Ion selective electrode. Methods of Soil Analysis 2nd Edition, Pt2, 26-4.3.3.
Total Organic Carbon	Acid pretreatment to remove carbonates present followed by Catalytic Combustion (900 °C, O2), separation, Thermal Conductivity Detector [Elementar Analyser].

Appendix 2

The Appendix 2 is supporting the chapter 1 of Results and is presenting the excess data not shown in that chapter.

A2.1. Physicochemical Properties of the Soil Sampling Sites.

As was discussed in Section 4.1 of the chapter 1 of result, physicochemical properties of the soil samples collected from WR and BRP were assessed and continue of the result are listed in Table A2.1 for WR and A2.2 for BRP soil samples.

Table A2.1. Continue of physicochemical properties of WR's soil samples

Site No.	**73**	**85**	**86**	**69**	**135**
Anaerobically Mineralised N (mg kg^{-1})	165	31.2	24.9	222	176
Total porosity (%, v/v)	64.3	60.6	62.2	58.4	60.0
Bulk density t/m^3	0.76	1.03	1.11	0.84	0.86
F *	136	340	460	340	530
Al *	36000	47000	68000	38000	59000
Sb *	0.07	0.06	0.05	0.07	0.08
As *	6.20	9.40	10.80	7.00	8.90
Ba *	240	138	114	128	166
Bi *	0.36	0.35	0.28	0.19	0.33
B *	5.00	3.00	2.00	4.00	4.00
Cs *	6.30	3.60	3.50	3.80	5.30
Ca *	2200	4600	2900	5800	5100
Cr *	13.2	18.1	23.0	56.0	19.7
Co *	10.9	3.40	4.30	10.4	12.7
Cu *	10.8	16.3	20.0	23.0	22.0
La *	5.60	7.40	8.20	12.4	9.20
Pb *	28.0	25.0	24.0	21.0	32.0
Li *	8.50	10.7	16.0	9.50	15.8
Mg *	890	470	680	1120	590
Mn *	2400	600	1080	2300	3700
Mo *	1.09	1.17	1.46	1.13	1.55
Ni *	5.20	7.10	6.00	17.70	6.70
K *	530	770	720	1380	730
Rb *	13.4	9.10	7.00	15.1	14.2
Se *	< 2.00	3.00	3.00	2.00	3.00
Ag *	0.10	0.08	0.12	0.19	0.12
Na *	250	91.0	83.0	146	120
Sr *	36.0	21.0	22.0	32.0	23.0
Ti *	0.72	0.15	0.34	0.39	0.65
Sn *	2.20	2.70	2.80	2.40	2.50
U *	1.52	2.50	2.60	2.30	2.70
V *	71.0	101	151	113	106
Hot Water C (mg Kg^{-1})	3359	1018	688	5010	4184
Hot Water N (mg Kg^{-1})	291	130	250	611	528

Hot Water C/Hot Water N	12.0	8.00	3.00	8.00	8.00
P-retension	44.0	43.0	48.0	45.0	48.0
15N	1.46	7.11	6.37	6.28	6.29

*mg kg^{-1} of dry soil

Table A1.2. Continue of physicochemical properties of BRP soil samples.

Site No.	**B10**	**B11**	**B12**	**B13**	**B14**	**B15**	**B16**	**B17**
Dry Matter (g/100g)	70.0	74.0	73.0	73.0	73.0	73.0	73.0	73.0
F *	540	570	880	1040	1260	1580	2500	4500
Al *	21000	13500	22000	18600	19700	19300	23000	21000
Sb *	0.06	0.06	0.09	0.08	0.09	0.10	0.19	0.40
As *	3.30	3.10	3.70	3.40	3.60	3.40	3.90	4.30
Ba *	33.0	33.0	35.0	36.0	38.0	39.0	55.0	75.0
Bi *	0.16	0.15	0.15	0.14	0.15	0.16	0.17	0.13
B *	4.00	4.00	4.00	4.00	5.00	4.00	5.00	6.00
Cs *	2.00	1.82	2.20	1.97	2.00	1.96	1.98	2.10
Ca *	6900	10600	10000	35000	35000	20000	18900	65000
Cr *	15.9	16.2	18.8	18.6	20.0	22.0	27.0	37.0
Co *	5.00	5.20	6.10	6.70	8.00	8.20	7.30	13.5
Cu *	10.4	9.90	15.4	18.9	22.0	27.0	13.6	18.8
La *	9.80	10.0	10.9	11.5	11.8	11.6	14.8	25.0
Pb *	17.0	14.9	16.4	15.5	16.3	16.0	18.5	16.4
Li *	23.0	15.3	24.0	21.0	23.0	21.0	24.0	18.4
Mg *	2500	2100	3100	3100	3400	2800	2600	3200
Mn *	320	200	330	340	370	350	290	370
Mo *	0.72	0.56	0.77	0.75	1.00	1.04	1.43	1.71
Ni *	8.00	8.80	9.20	10.2	10.2	10.5	9.20	11.8
K *	880	560	950	960	940	1100	1230	1810
Rb *	13.8	12.0	13.9	12.7	13.7	13.5	15.8	16.0
Se *	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00	<2.00
Ag *	0.10	0.08	0.10	0.10	0.11	0.11	0.15	0.21
Na *	197	146	230	310	310	340	390	510
Sr *	37.0	55.0	55.0	123	131	103	118	280
Ti *	0.14	0.13	0.14	0.13	0.14	0.14	0.16	0.15
Sn *	1.29	1.21	1.28	1.22	1.19	1.24	1.33	1.06
U *	2.20	2.10	3.60	4.00	4.90	5.60	9.90	17.0
V *	38.0	35.0	38.0	34.0	39.0	37.0	43.0	43.0

*mg kg^{-1} of dry soil

A2.2. Bacterial Total Counts of Waikato Region Soil Samples

As discussed in Section 4.1.4, the number of viable bacterial cells in pastoral and arable cropping soils was higher compared to the soil from the control (native bush) site ($P < 0.05$) for each WR region soil sample sets (WRSS2, the second sampling date of August 2014; WRSS3, the third sampling date of June 2015) (Figure A2.1). There were higher number of bacteria in pasture soils compared to arable soils for each of these soil sample sets (Table A2.1).

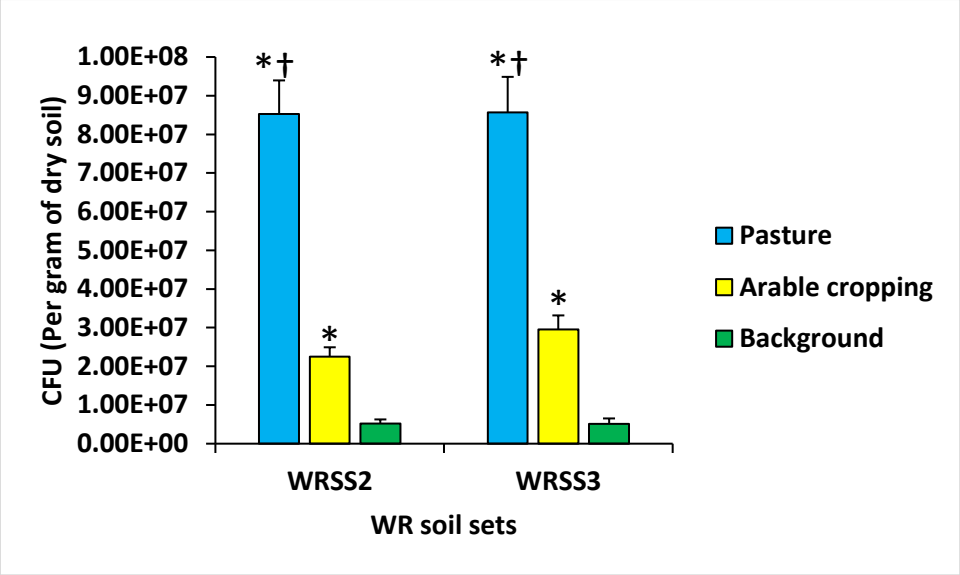

Fig. A2.1. Total number of CFU (per gram of dry soil) from WRSS2 and WRSS3 pastoral, arable and native bush soil samples, and selected on R2A agar. *$p < 0.05$ compared to background soil bacteria total CFU; †$p < 0.05$ compared to arable soil bacteria total CFU.

A2.2.1. Resistant Bacterial Counts of Waikato Region's Soil Samples on Plates with HM additives

Plate culturing of WRSS2 and WRSS3 on a range of Cd, Zn and Hg showed there were higher HMR/total bacterial CFU ratios of pastoral soil bacteria compared to those from background soil ($p < 0.05$). These results were similar to the results determined for those bacteria from WRSS1 (Figures A2.2-A2.4).

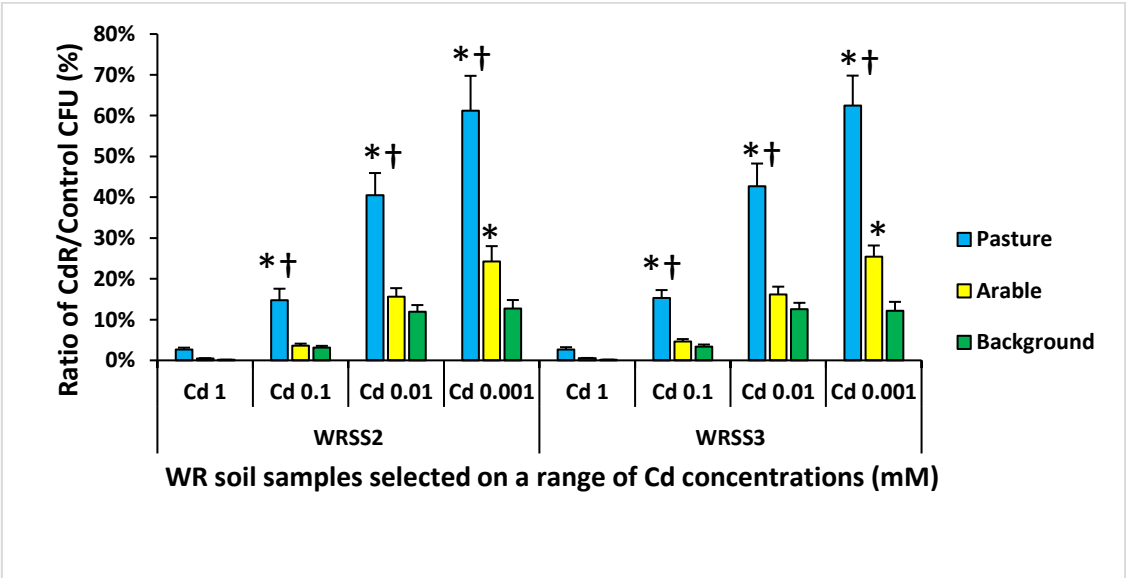

Fig. A2.2. Mean ratios of CdR/total bacterial CFU, selected on a range of Cd concentrations, for WRSS2 and WRSS3. *$p < 0.05$ compared to background soil CdR/total bacterial CFU ratio

selected on the same Cd concentration; †$p < 0.05$ compared to arable CdR/total bacterial CFU ratio selected on the same Cd concentration.

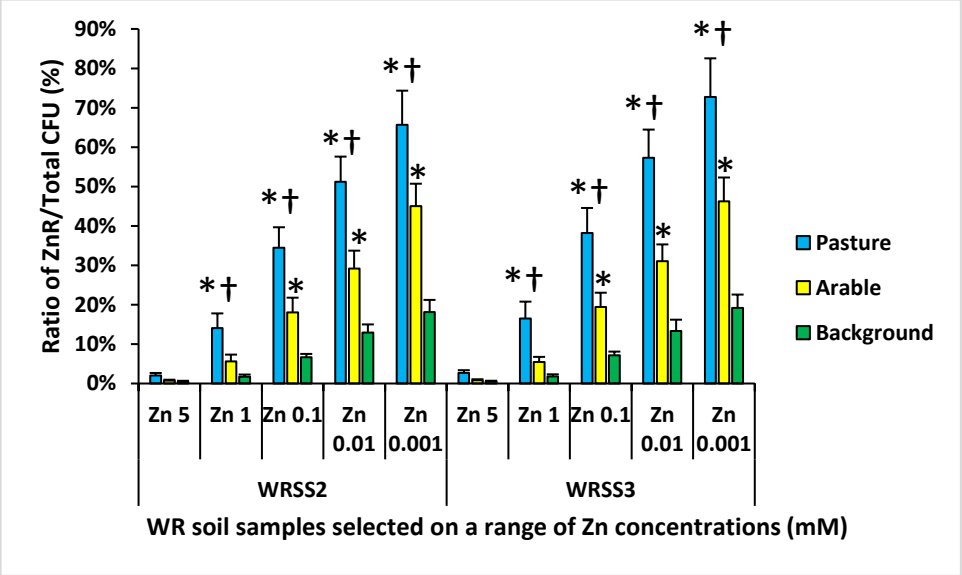

Fig. A2.3. Mean ratios of ZnR/total bacterial CFUs, selected on a range of Zn concentrations, for the WRSS2 and WRSS3. *$p < 0.05$ compared to background ZnR/total bacterial CFU ratio selected on the same HM concentration; †$p < 0.05$ compared to arable ZnR/total bacteria CFU ratio selected on the same HM concentration.

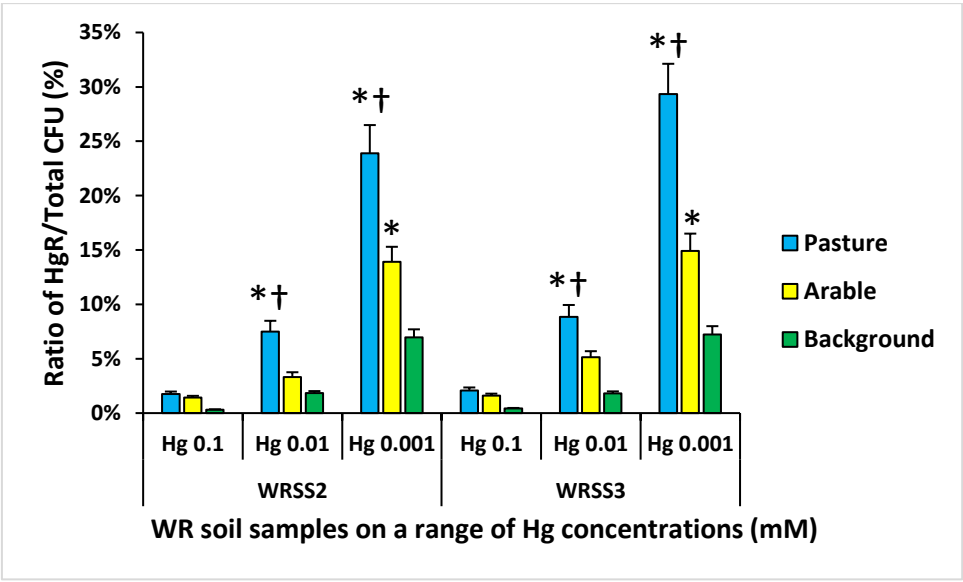

Fig. A2.4. Mean ratios of HgR/total bacterial CFUs, selected on a range of Hg concentrations, for the WRSS2 and WRSS3. *$p < 0.05$ compared to background soil HgR/total bacterial CFU ratio selected on the same HM concentration; †$p < 0.05$ compared to arable soil HgR/total bacterial CFU ratio selected on the same HM concentration.

A2.2.2. Resistant Bacterial Counts of Waikato Region's Soil Samples on Plates with Ab additives

Similar to what discussed in Section 4.1.3.2 for WRSS1, the pastoral and arable soils of WRSS2 and WRSS3 had significantly higher AbR/total bacterial CFUs compared to background, and the pastoral soils had higher AbR/total bacterial CFUs compared to arable soils.

In addition, similar to the HM plates, the lower concentrations of Abs have significantly higher ratios of AbR/total bacterial CFUs compared to the higher concentrations (Figure A2.5).

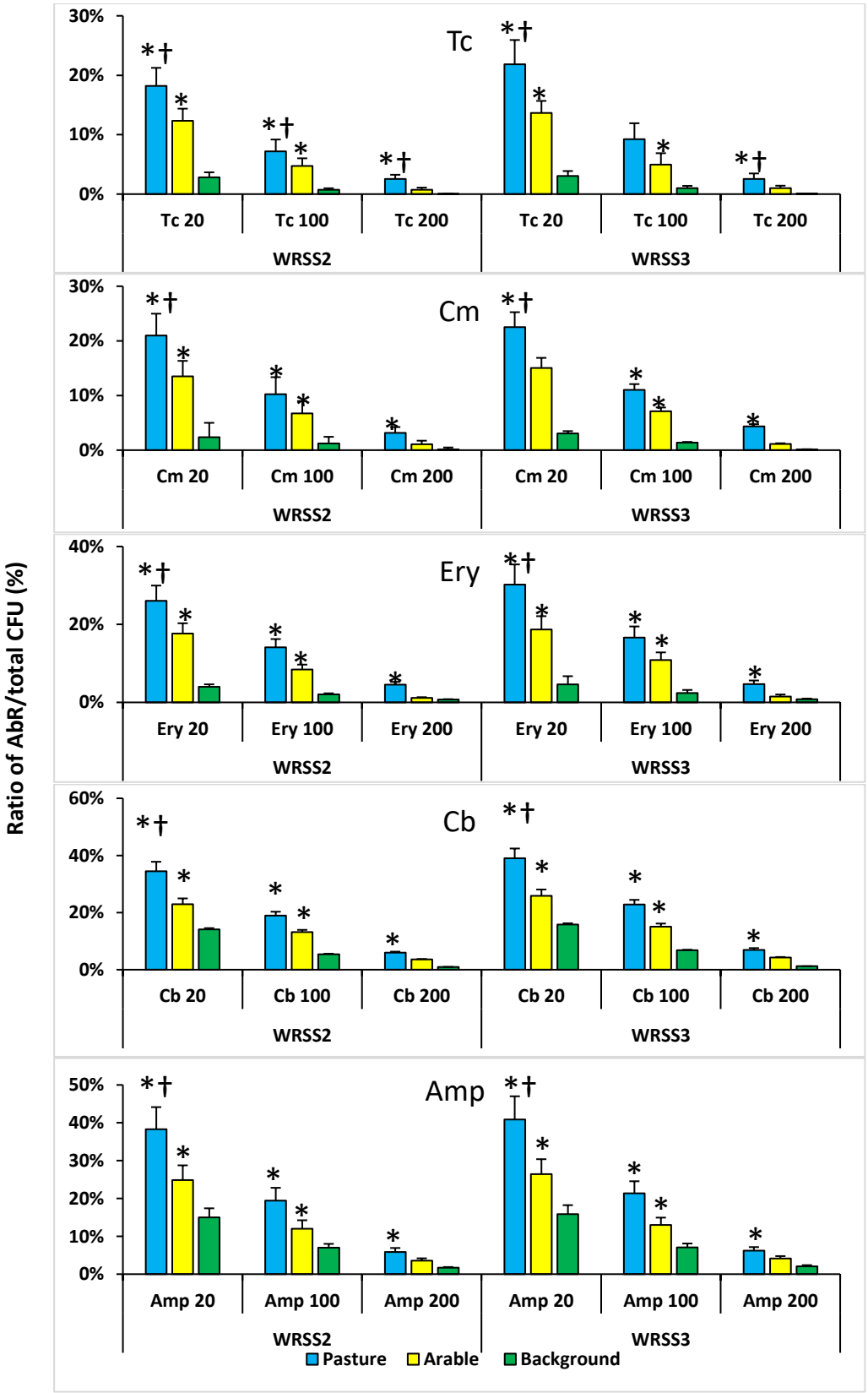

Fig A2.5. Mean ratios of AbR/total bacterial CFUs, selected on a range of Abs concentrations, for the WRSS2 and WRSS3. The Ab concentrations are per µg mL^{-1}. *$p < 0.05$ compared to background soil AbR/total bacterial CFU ratio selected on the same Ab concentration; †$p < 0.05$ compared to arable soil AbR/total bacterial CFU ratio selected on the same Ab concentration.

A2.3. PICT Analysis for WR Bacterial Isolates

According to Section 4.1.5, it was revealed that there were significant differences between HMs MIC/EC50 values of PICT test for bacterial communities from WRSS2 and WRSS3 pastoral soils compared to those from control soil. In addition, the result showed there were greater ABs MIC/EC50 values of PICT test for bacterial communities from WRSS2 and WRSS3 pastoral and arable cropping soils compared to those from control soil (Figures A2.38-A2.45).

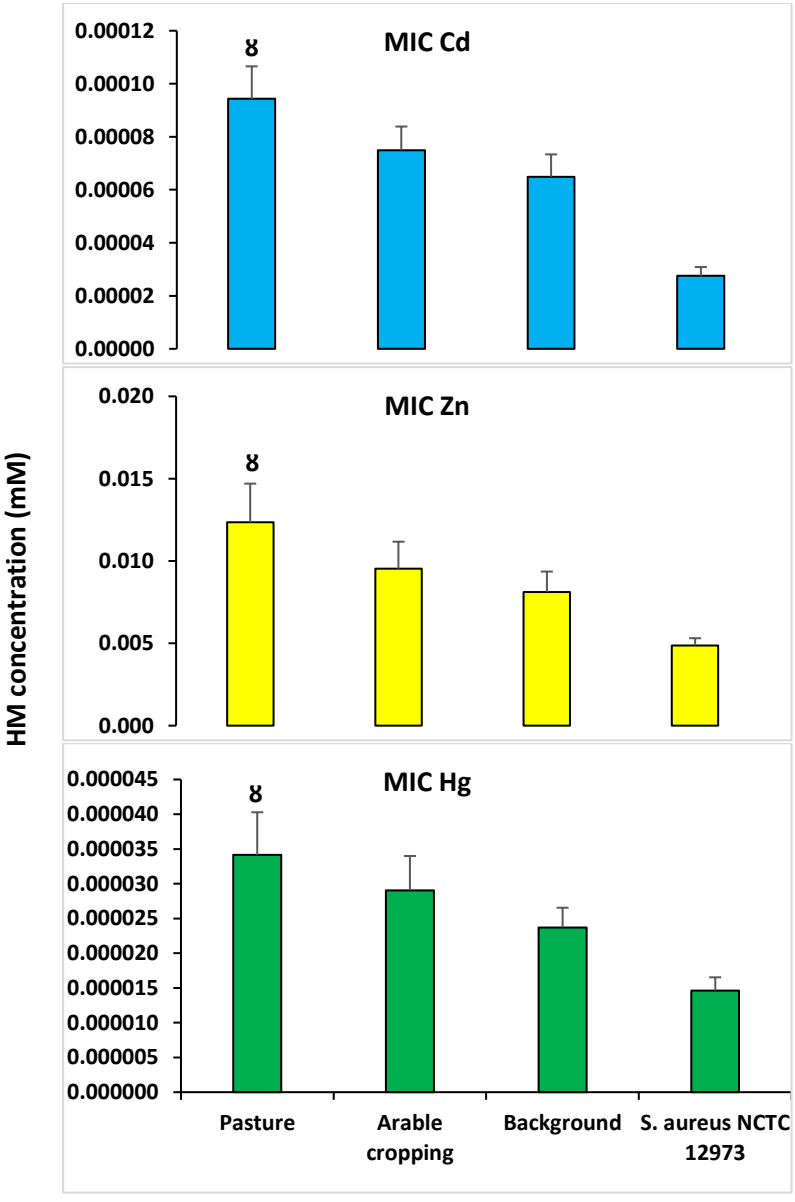

Fig. A2.6 Mean MIC values of PICT assay with Cd, Zn and Hg for bacteria from WRSS2. ϑ$p < 0.05$ compared to HM MIC values for bacteria from background soil.

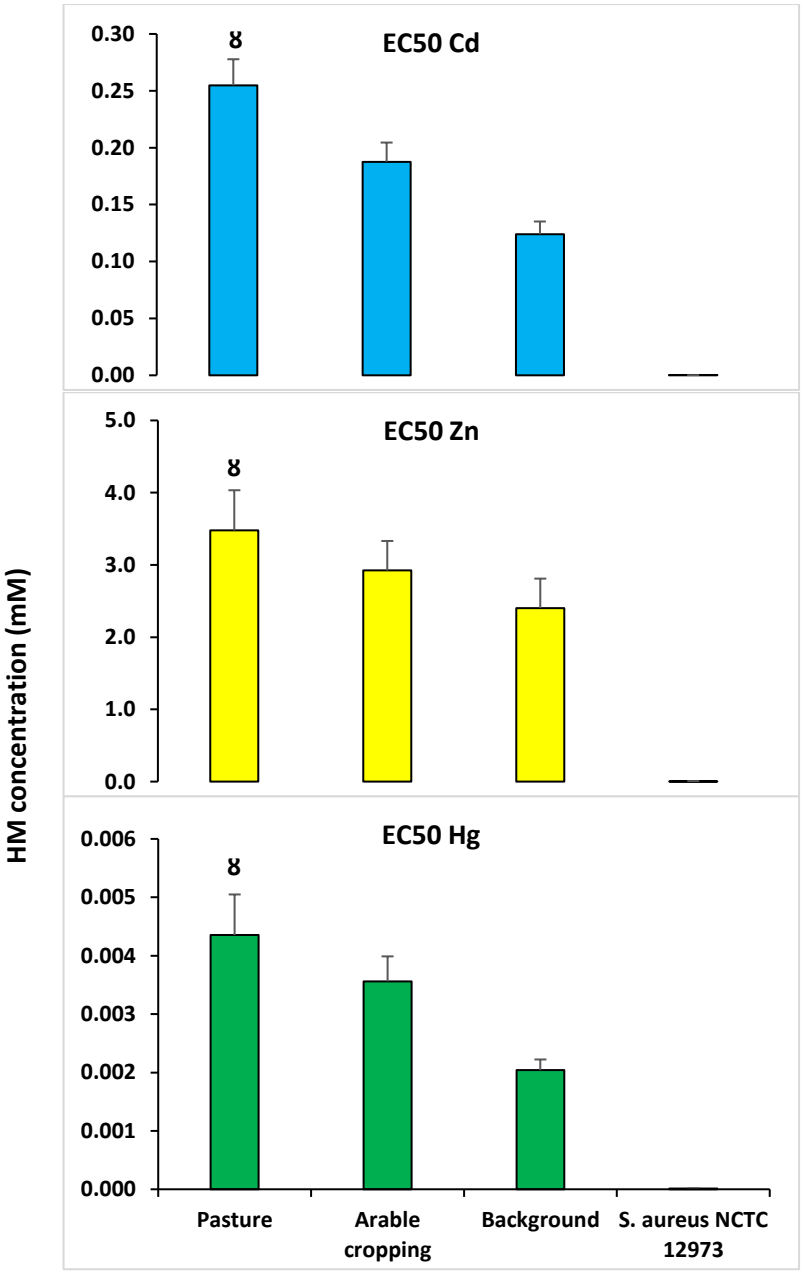

Fig. A2.7. Mean EC50 values of PICT assay with Cd, Zn and Hg for bacteria from WRSS2. ϐ$p < 0.05$ compared to HM MIC values for bacteria from background soil.

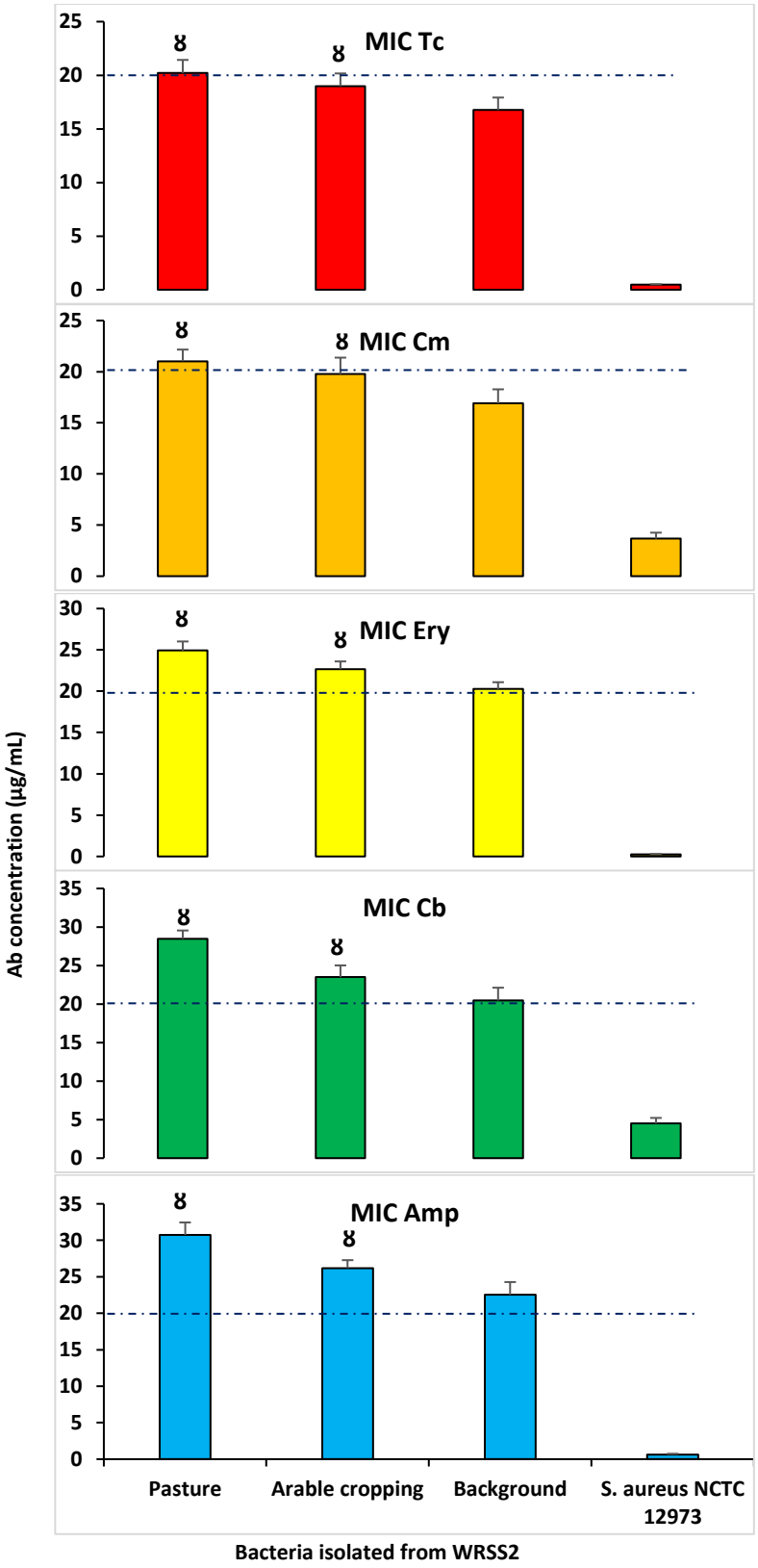

Fig. A2.8. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS2. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from control soil. The dash line defines AbR level in soil bacteria.

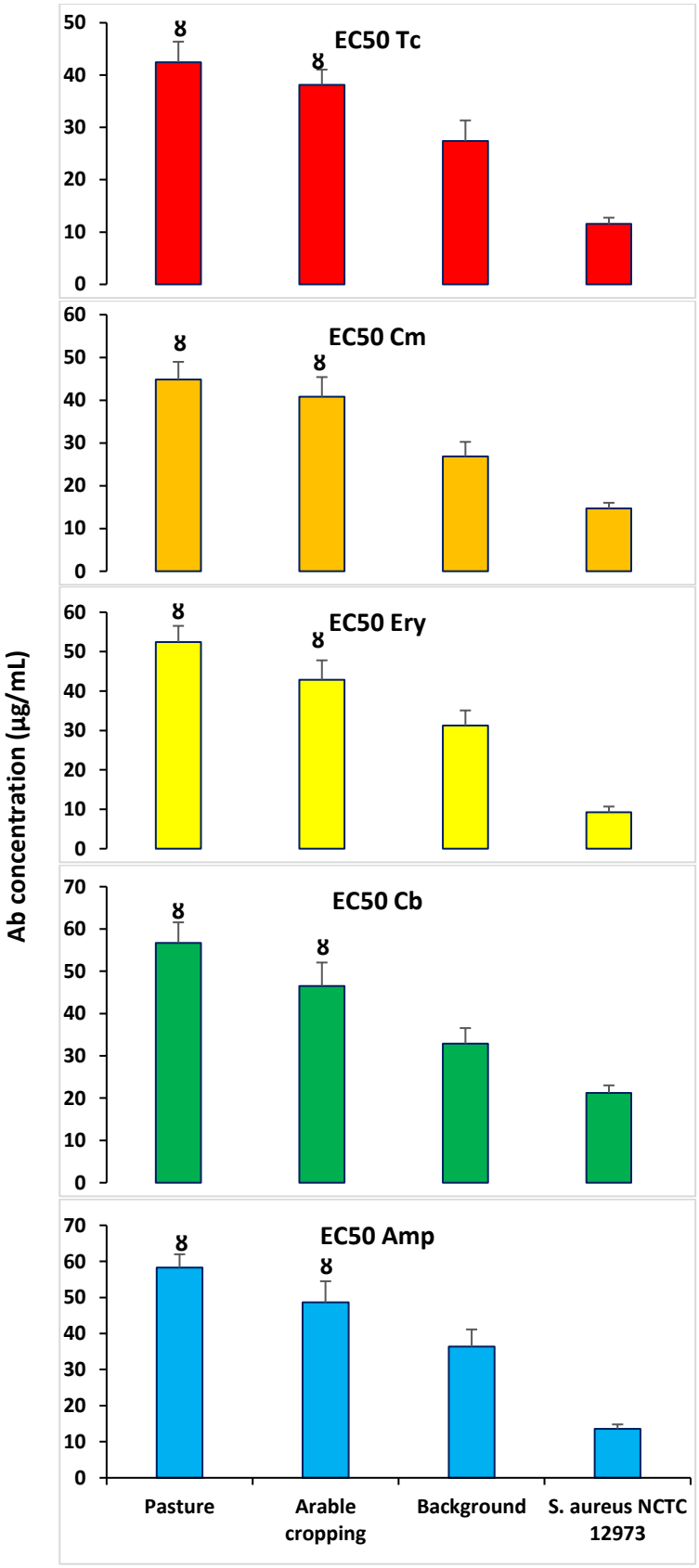

Fig. A2.9. Mean EC50 values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS2. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from control soil.

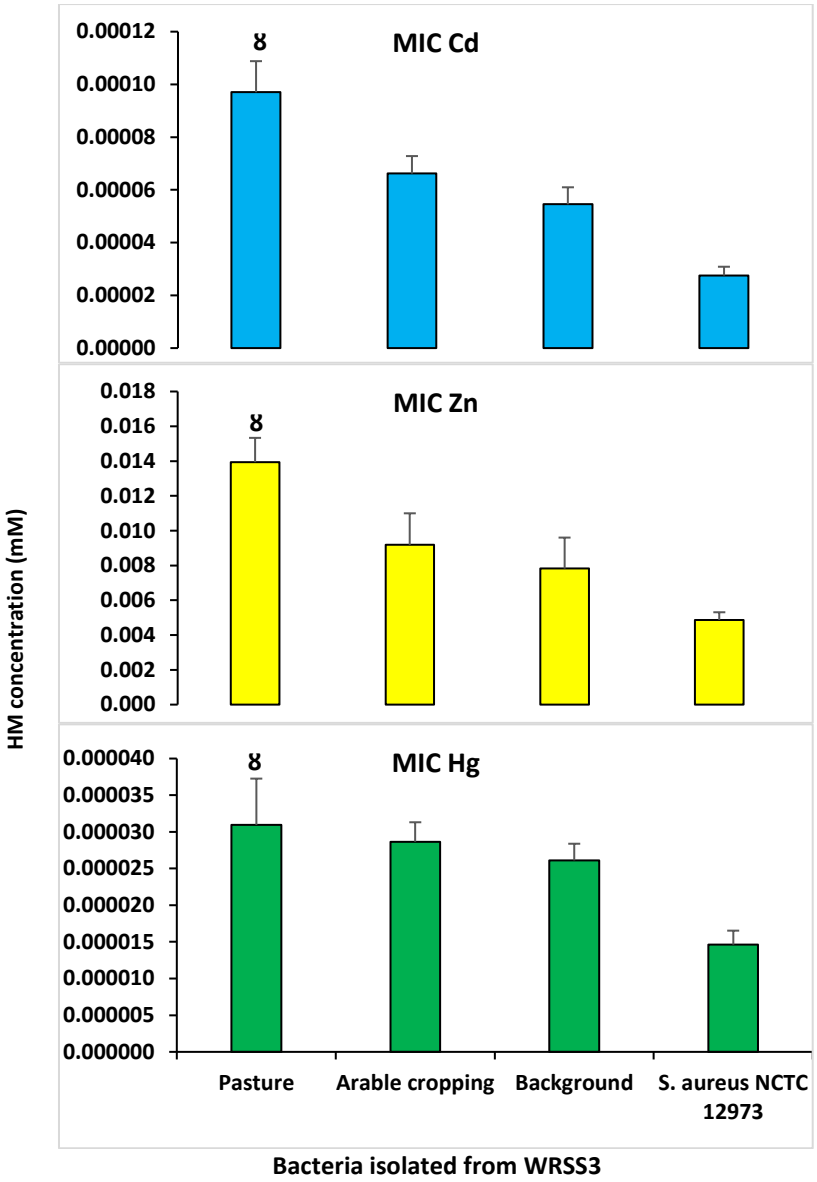

Fig. A2.10. Mean MIC values of PICT assay with Cd, Zn and Hg for bacteria from WRSS2. ϐ$p < 0.05$ compared to HM MIC values for bacteria from control soil.

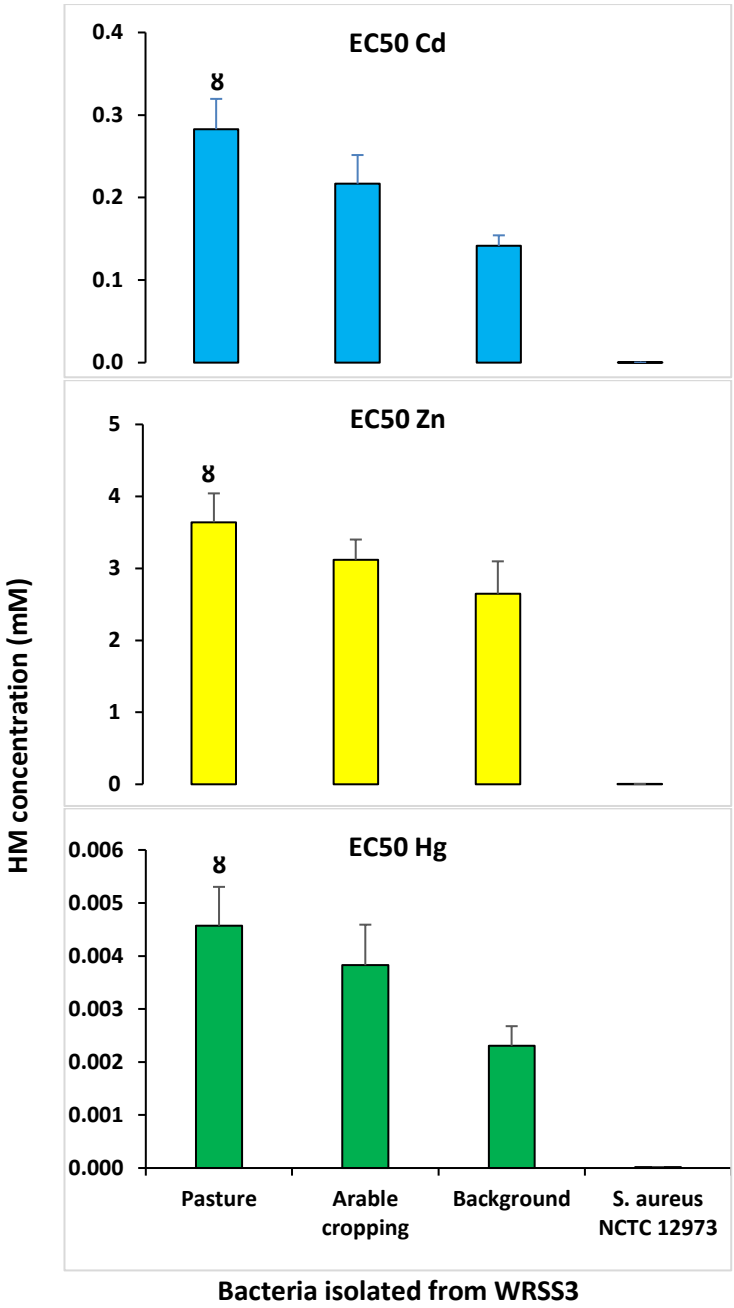

Fig. A2.11. Mean EC50 values of PICT assay with Cd, Zn and Hg for bacteria from WRSS2. ϐ$p < 0.05$ compared to HM MIC values for bacteria from control soil.

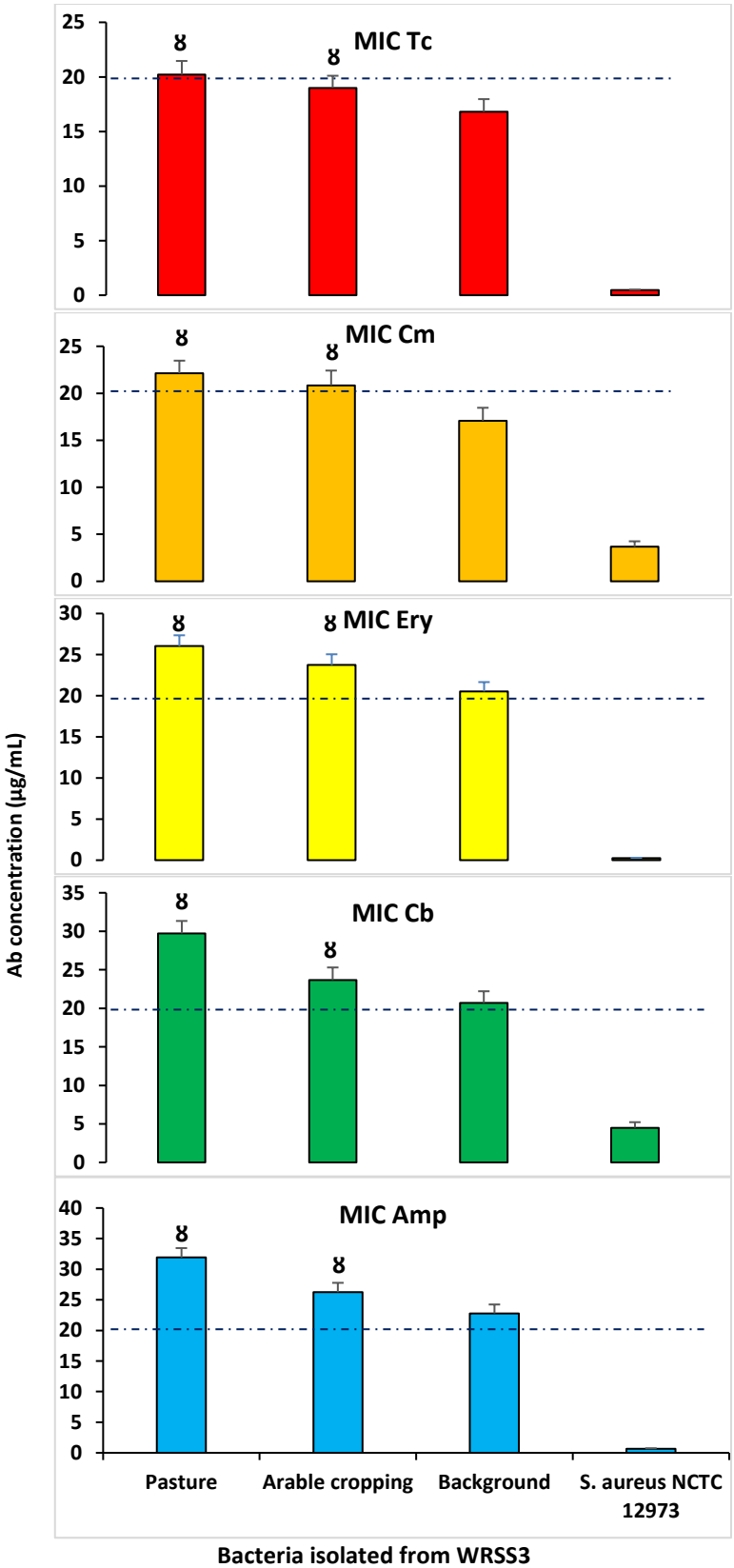

Fig. A2.12. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS3. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from control soil. The dash line defines AbR level of soil bacteria.

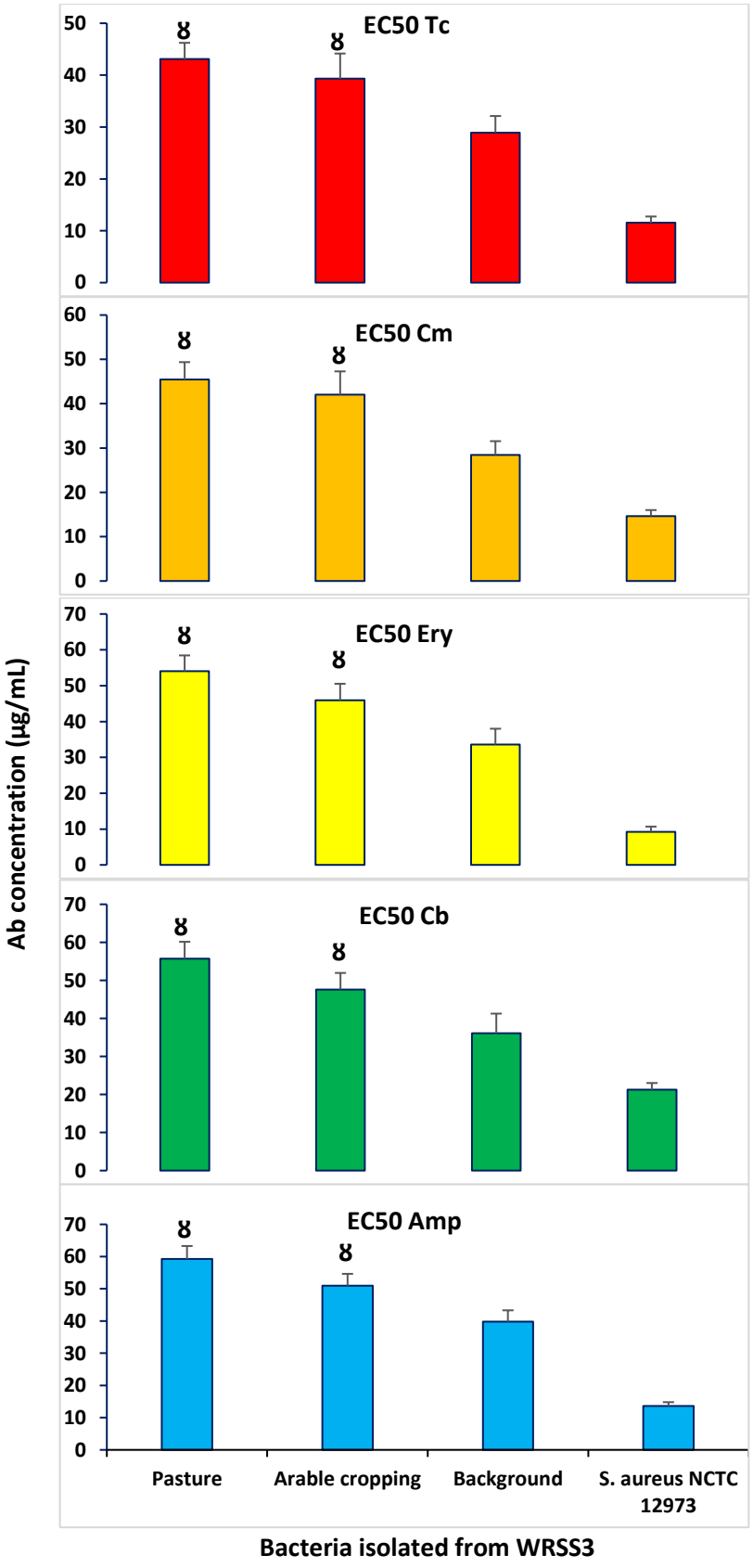

Fig. A2.13. Mean EC50 values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from WRSS3. 8$p < 0.05$ compared to Abs MIC values for bacteria from control soil.

A2.4. Pollution Induced Community Tolerance test for Belmont Regional Park soils

According to Section 4.1.5.2, there were higher HM's MIC/EC50 values of PICT test for bacteria from B16-B15 soils compared to those from B10 soil. There were some exceptions, including for Hg MIC value for bacteria from B15 soil, as well as the significant differences determined between EC50 values for bacteria B16 compared to those from B10. There were greater Ab's MIC/EC50 values of bacteria from B16-B15 soils compared to those from B10 soil, as well as Tc and Cm MIC values of bacteria from B13-B12 soils compared to those from B10 soil (Figures A2.14-A2.17).

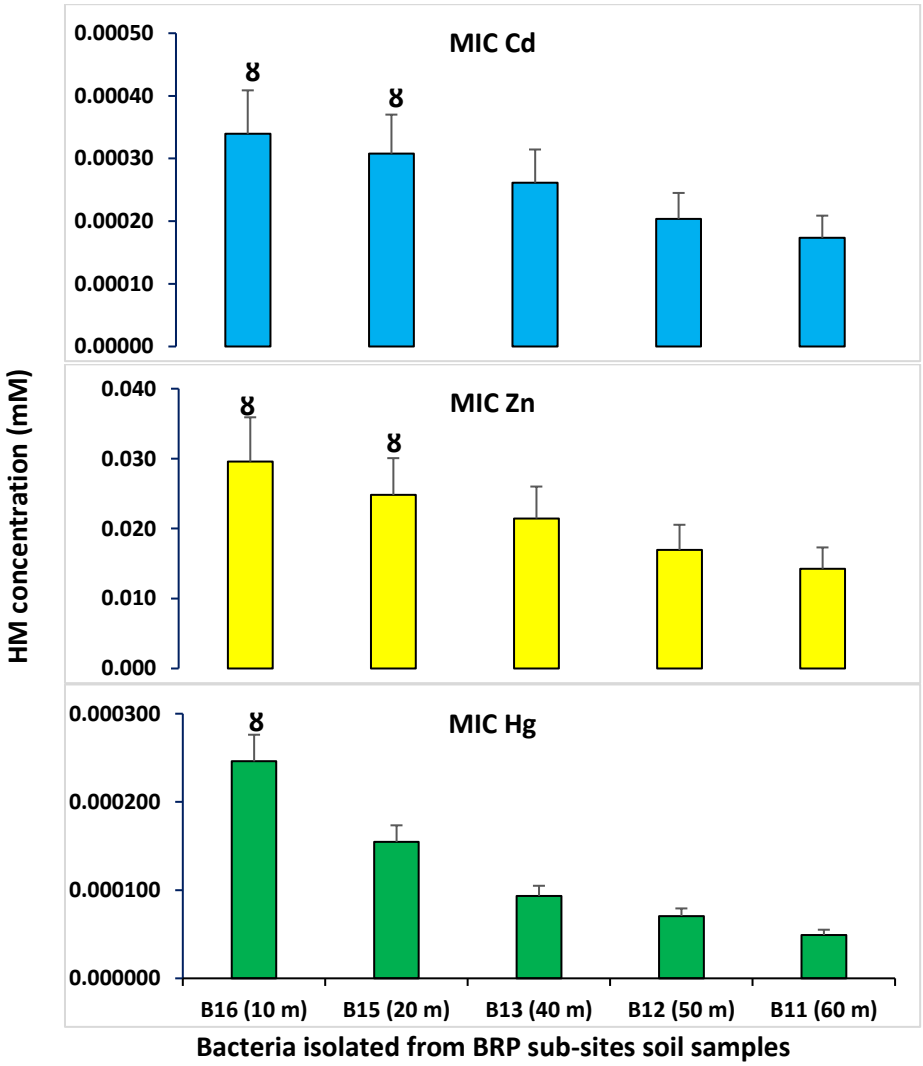

Fig. A2.14. Mean MIC values of PICT assay with Cd, Zn and Hg for bacteria from BRP B16, B15, B13, B12 and B11 soils. ϑ$p < 0.05$ compared to HM MIC values for bacteria from B10 soil.

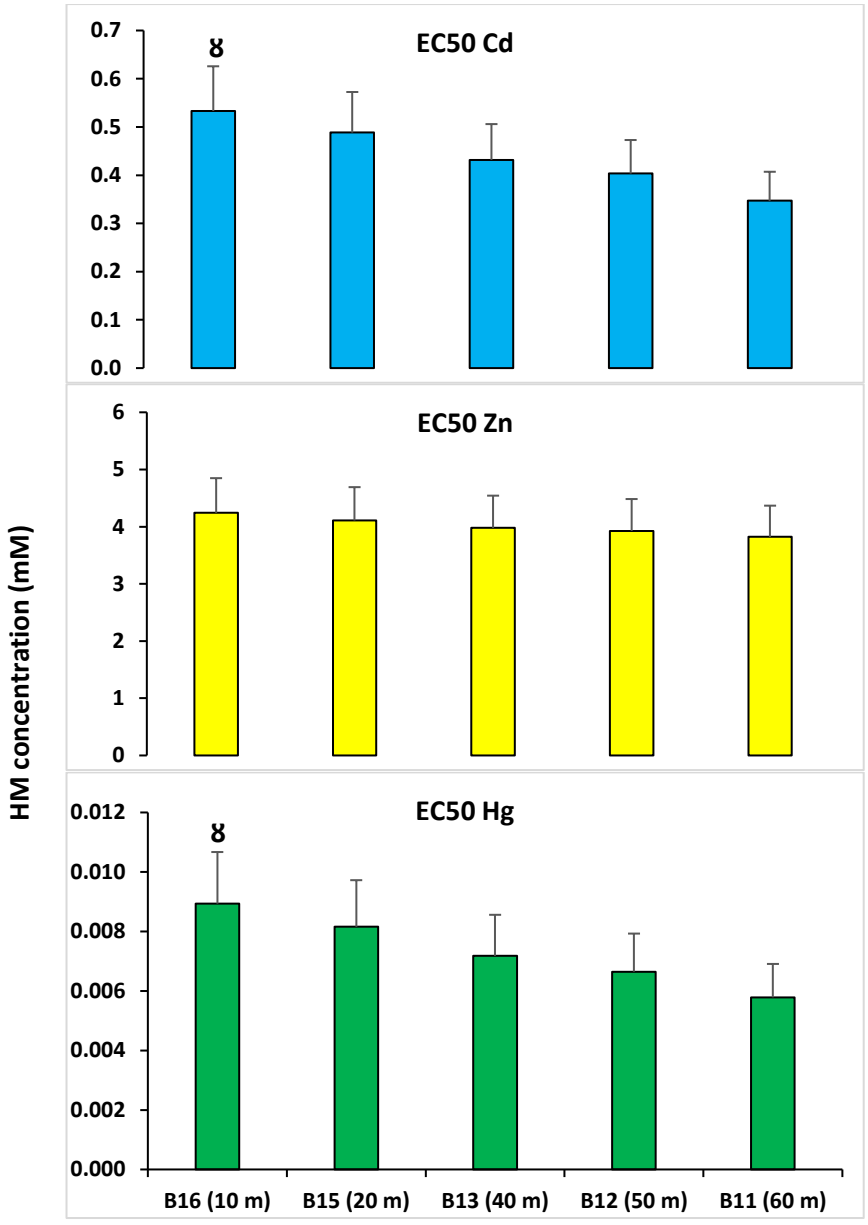

Fig. A2.15. Mean EC50 values of PICT assay with Cd, Zn and Hg for bacteria from BRP B16, B15, B13, B12 and B11 soils. ϐ$p < 0.05$ compared to HM MIC values for bacteria from B10 soil. The dash line defines AbR level of soil bacteria.

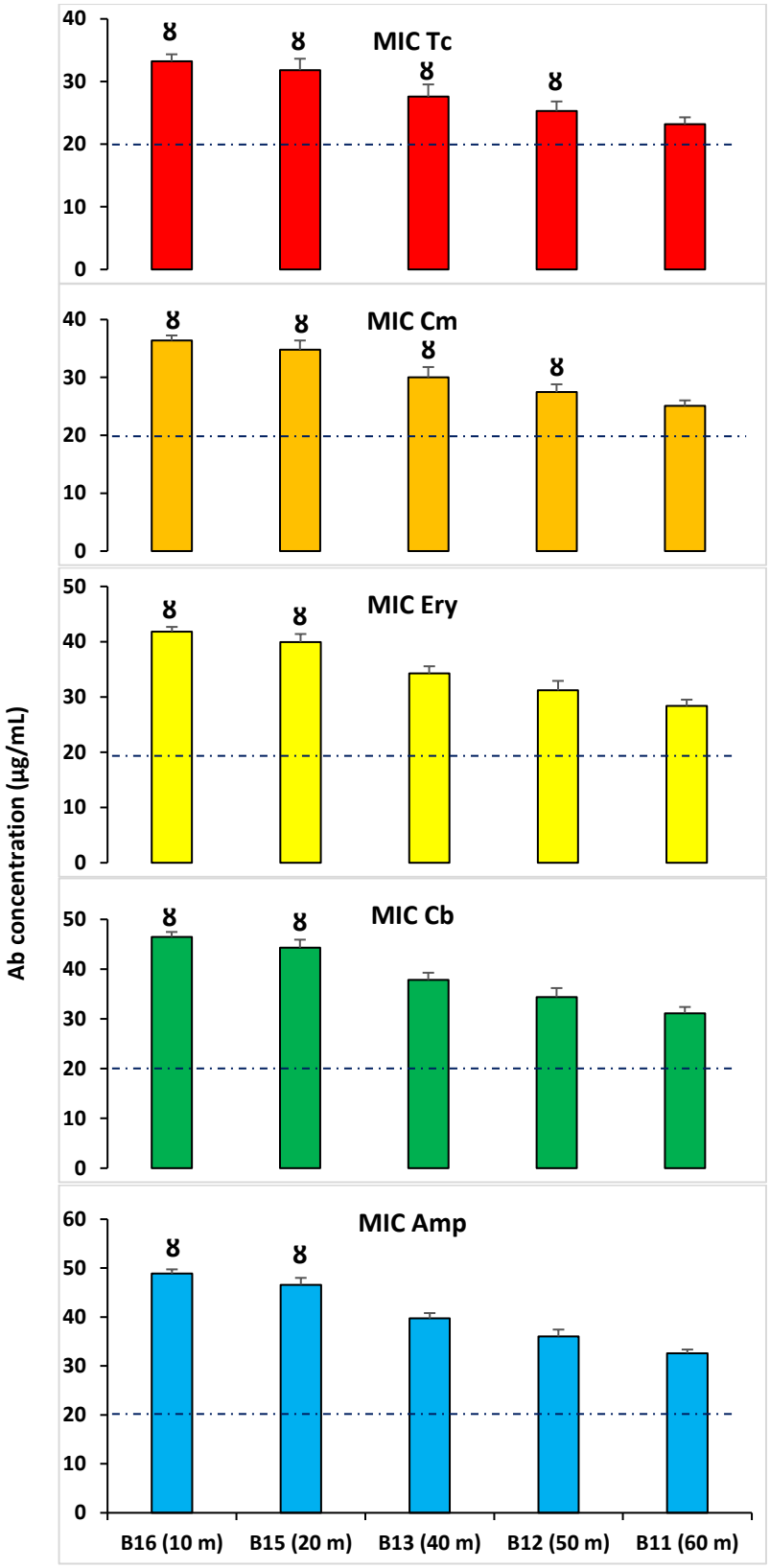

Fig. A2.16. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from BRP B16, B15, B13, B12 and B11 soils. ϑ$p < 0.05$ compared to Abs MIC values for bacteria from B10 soil.

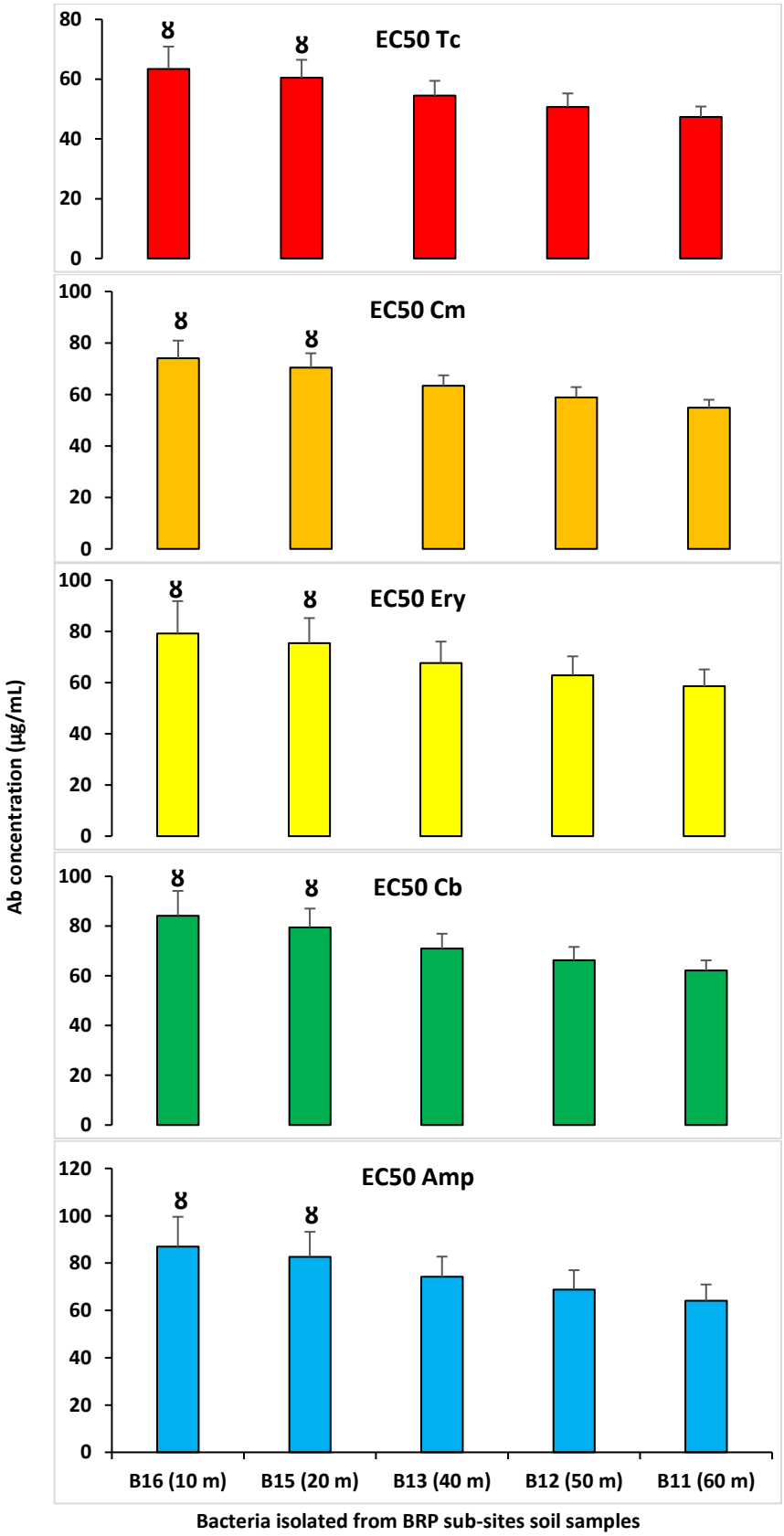

Fig. A2.17. Mean MIC values of PICT assay with Tc, Cm, Ery, Cb and Amp for bacteria from BRP B16, B15, B13, B12 and B11 soils. ϐ$p < 0.05$ compared to Abs MIC values for bacteria from B10 soil.

A2.5. Broth Microdilution Analysis for Waikato Region Soil Samples.

According to WRSS1 BM test (Section 4.1.6), there were lower HM and Ab's MIC and EC50 values for bacteria from WRSS2 and WRSS3, especially for those from control and arable cropping soils selected on plates containing Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those selected on plates with a higher concentration of each HM (Figures A2.18-A2.21).

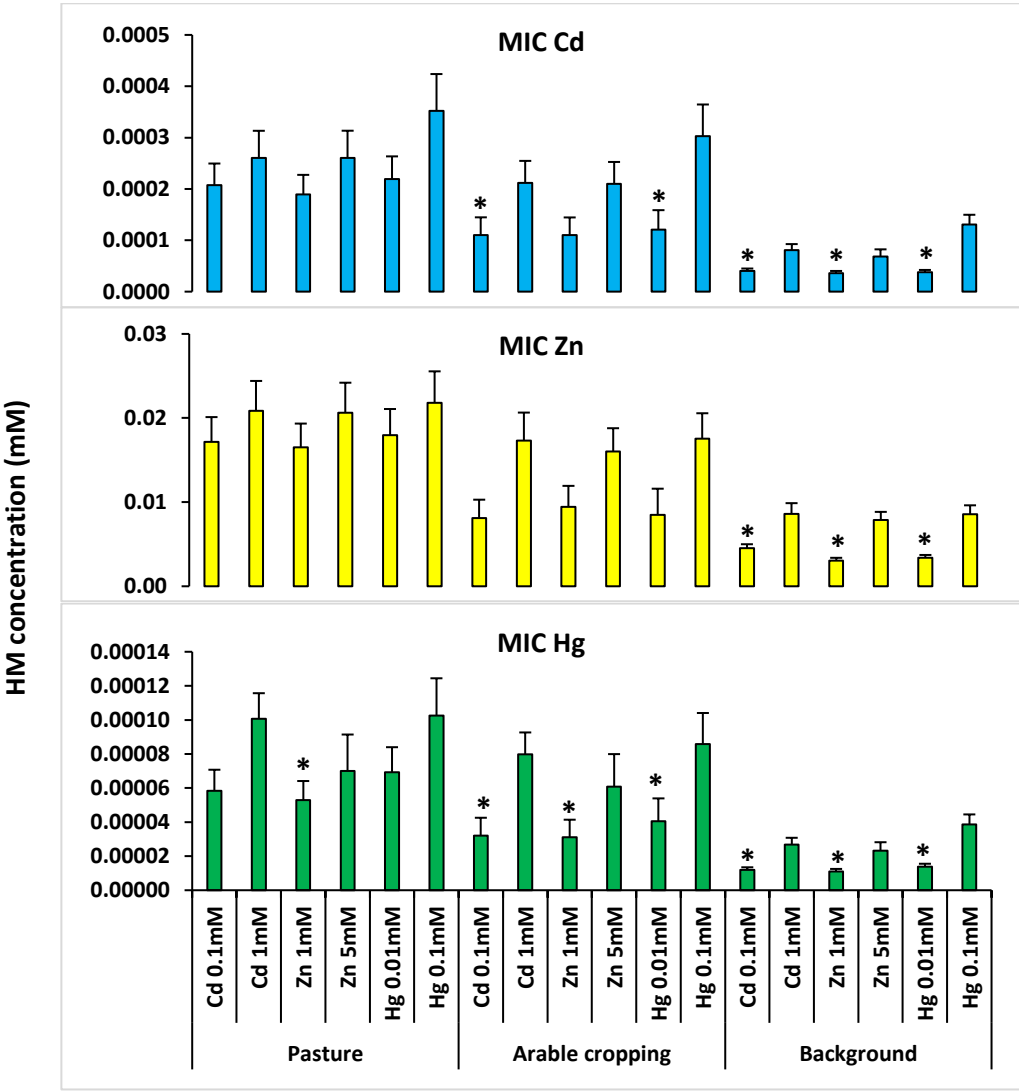

Fig. A2.18. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS1 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same HM's MIC value for the isolates from the same soil and selected on the higher concentration of each HM.

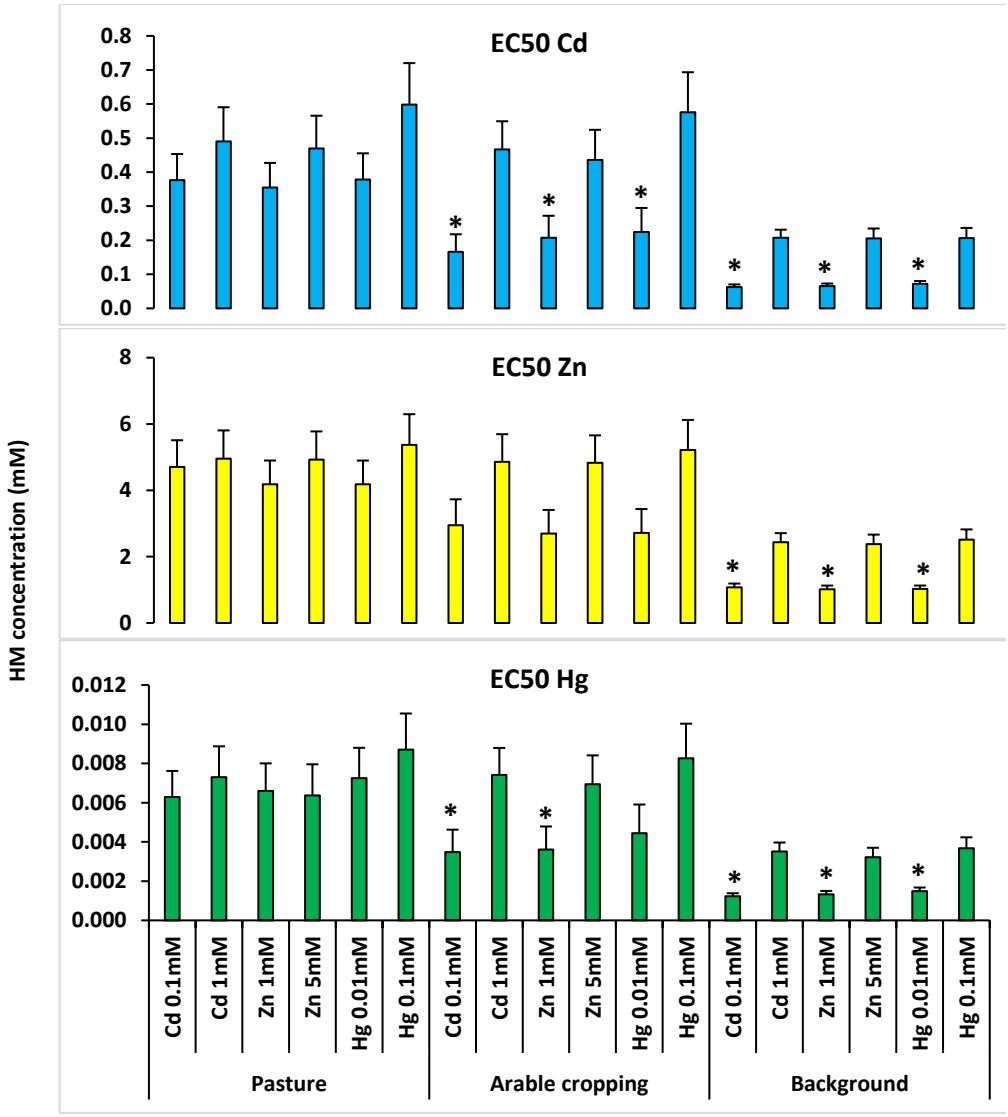

Fig. A2.19. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS1 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same HM's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

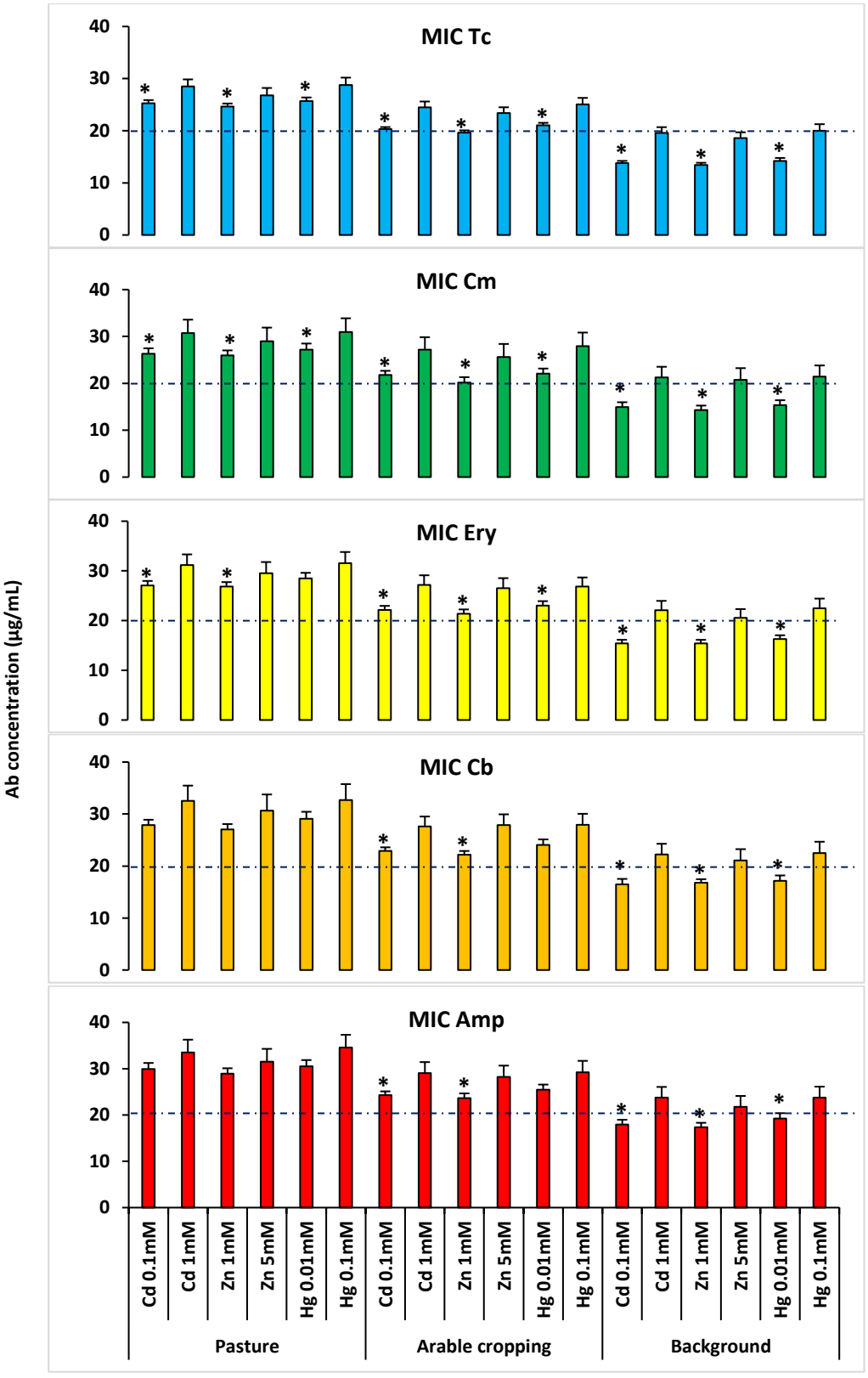

Fig. A2.20. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS1 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's MIC value for the isolates from the same soil and selected on the higher concentration of each HM. The dash line defines AbR level of soil bacteria.

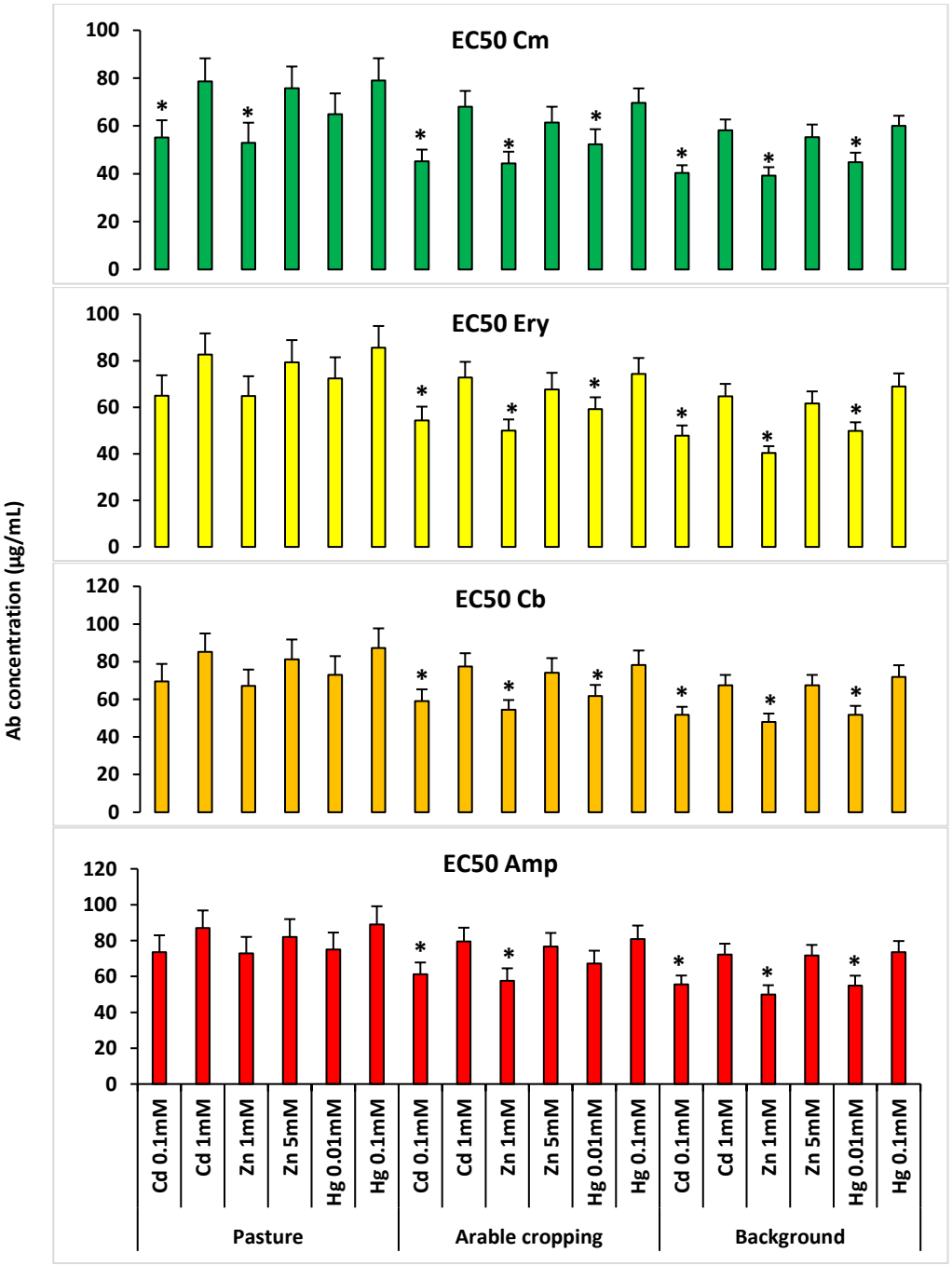

Fig. A2.21. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS1 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

According to Section 4.1.5, there were higher HM MIC and EC50 values for HMR bacteria from WRSS2 and WRSS3 pastoral and arable cropping soils compared to the HMR isolates from the

same set's control soil. In addition, there were greater HM MIC and EC50 values for HMR isolates from WRSS2 and WRSS3 soils compared to the HM-sensitive bacteria from the same soil.

There were lower HM MIC and EC50 values for bacteria from WRSS2 and WRSS3 control and arable cropping soils which were selected on plates containing Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those selected on plates with the higher concentration of each HM.

There were greater Ab MIC and EC50 values for HMR isolates from WRSS2 and WRSS3 pastoral and arable cropping soils compared to those from the same soil set's control soil. In addition, there were higher Ab MIC and EC50 values for HMR isolates from WRSS2 and WRSS3 pastoral and arable cropping soils compared to the HM-sensitive isolates from the same soil (Figures A2.22-A2.25 and A2.30-A2.33).

Result showed there were greater Ab MIC and EC50 values for HMR isolates from WRSS2 and WRSS3 pastoral, arable cropping and control soils and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to the HM-sensitive isolates from the same soil sample (Figures A2.26-A2.29 and A2.34-A2.37).

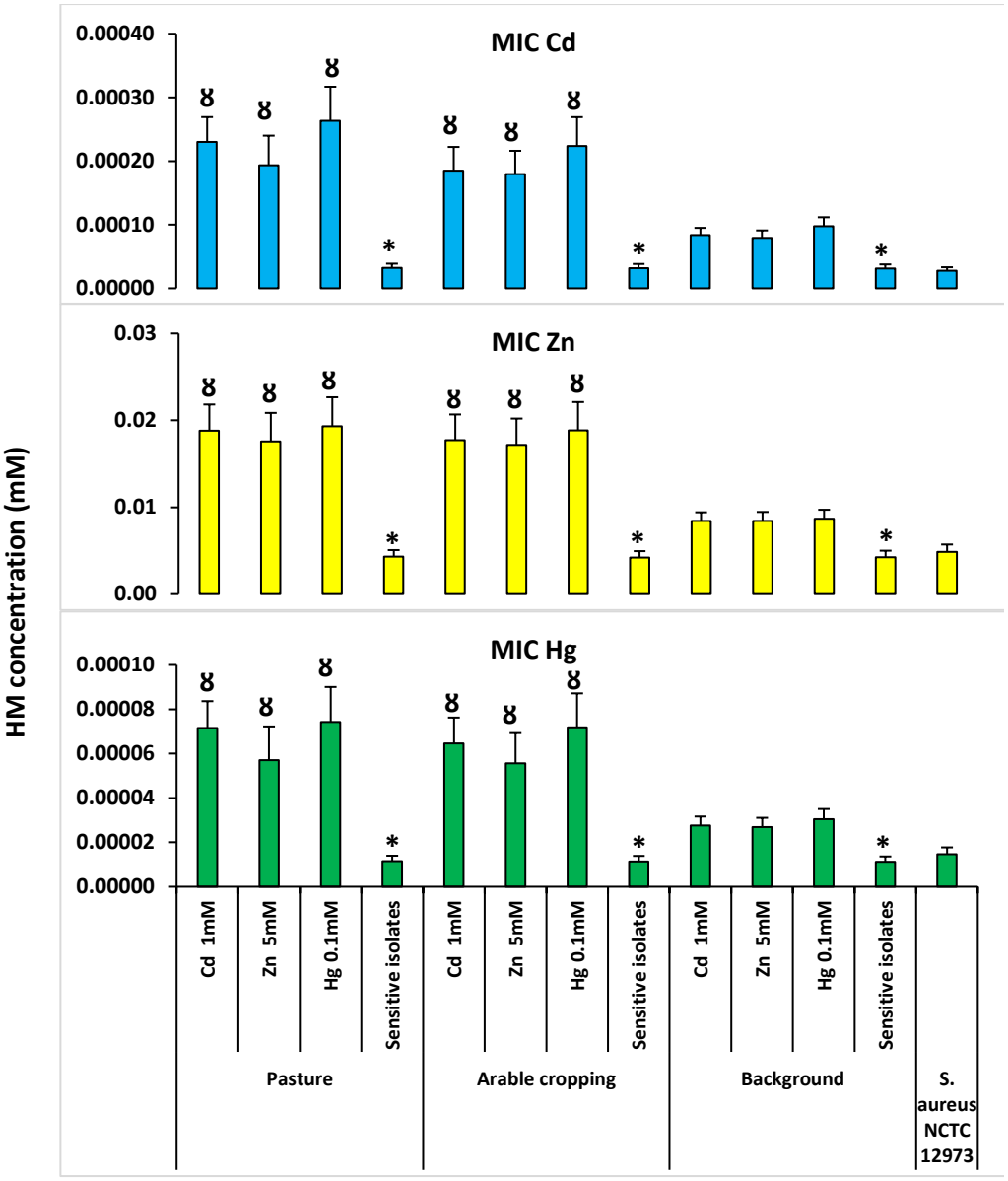

Fig A2.22. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS2. *$p < 0.05$ compared to HM MIC value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to HM MIC value for HMR isolates from control soil.

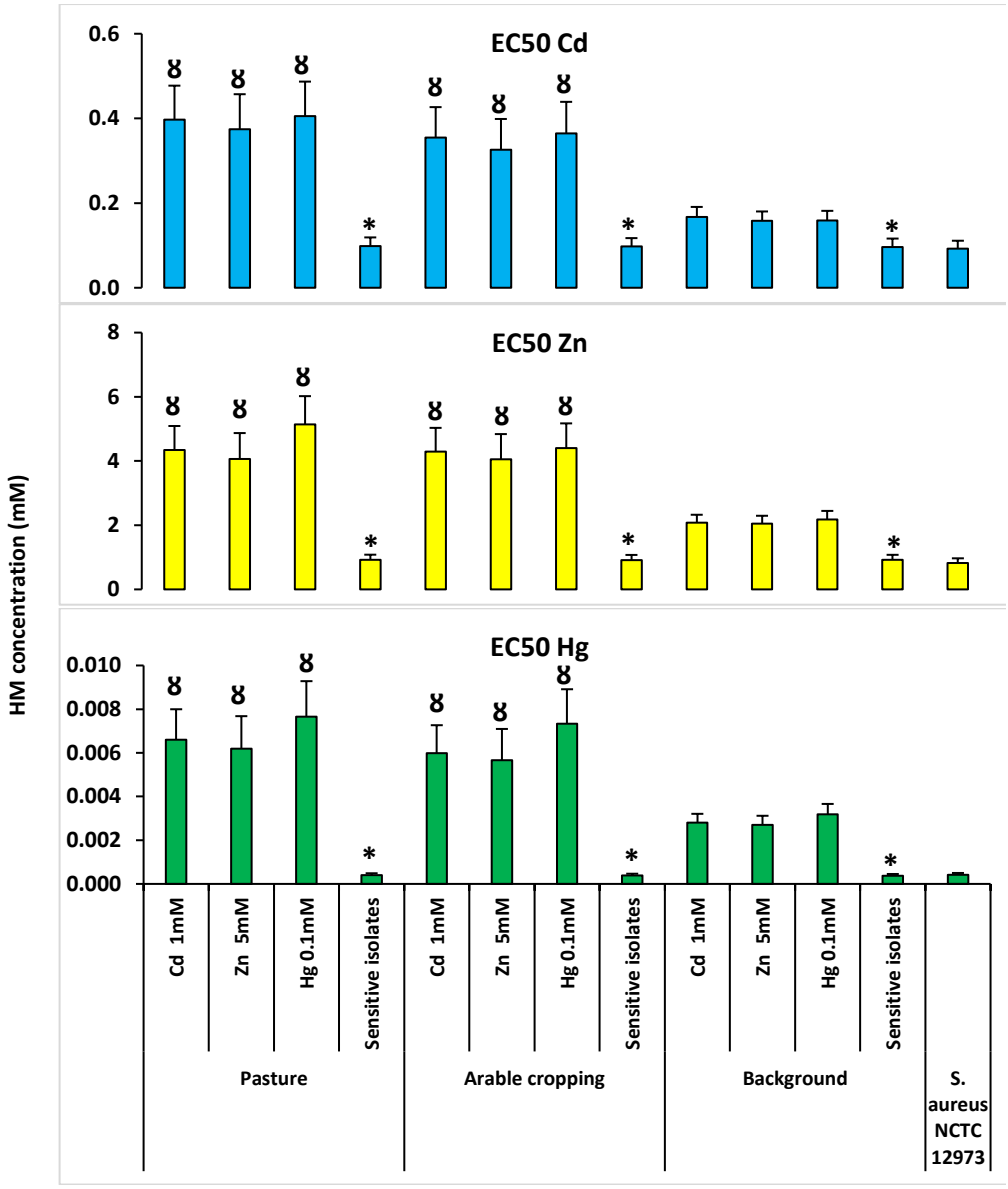

Fig. A2.23. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS2. *$p < 0.05$ compared to HM EC50 value for HMR isolates from the same soil; ϐ$p < 0.05$ compared to HM EC50 value for HMR isolates from background soil.

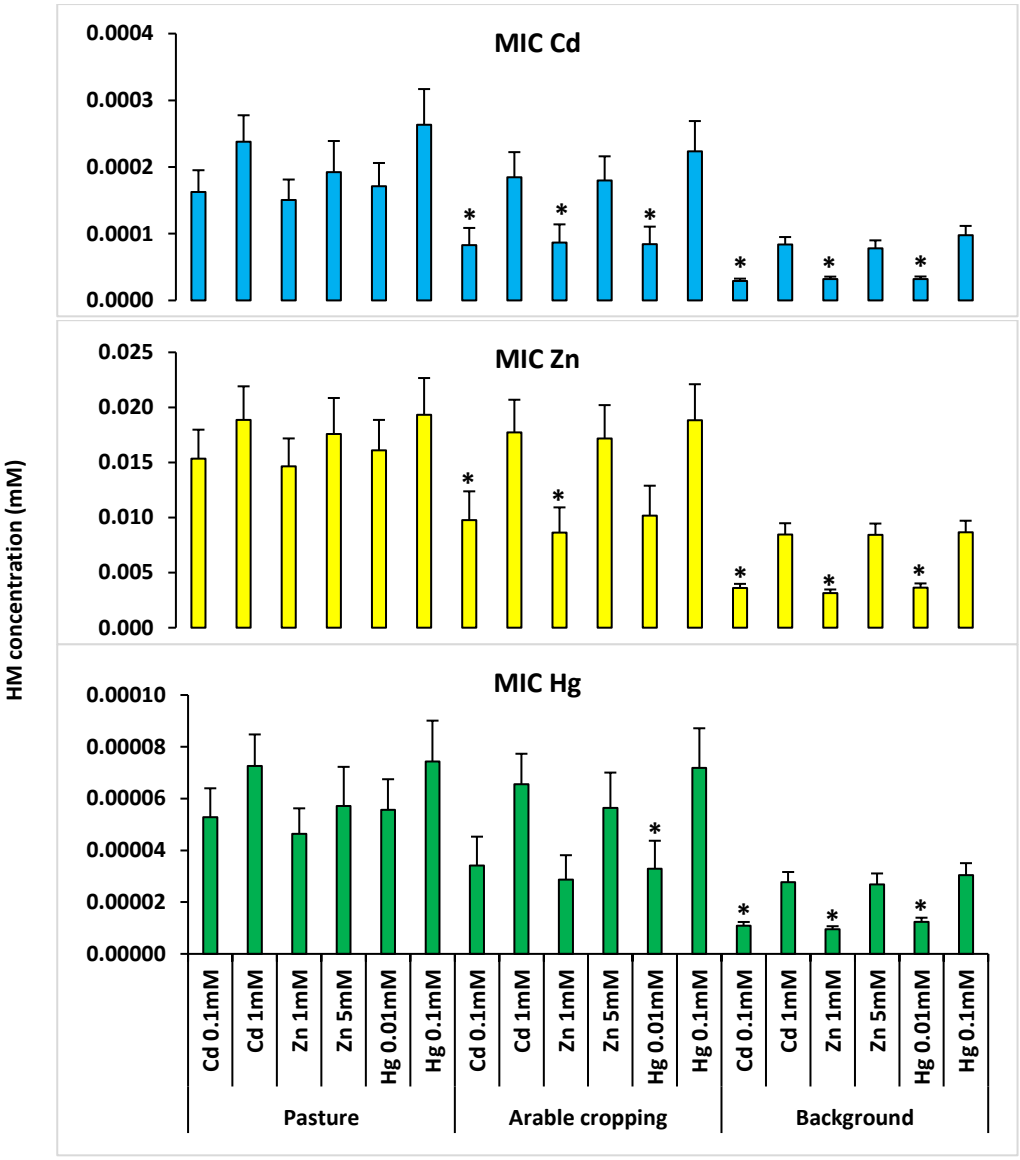

Fig. A2.24. Mean MIC values of BM test with Cd, Zn and Hg for HMR isolates from WRSS2. *$p < 0.05$ compared to the same HM's MIC value for the isolates from the same soil and selected on the higher concentration of each HM.

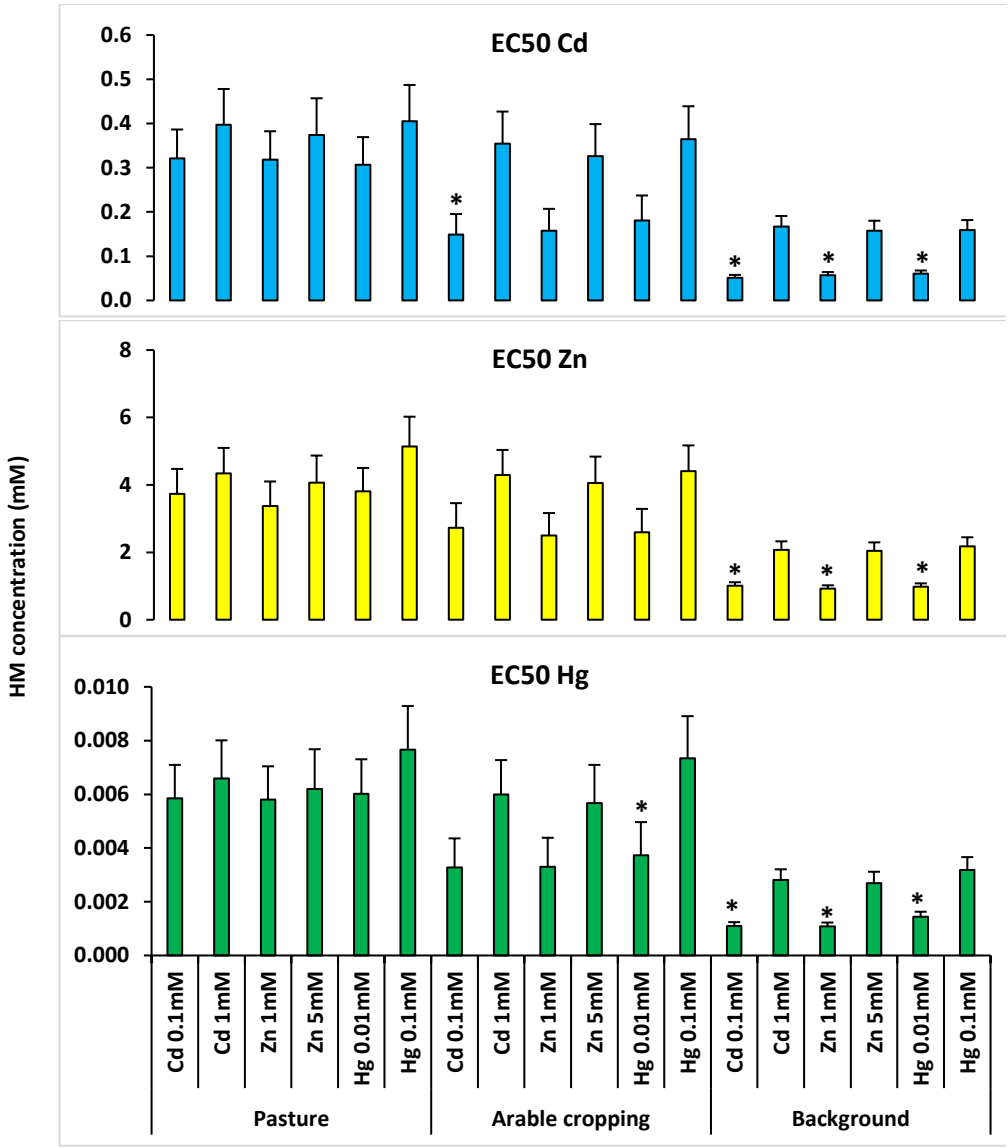

Fig. A2.25. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS2. *$p < 0.05$ compared to the same HM's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

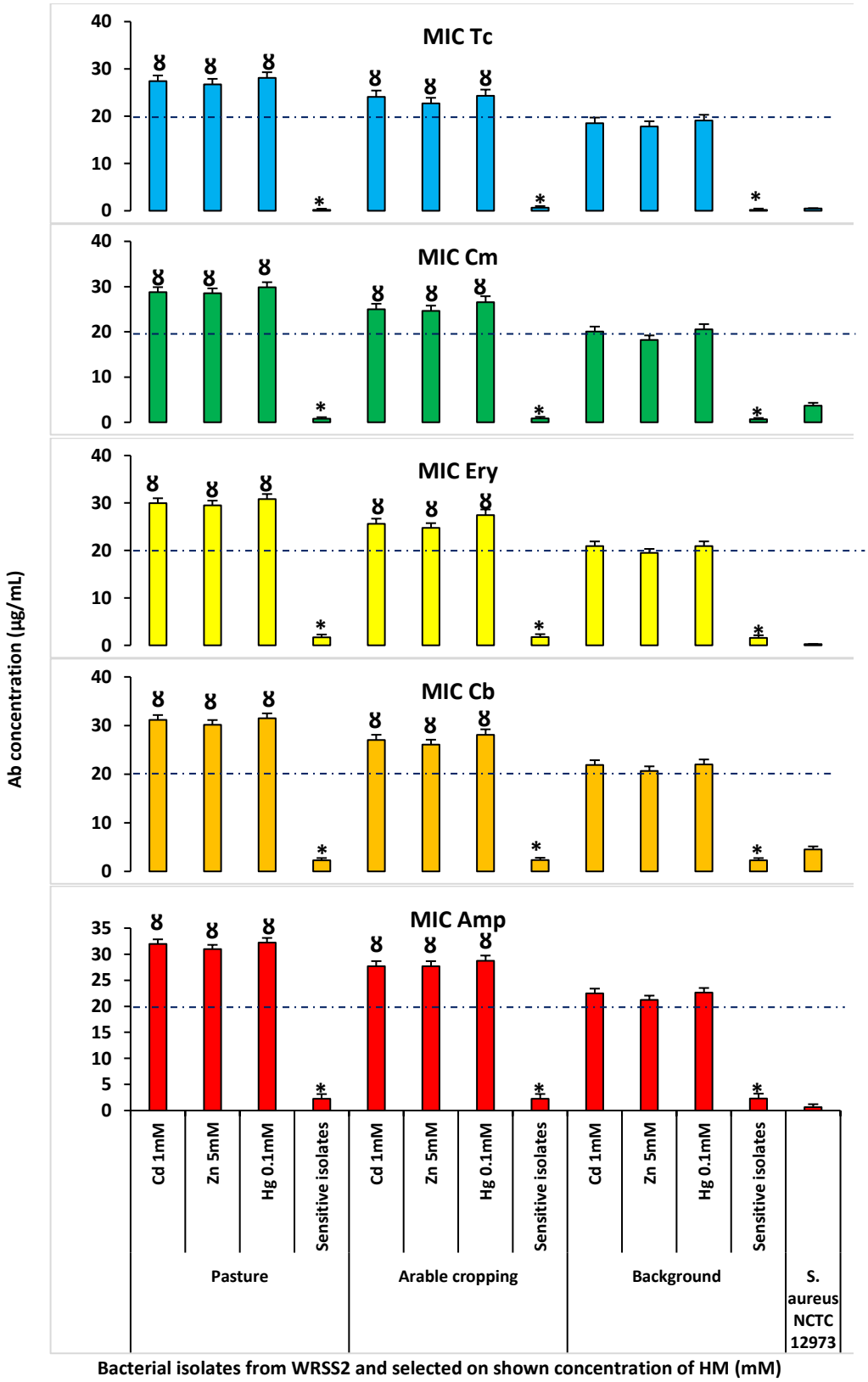

Fig. 2.26. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS2. *$p < 0.05$ compared to Ab MIC value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for isolates from control soil and selected on the same HM concentration. The dash line defines AbR level of soil bacteria.

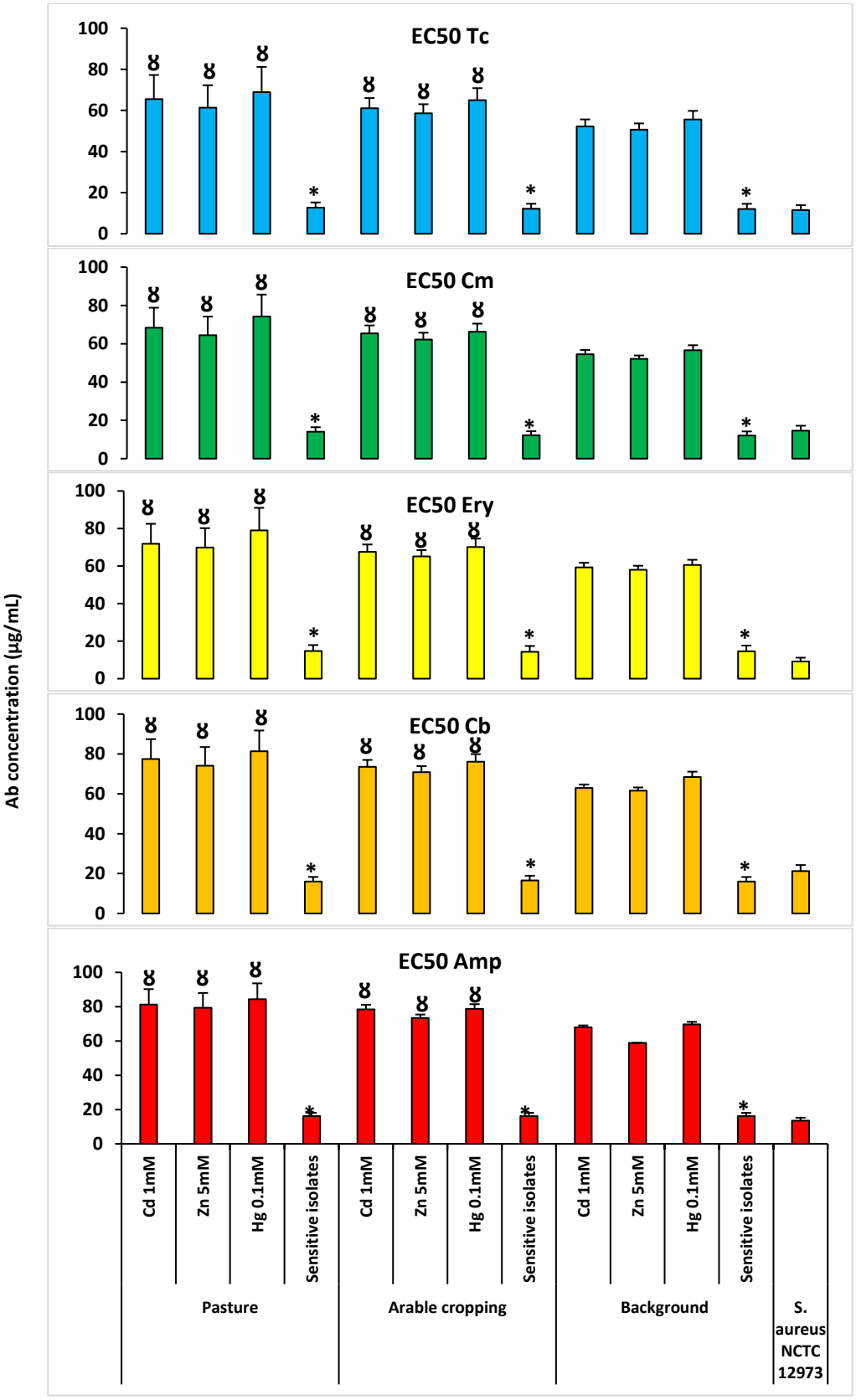

Fig. 2.27. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS2. *$p < 0.05$ compared to Ab EC50 value for HMR isolates from the same soil; ϑ$p < 0.05$ compared to Ab EC50 value for isolates from background soil and selected on the same HM concentration.

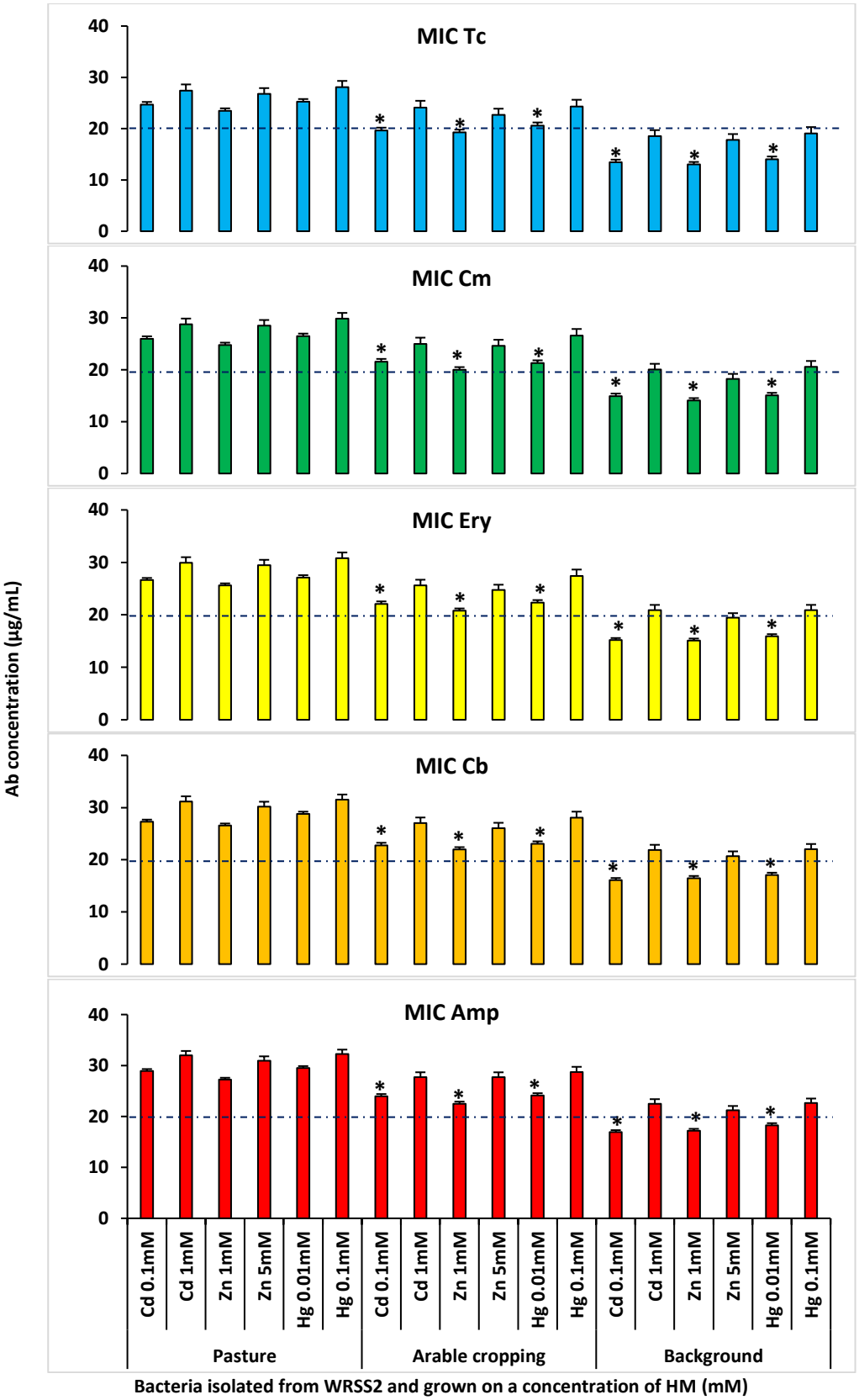

Fig. 2.28. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS2 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's MIC value for the isolates from the same soil and selected on the higher concentration of each HM. The dash line defines AbR level of soil bacteria.

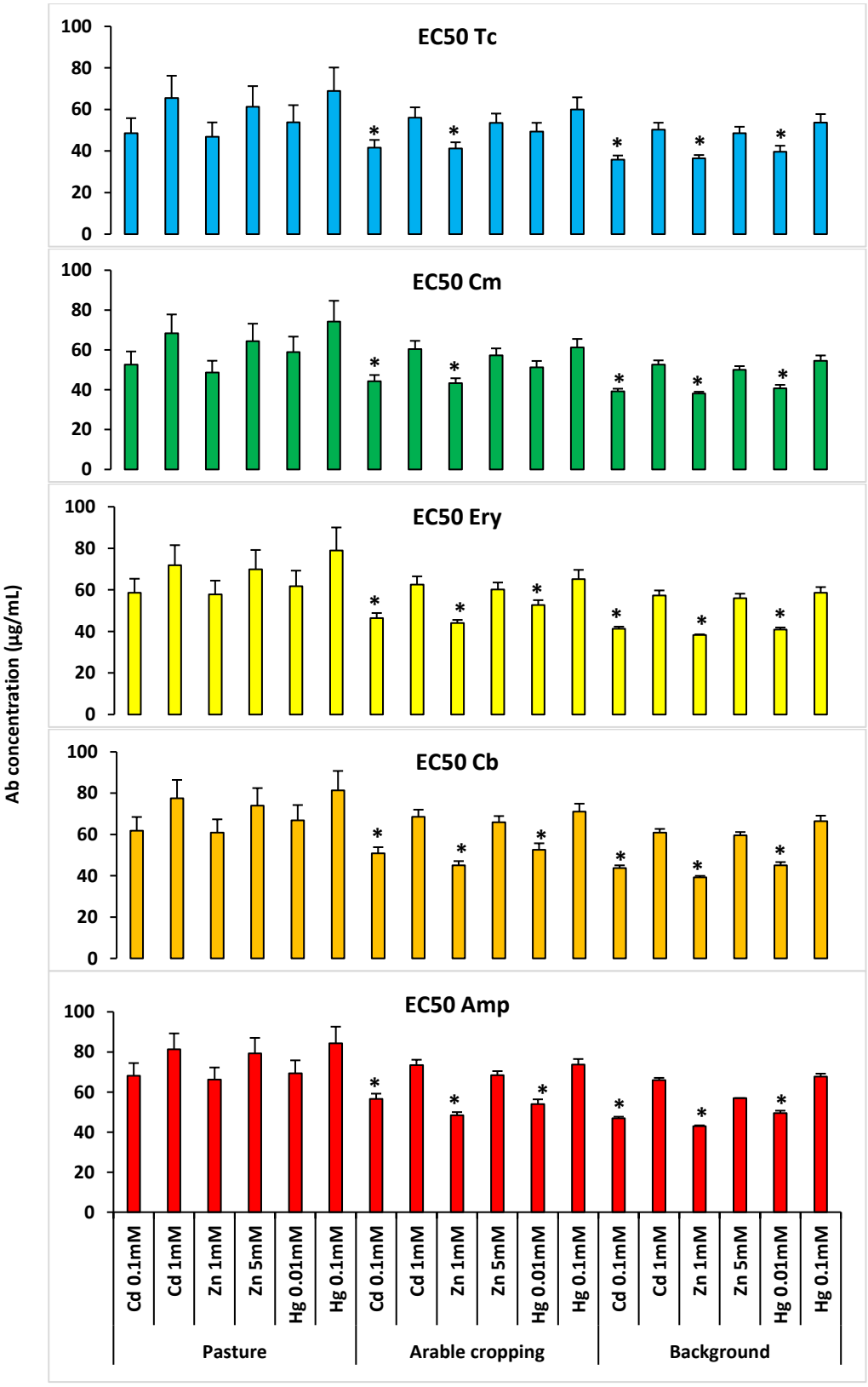

Fig. 2.29. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS2 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

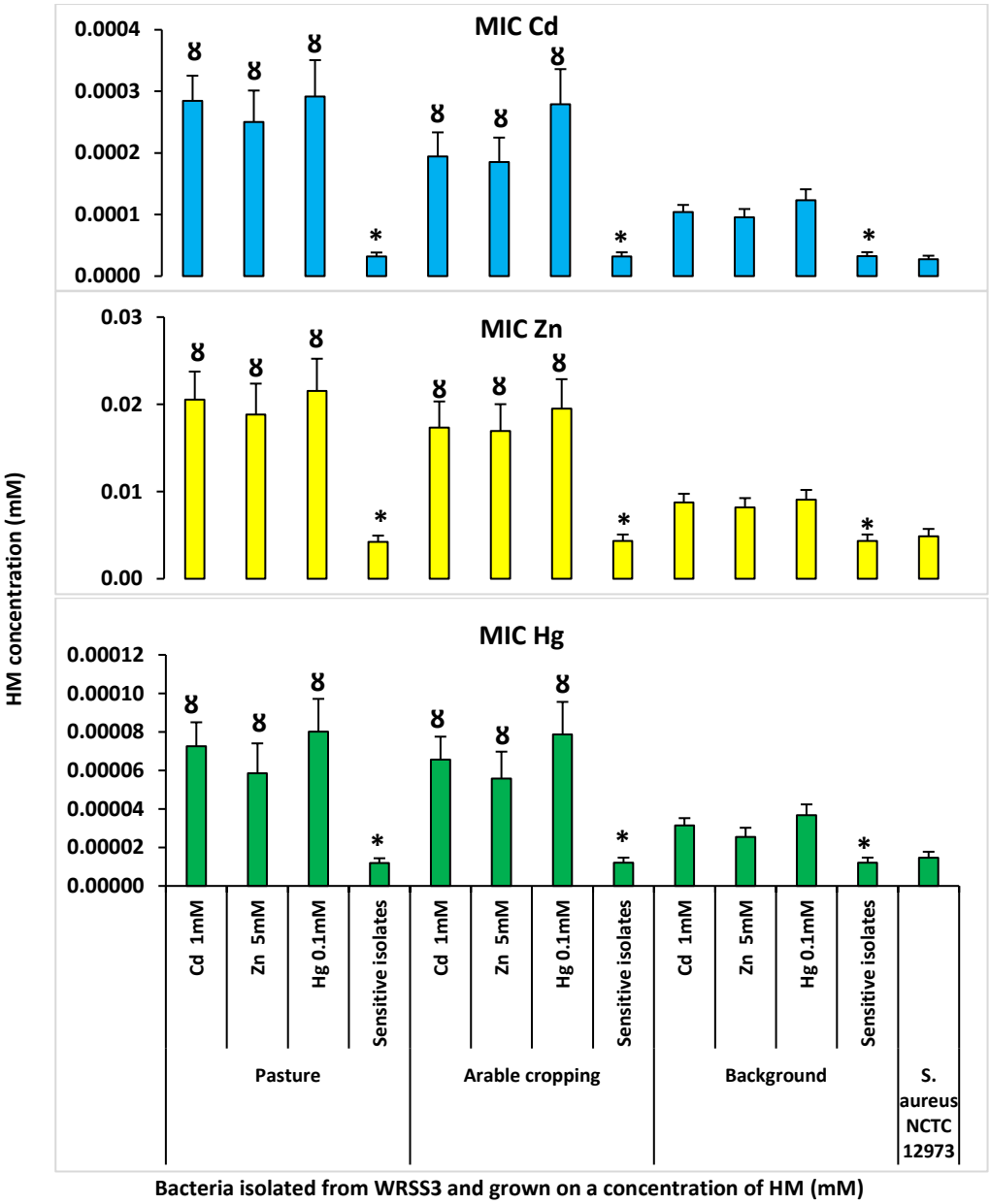

Fig. A2.30. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS3. *$p < 0.05$ compared to HM MIC value for HM-sensitive isolates from the same soil; ϐ$p < 0.05$ compared to HM MIC value for HMR isolates from control soil.

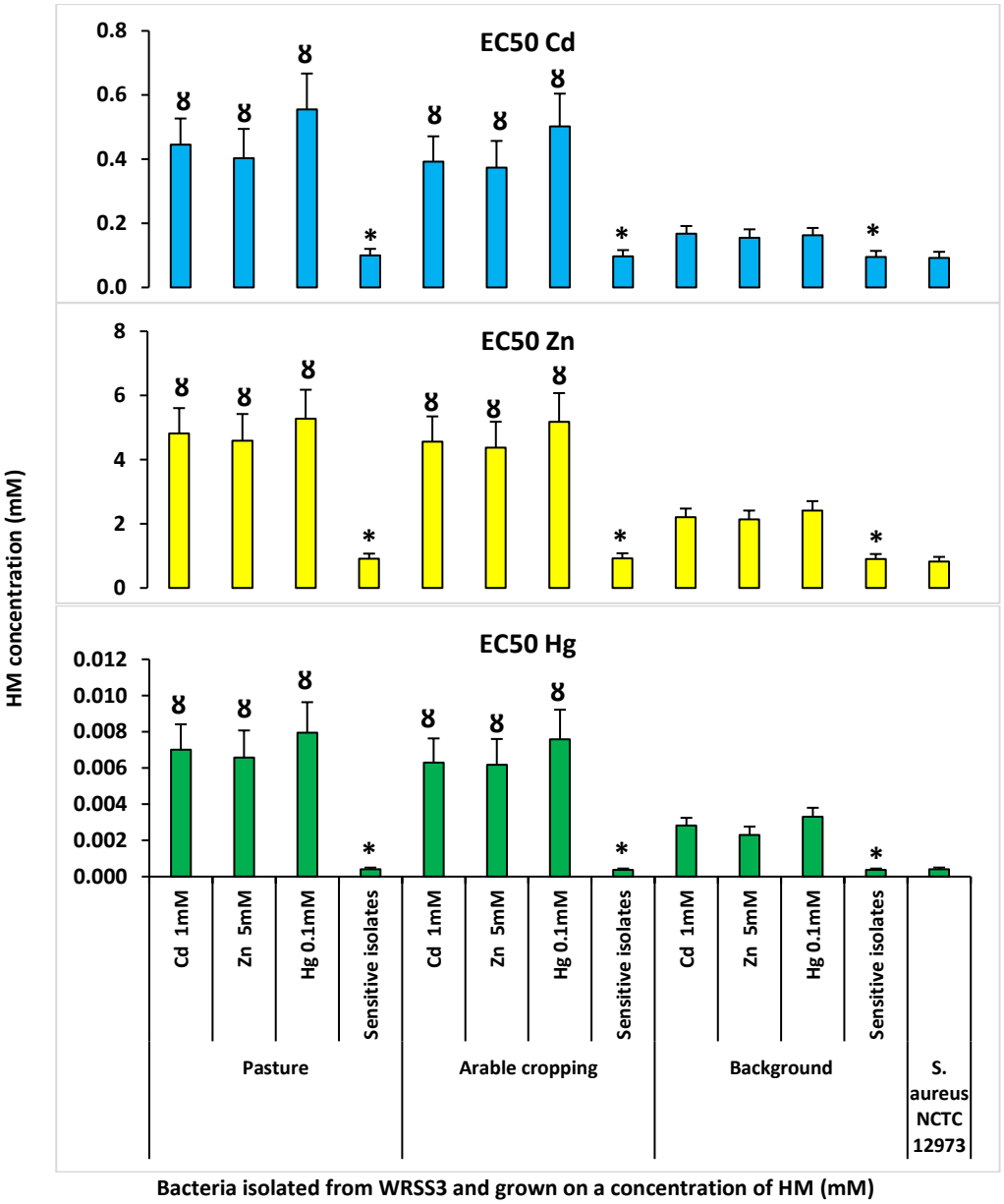

Fig. A2.31. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS3. *$p < 0.05$ compared to HM EC50 value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to HM EC50 value for HMR isolates from control soil.

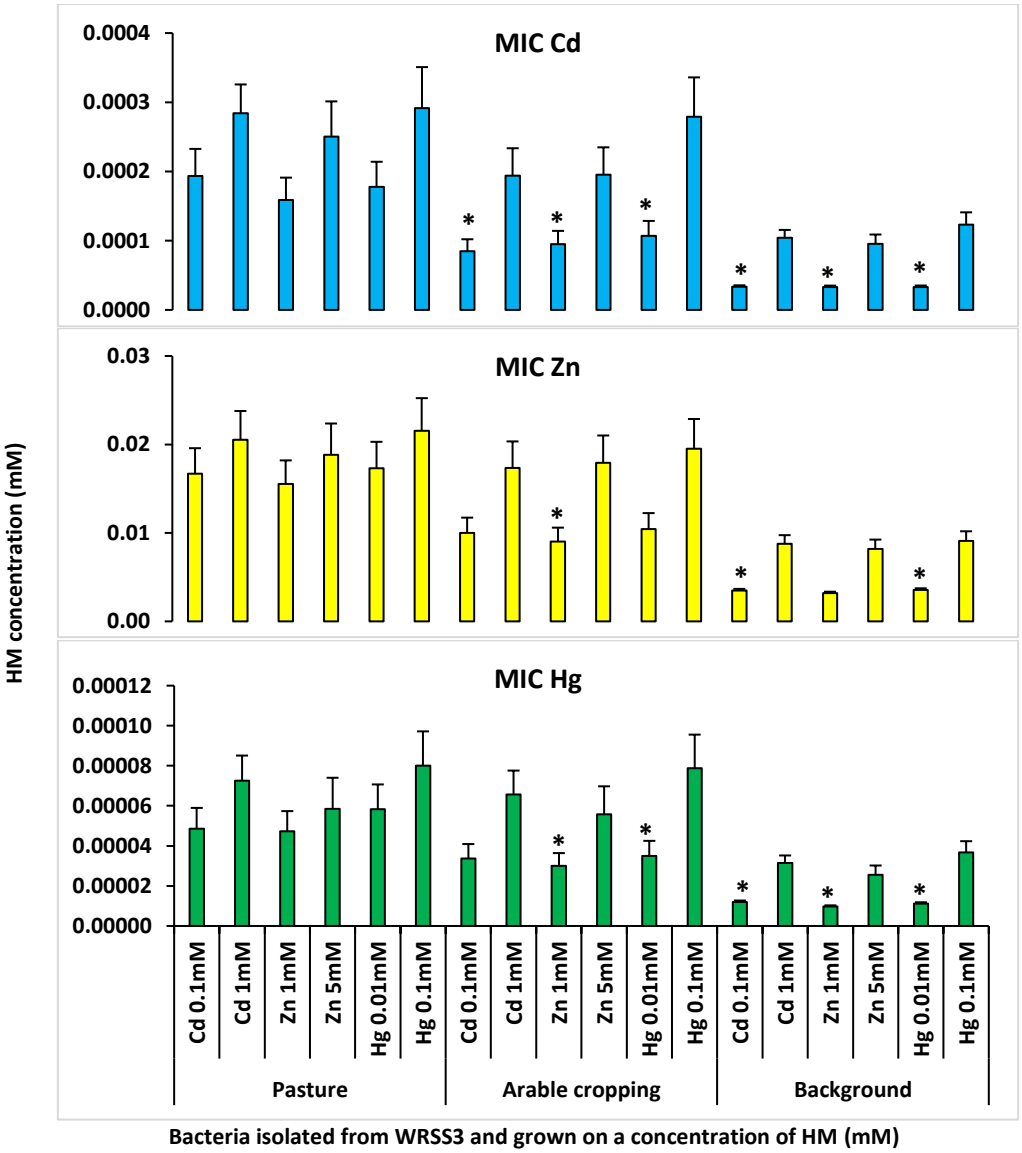

Fig A2.32. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from WRSS3. *$p < 0.05$ compared to the same HM's MIC value for the isolates from the same soil and selected on the higher concentration of each HM.

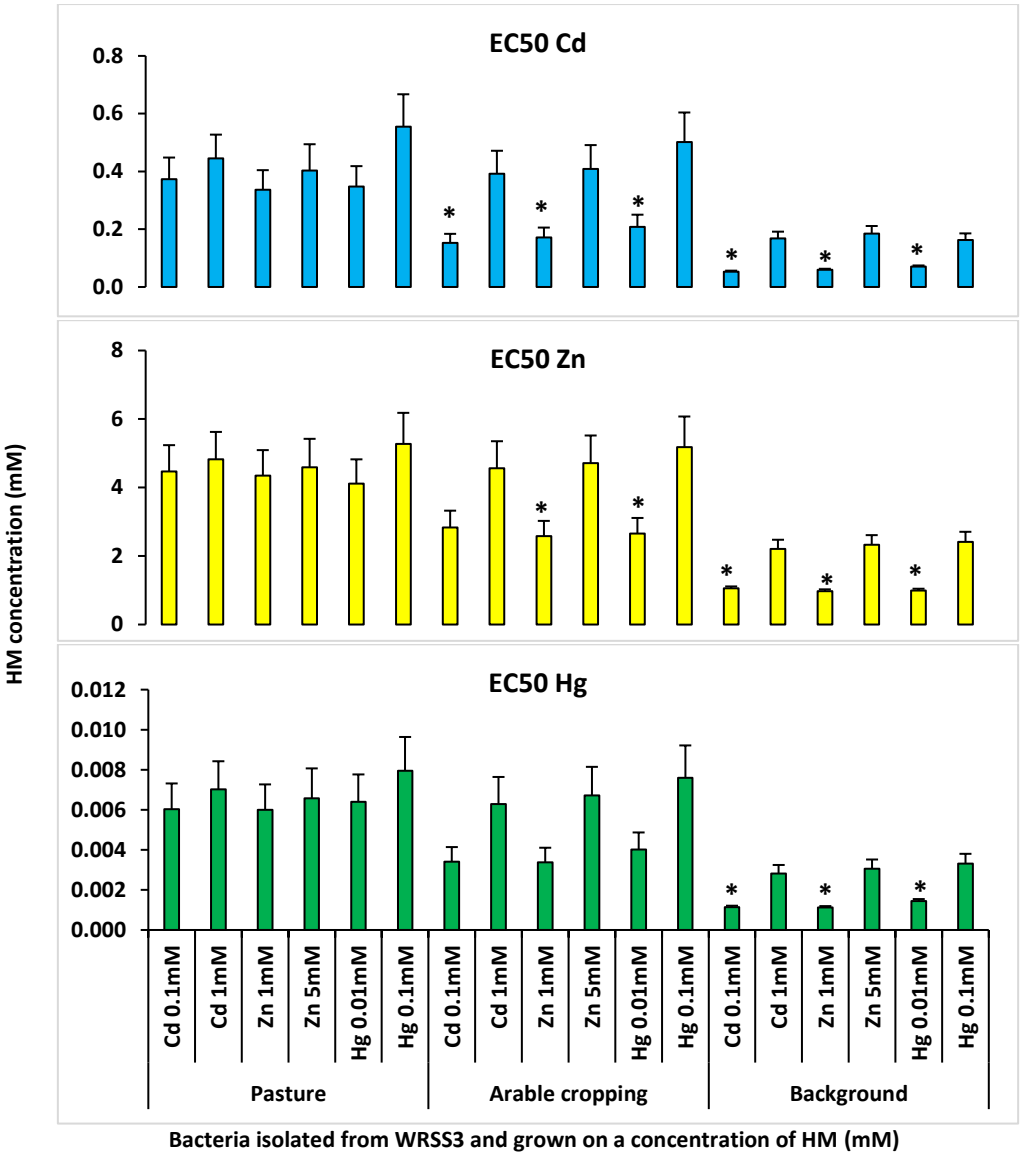

Fig. A2.33. Mean EC50 values of BM test with Cd, Zn and Hg for HMR isolates from WRSS3. *$p < 0.05$ compared to the same HM's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

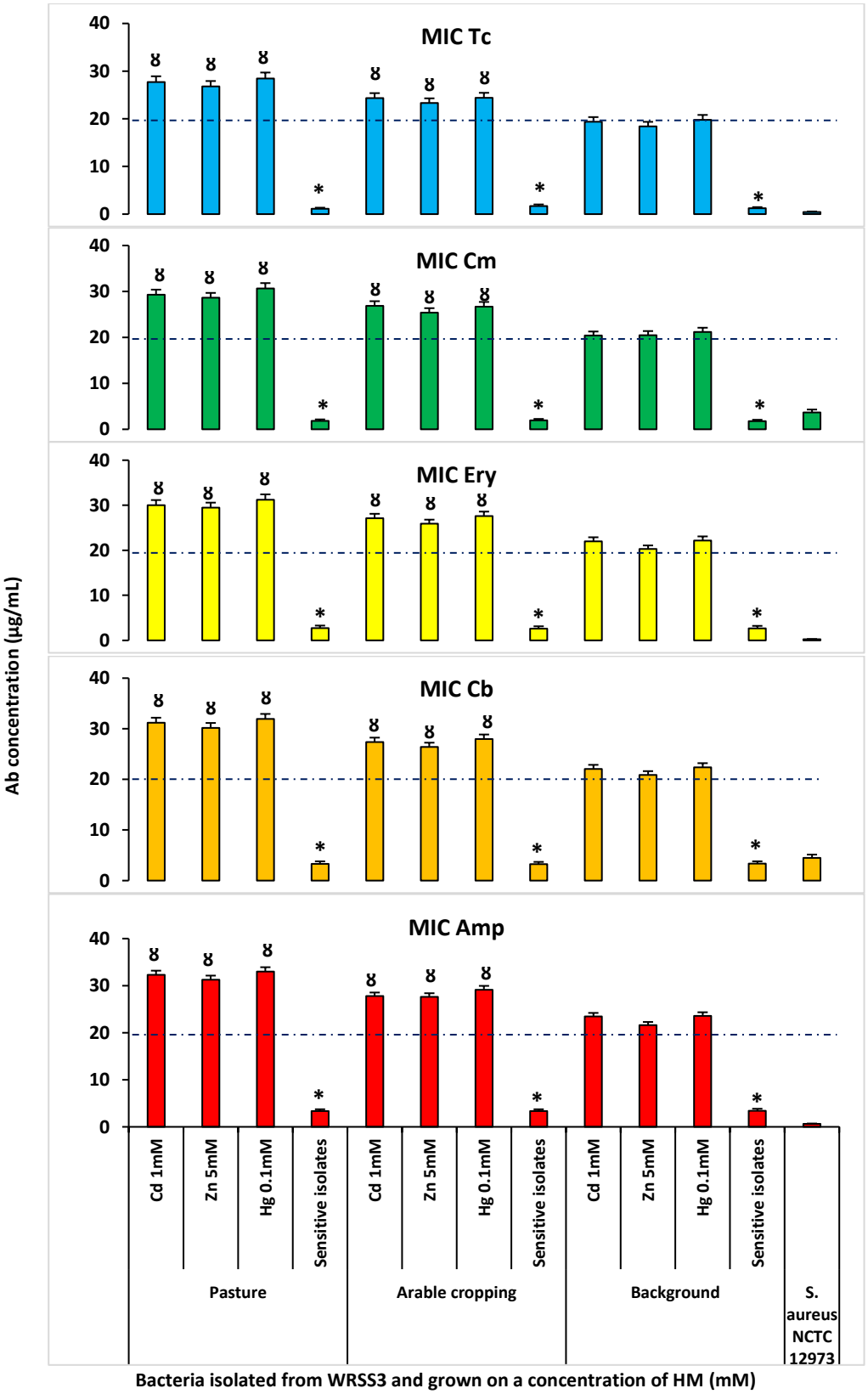

Fig. A2.34. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS3. *$p < 0.05$ compared to Ab MIC value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for isolates from control soil and selected on the same HM concentration. The dash line defines AbR level of soil bacteria.

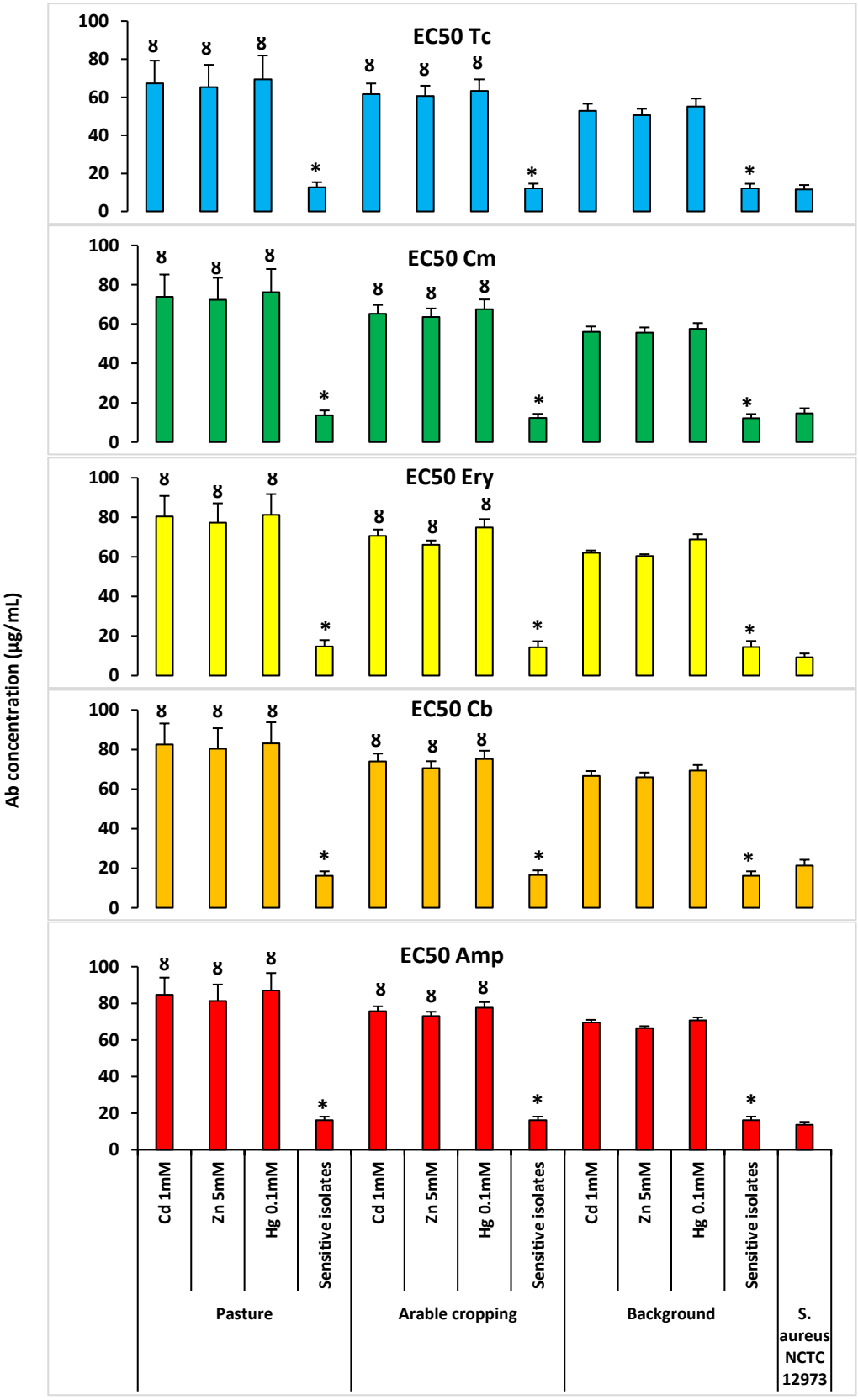

Fig. A2.35. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS3. *$p < 0.05$ compared to Ab EC50 value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to Ab EC50 value for isolates from control soil and selected on the same HM concentration.

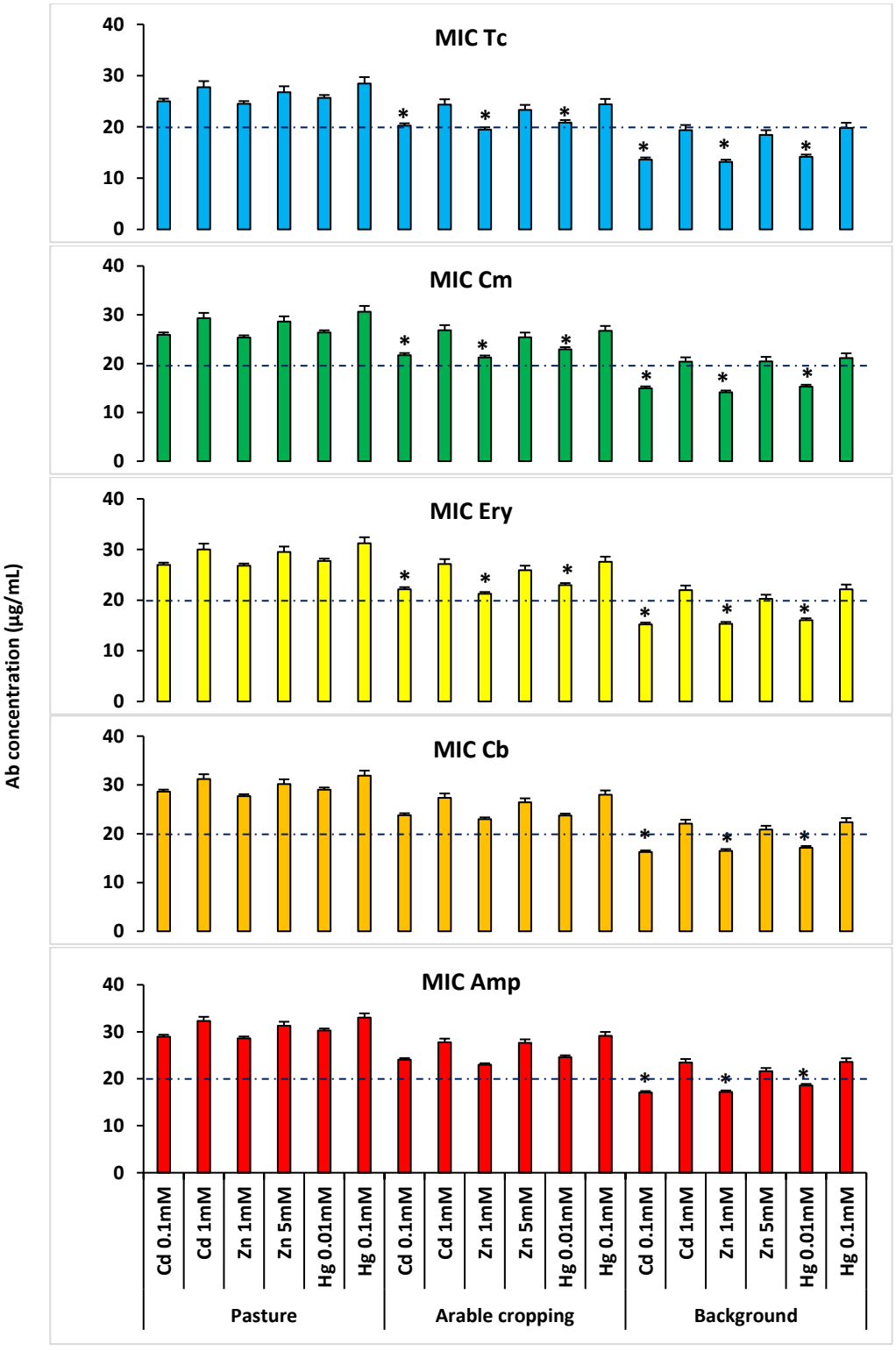

Fig. A2.36. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS3 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's MIC value for the isolates from the same soil and selected on the higher concentration of each HM. The dash line defines AbR level of soil bacteria.

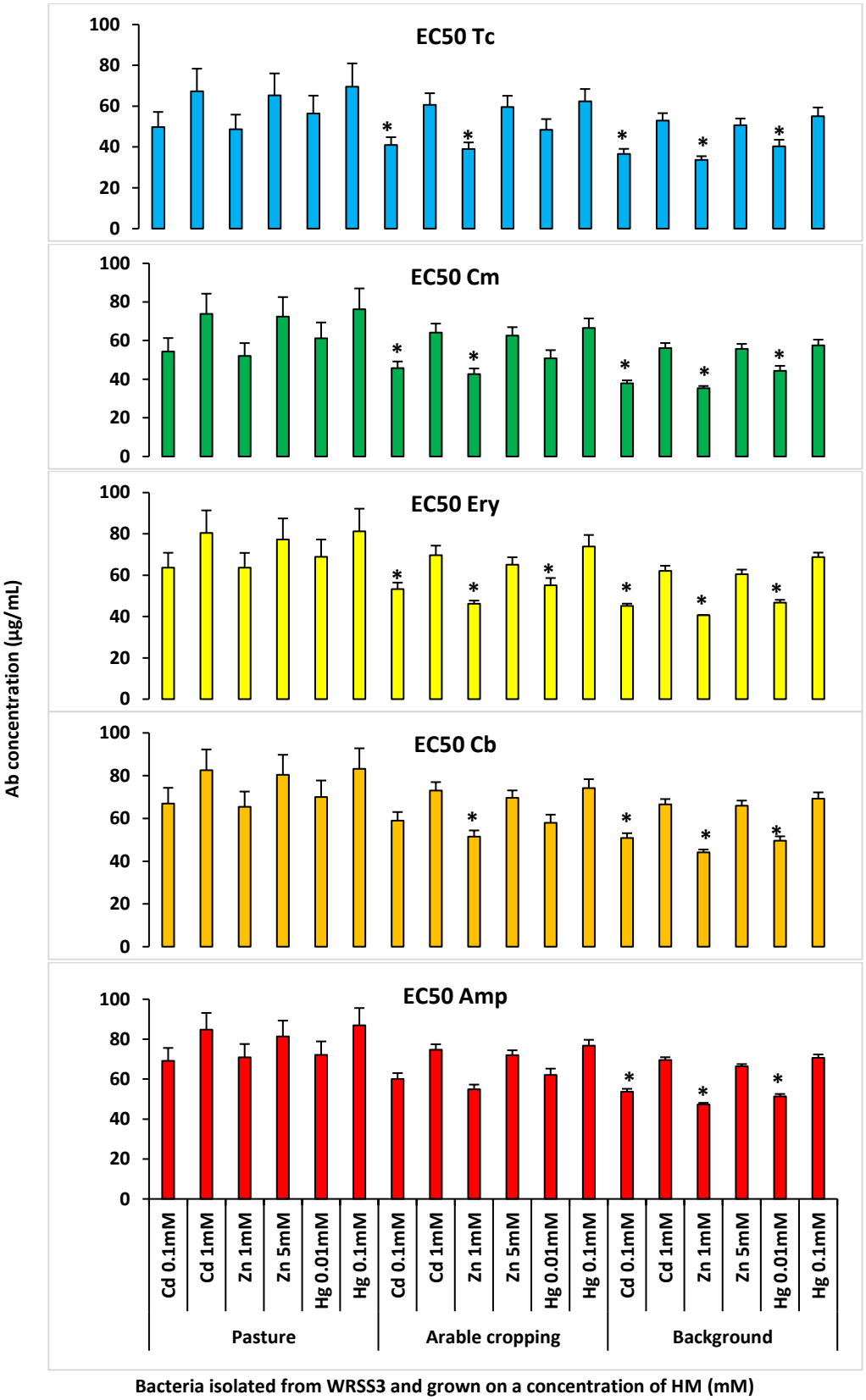

Fig. A2.37. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from WRSS3 and selected on plates with two concentrations of HMs. *$p < 0.05$ compared to the same Ab's EC50 value for the isolates from the same soil and selected on the higher concentration of each HM.

A2.6. Broth Microdilution Analysis for Belmont Regional Park Soil Samples.

According to the result of HMs and ABs BM test's MIC and EC50 determination (Section 4.3.2), there were no significant differences between bacteria from B17, B14 and B10 soils and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared those from the same soils and selected on plates with the higher concentrations of the HMs (Figures A2.38-A2.41).

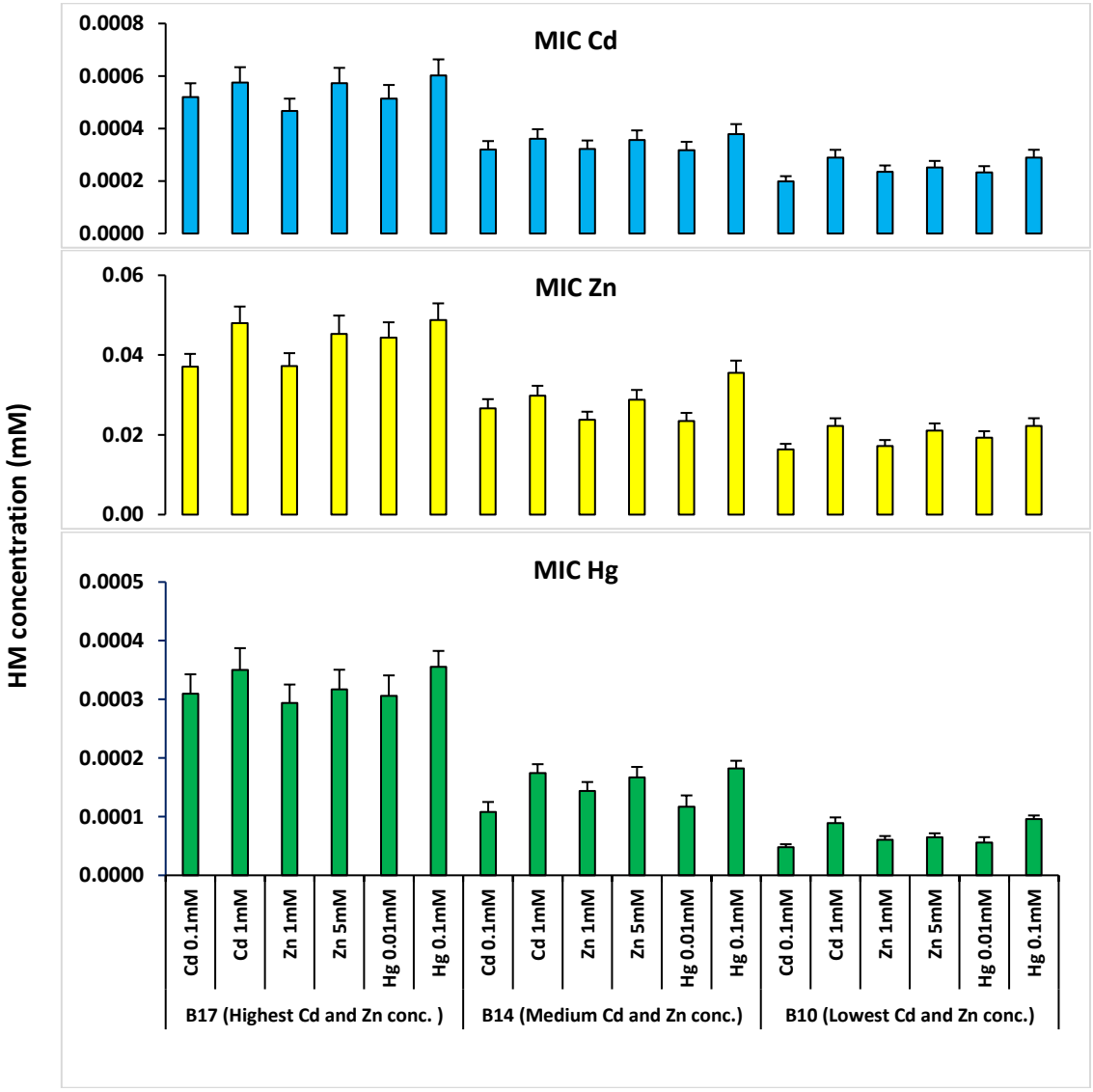

Fig. A2.38. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from BRP.

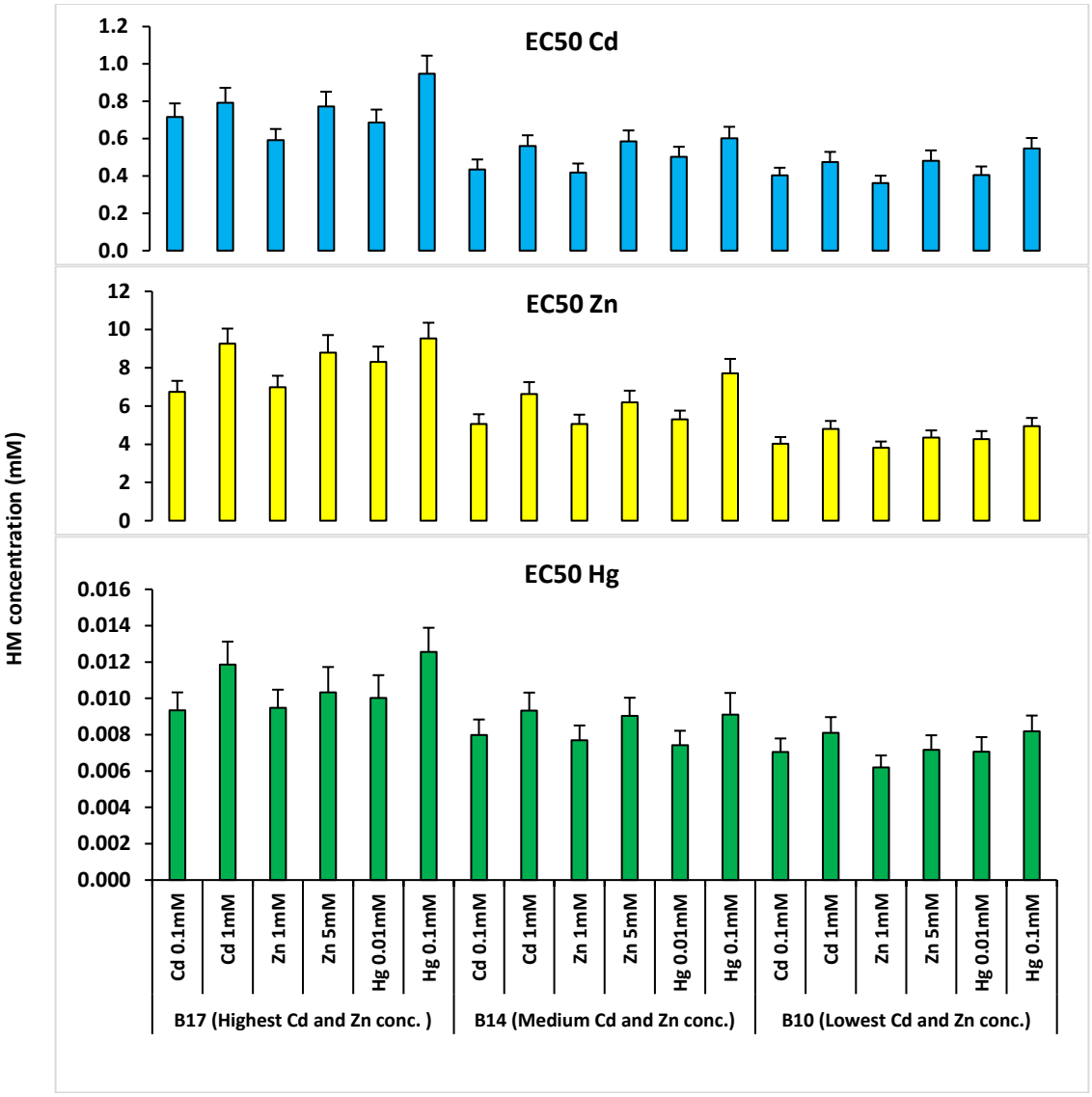

Fig. A2.39. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from BRP.

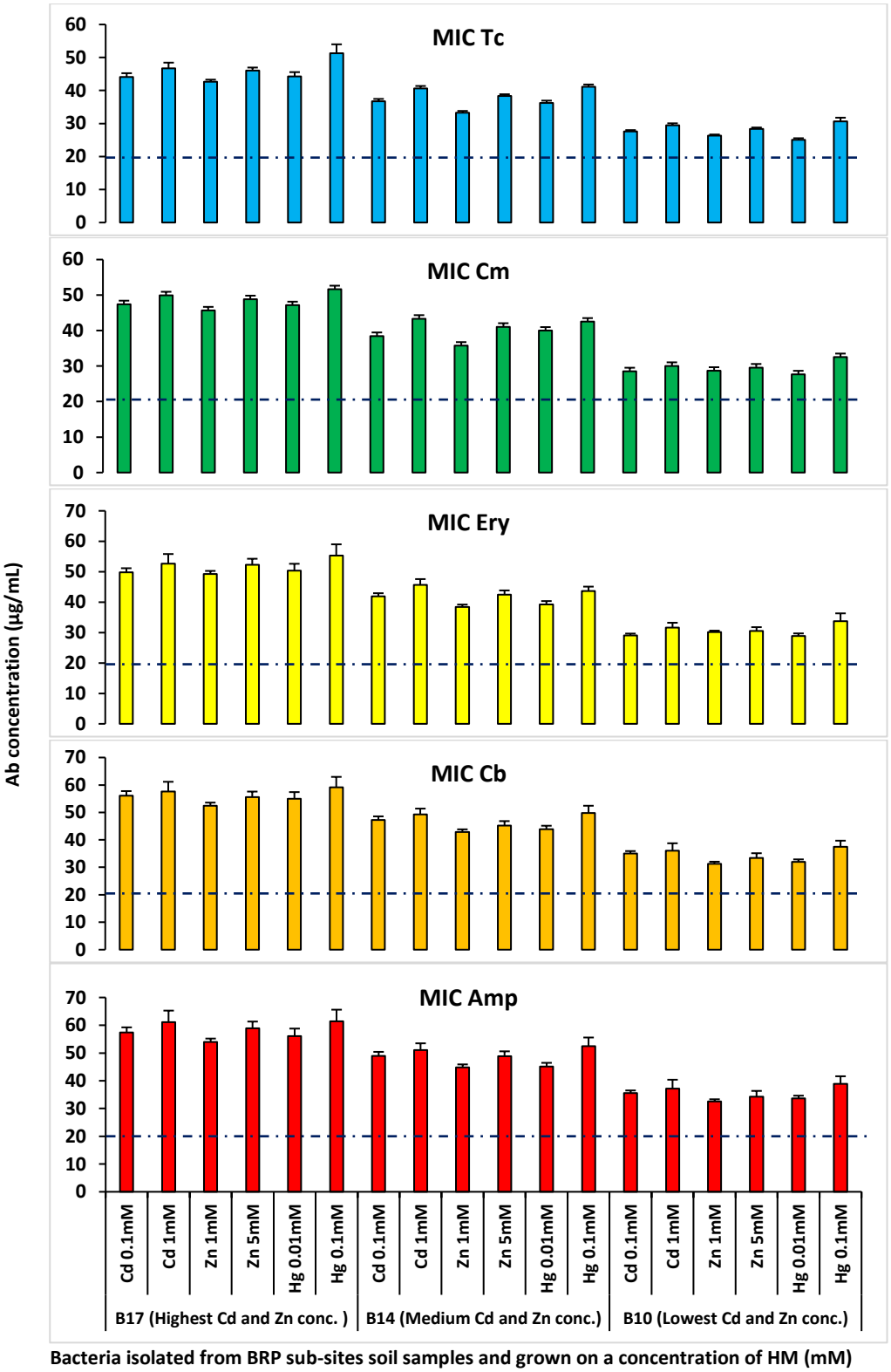

Fig. A2.40. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from BRP. The dash line defines AbR level of soil bacteria.

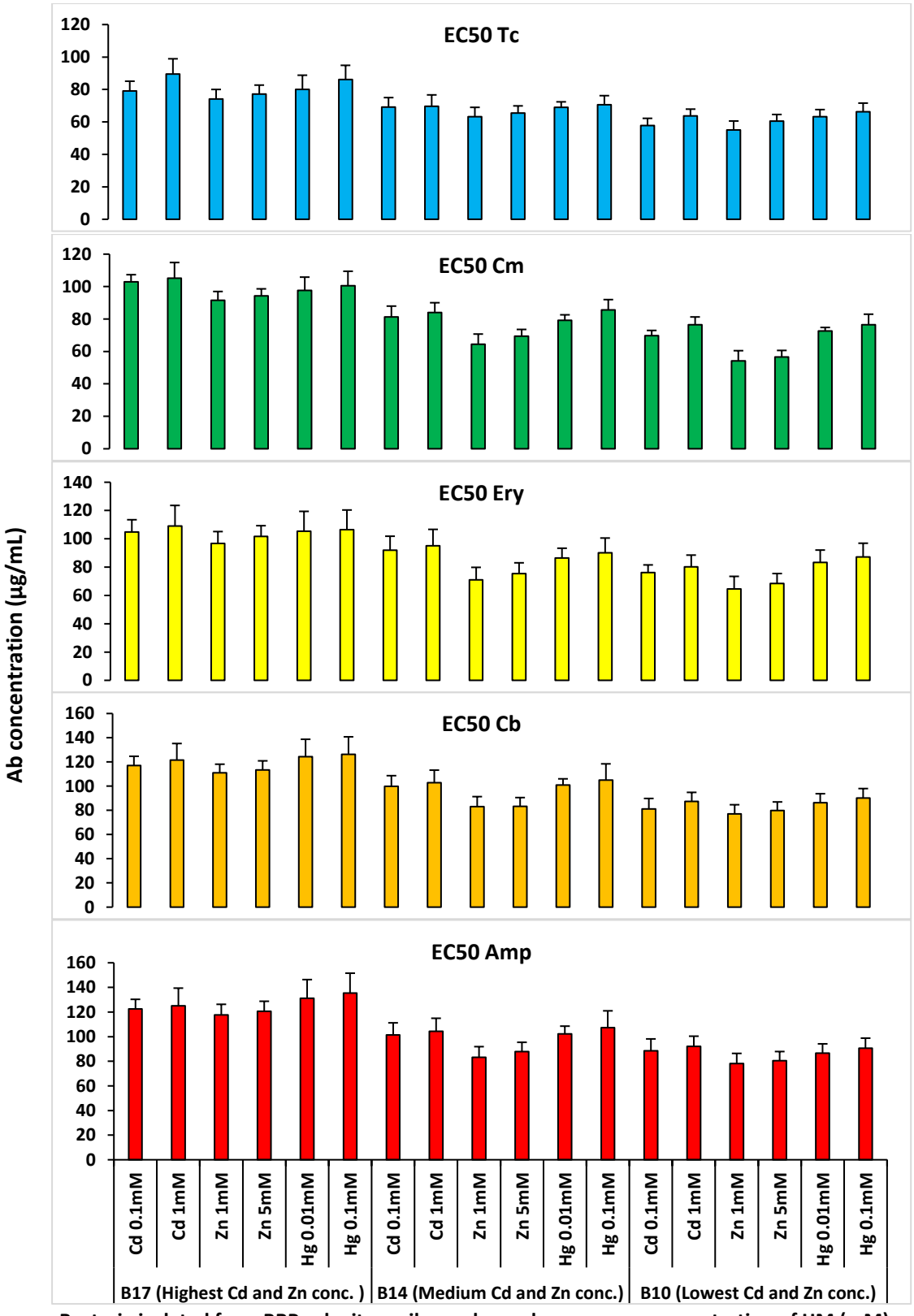

Fig. A2.41. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from BRP.

According to the result of HMs MIC and EC50 determination for the BM test of BRP (Section 4.1.6.2), there were greater HMs MIC and EC50 values for HMR isolates from B16 soil compared to those from B10. Also, there were higher HMs MIC and EC50 values for HMR isolates from B15 and B13 soils compared to those from B10 soil. The result showed there were higher HMR isolates' HMs MIC and EC50 values from B16, B15, B13 and B12 soils compared to the HM-sensitive isolates from the same soil samples. No significant differences between HMs MIC and EC50 values were determined for HMR bacteria from B16, B15, B13 and B12 soils compared to those from the same soil samples. The result of MIC and EC50 determination of the ABs BM test for BRP B16, B15, B13, B12 and B11 soil samples showed there were higher levels of these values for HMR isolates from these soils compared to the HM-sensitive isolates from each soil sample (Figures A2.42-A2.45). In addition, there were greater Abs MIC and EC50 values for HMR isolates from B16, B15, B13 and B12 soils compared to those from B10 soil sample; although, there were some exception for Cb and Amp MIC values for HMR bacteria from B13 soil. Additionally, there were no significant differences between ABs MIC/EC50 values for isolates from B16, B15, B13 and B12 soils and selected on plates with Cd 0.1mM, Zn 1mM and Hg 0.01mM compared to those from the same soil samples and selected on plates with the higher concentrations of the HMs (Figures A2.46-A2.49).

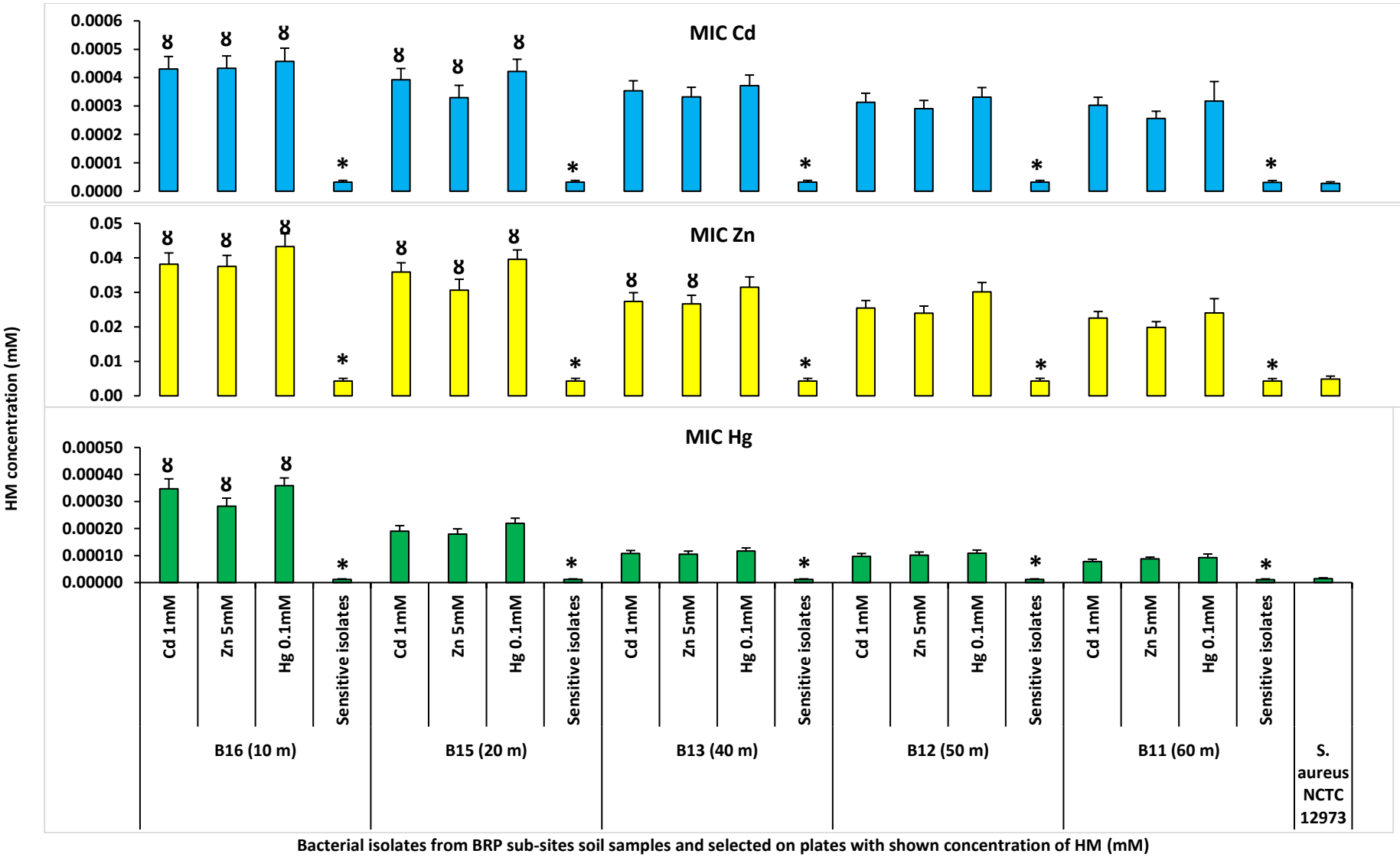

Fig. A2.42. Mean MIC values of BM assay with Cd, Zn and Hg for the HMR isolates from BRP. *$p < 0.05$ compared to HM MIC value for HM-sensitive bacteria from the same soil; ϑ$p < 0.05$ compared to HM MIC value for the bacteria from B10 soil and selected on the same HM.

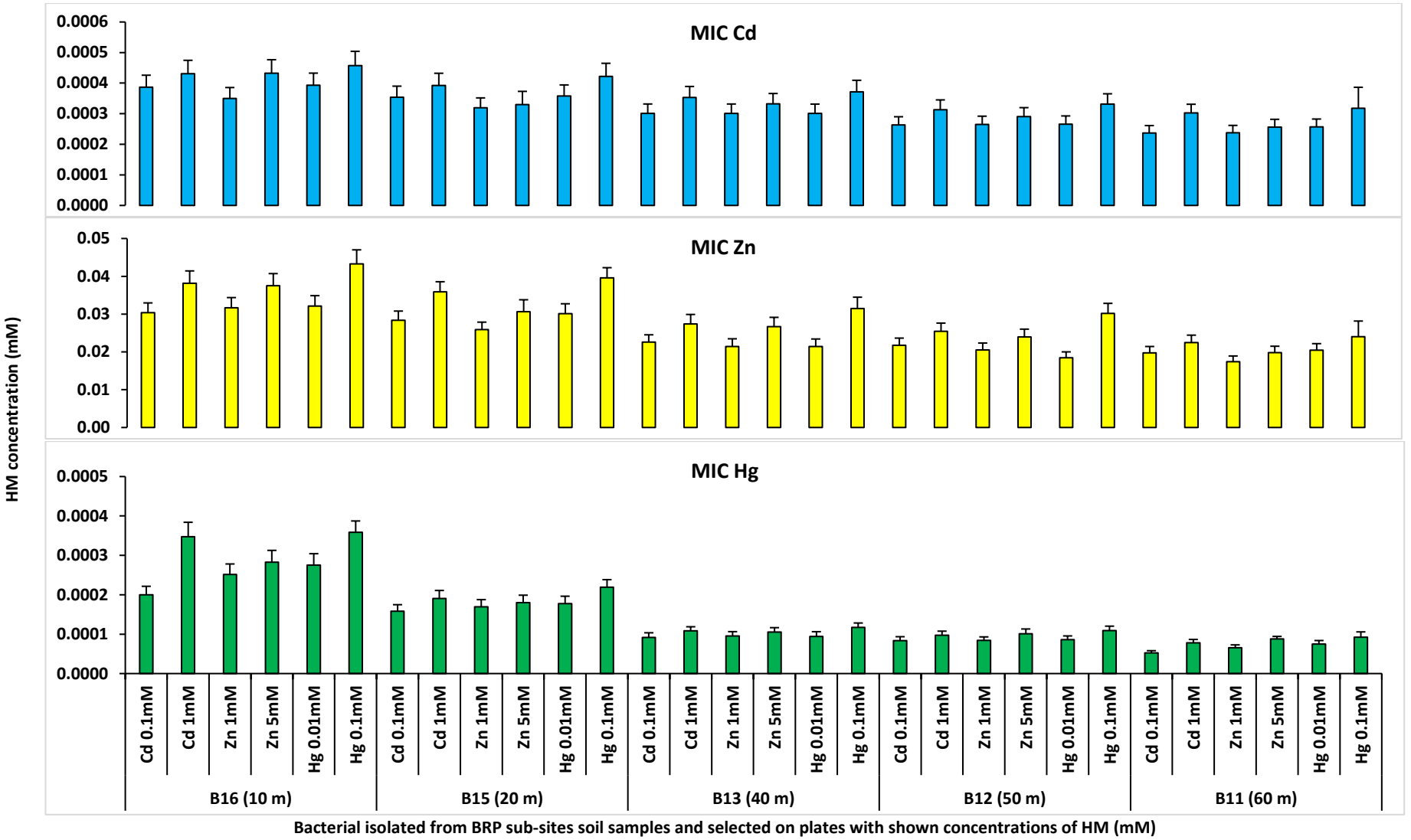

Fig. A2.43. Mean MIC values of BM assay with Cd, Zn and Hg for HMR isolates from BRP.

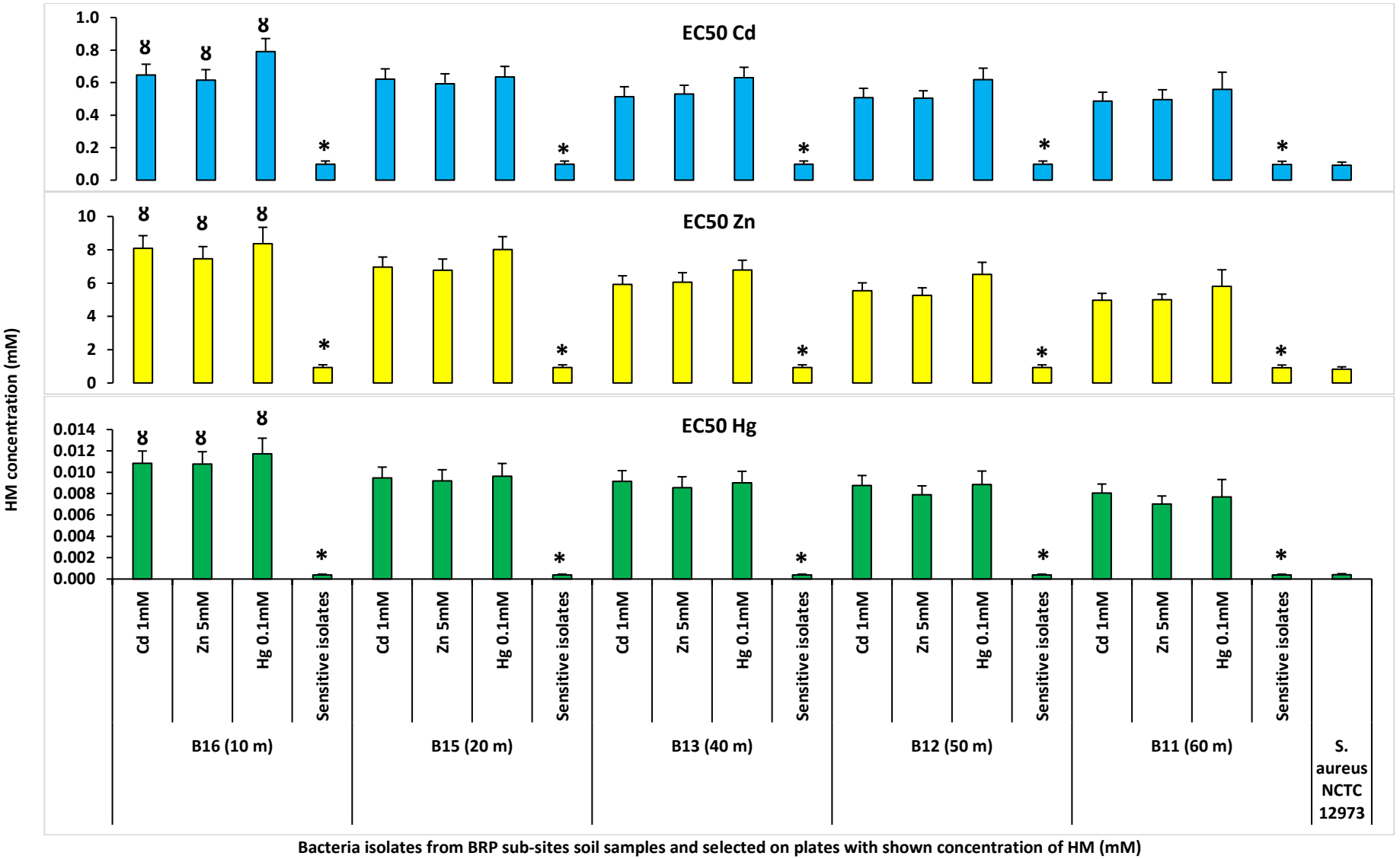

Fig. A2.44. Mean EC50 values of BM assay with Cd, Zn and Hg for the HMR isolates from BRP. *$p < 0.05$ compared to HM EC50 value for HM-sensitive bacteria from the same soil; ϐ$p < 0.05$ compared to HM EC50 value for the bacteria from B10 soil and selected on the same HM.

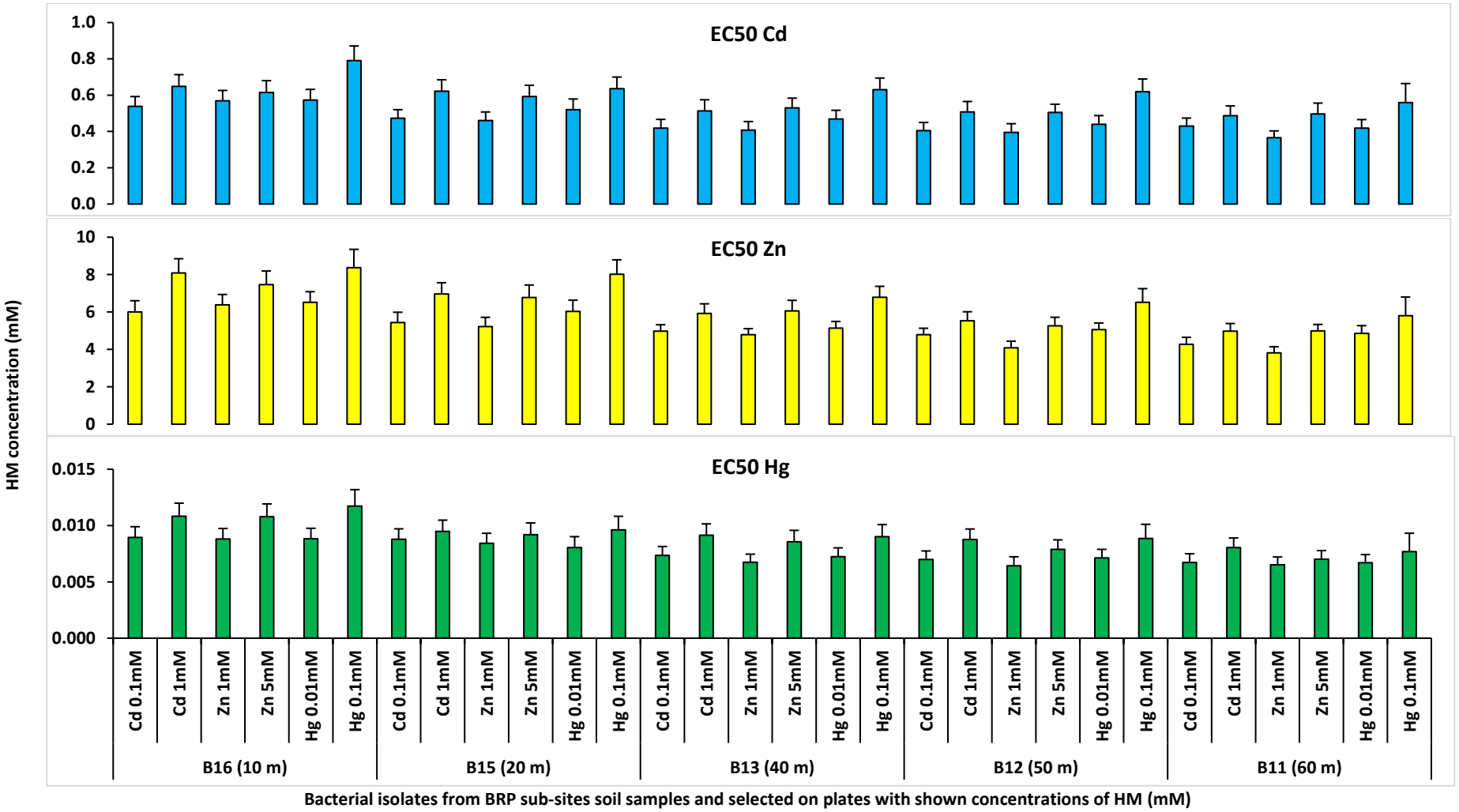

Fig. A2.45. Mean EC50 values of BM assay with Cd, Zn and Hg for HMR isolates from BRP.

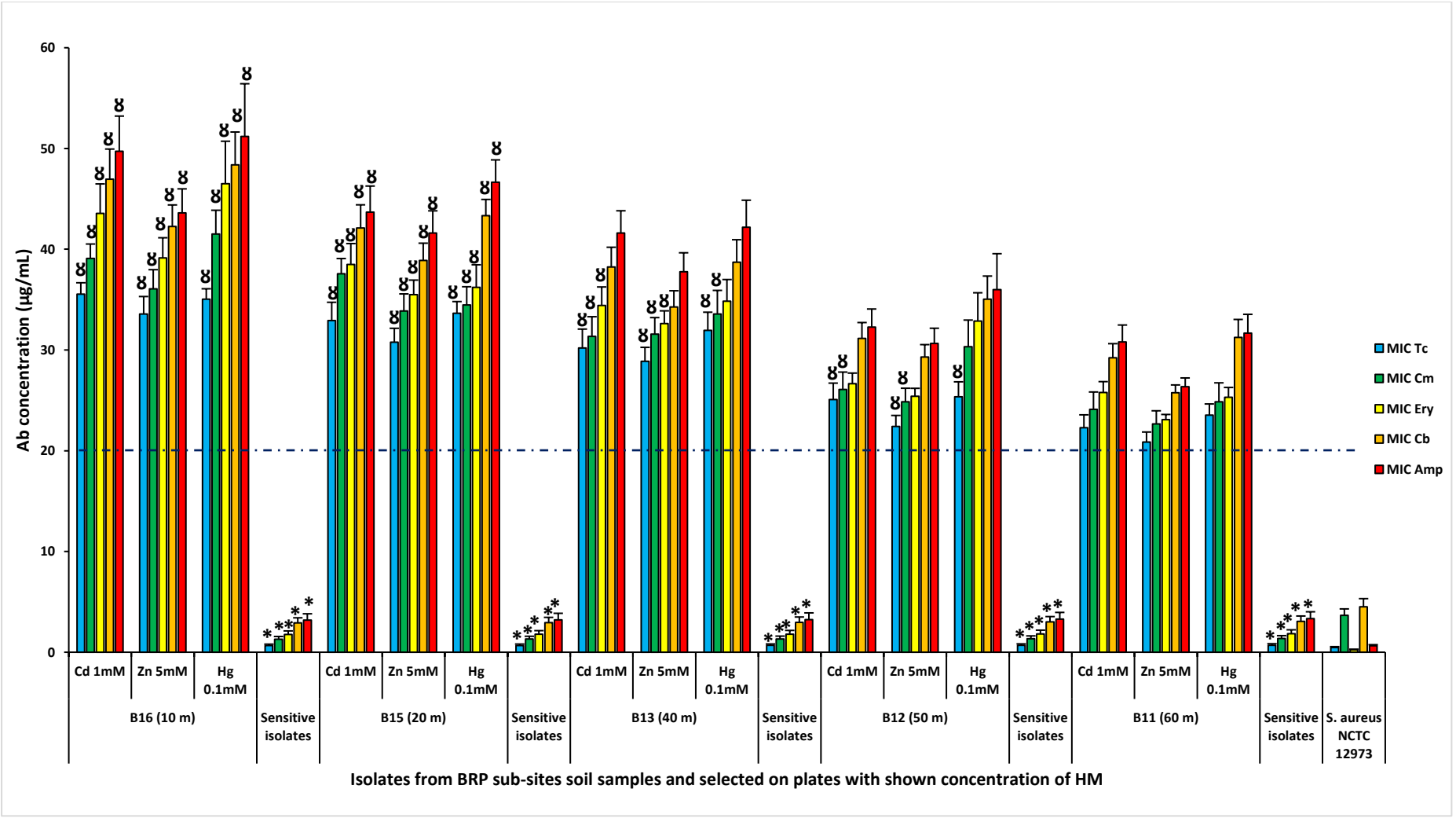

Fig. A2.46. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for the HMR isolates from BRP. *$p < 0.05$ compared to Ab MIC value for HM-sensitive isolates from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for the bacteria from B10 soil and selected on the same HM concentration. The dash line defines AbR level of soil bacteria.

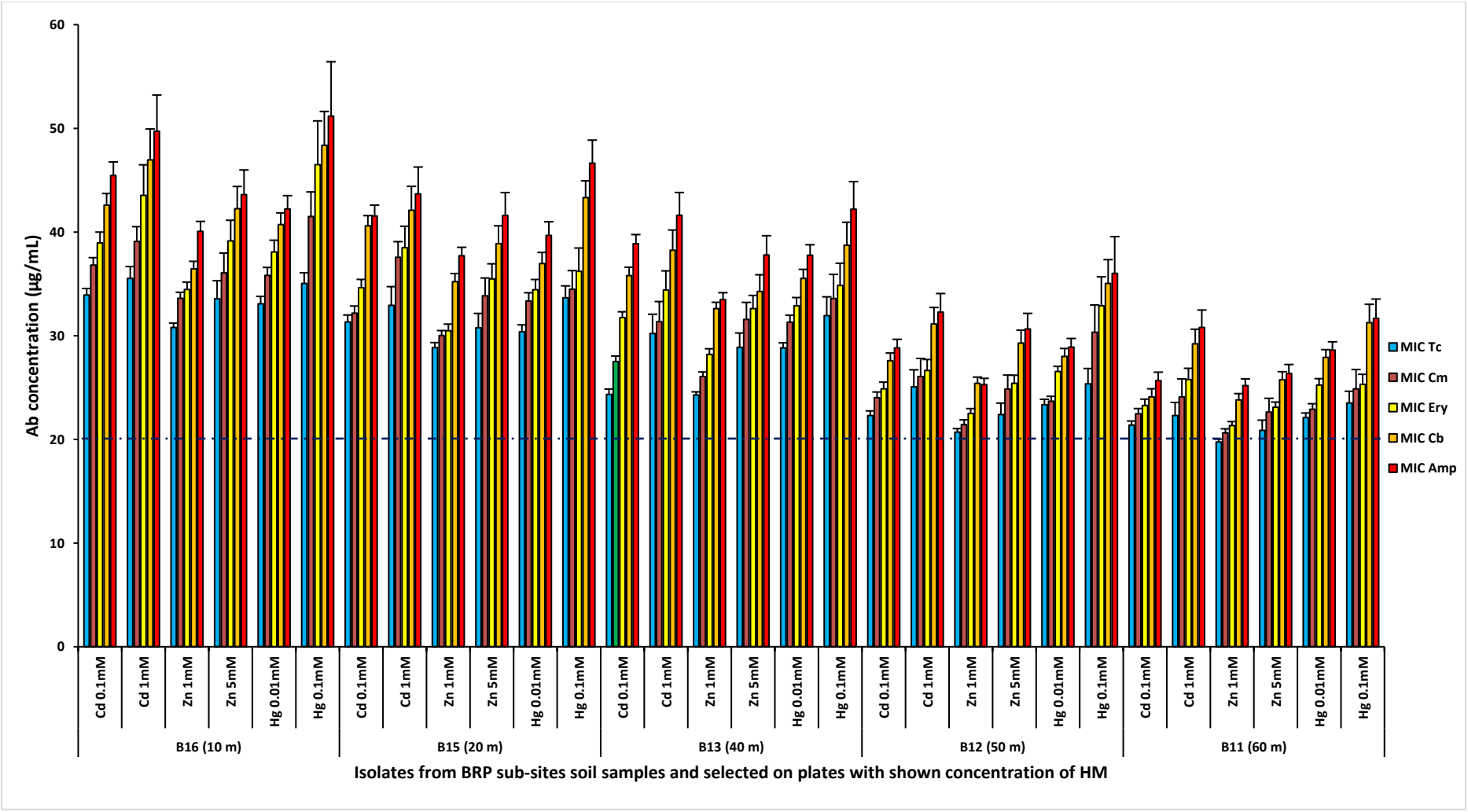

Fig. A2.47. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from BRP. The dash line defines AbR level of soil bacteria.

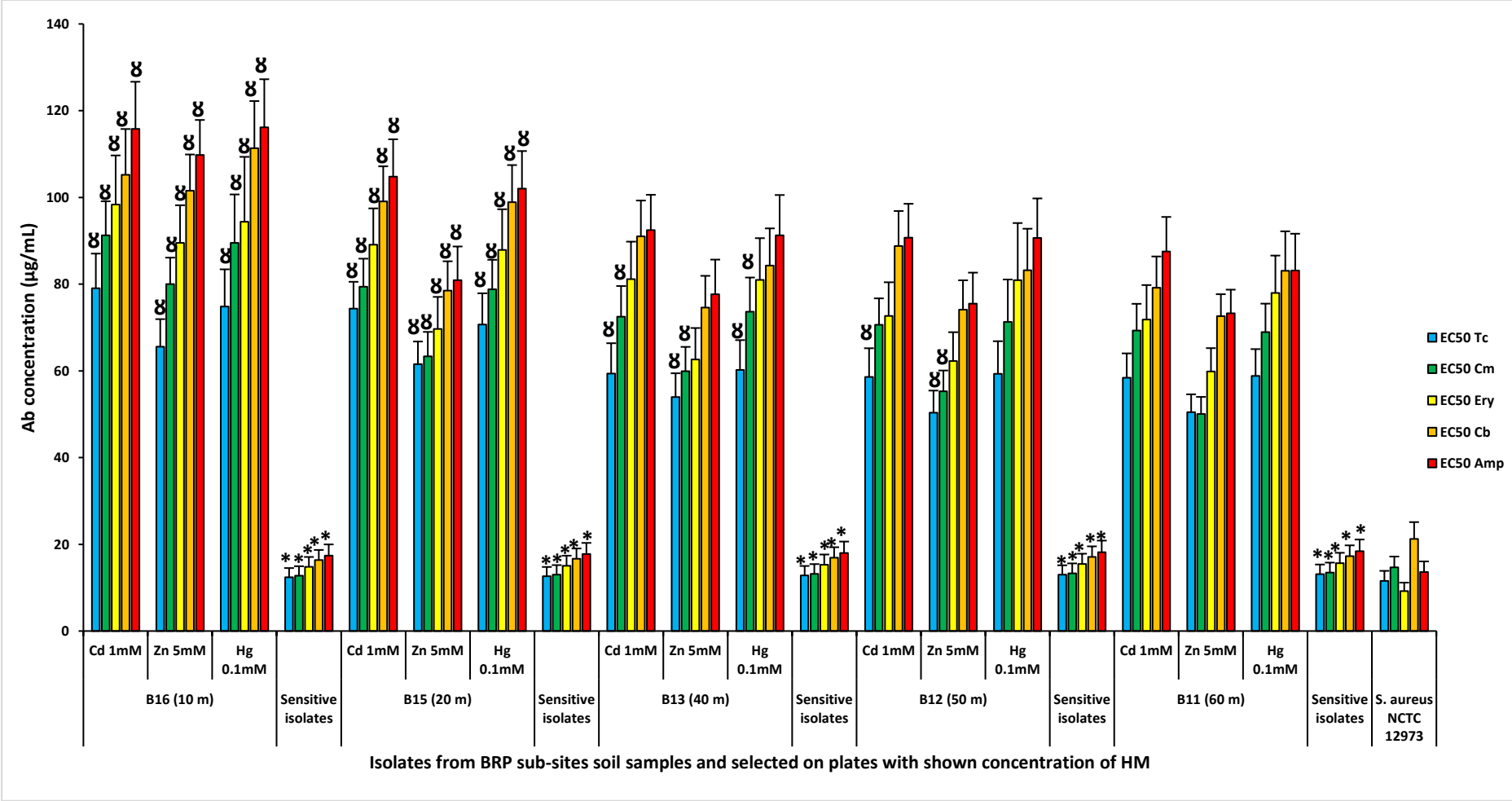

Fig. A2.48. Mean EC50 values of BM assay with Tc, Cm, Ery, Cb and Amp for the HMR isolates from BRP. *$p < 0.05$ compared to Ab MIC value for HM-sensitive bacteria from the same soil; ϑ$p < 0.05$ compared to Ab MIC value for the bacteria from B10 soil and selected on the same HM concentration.

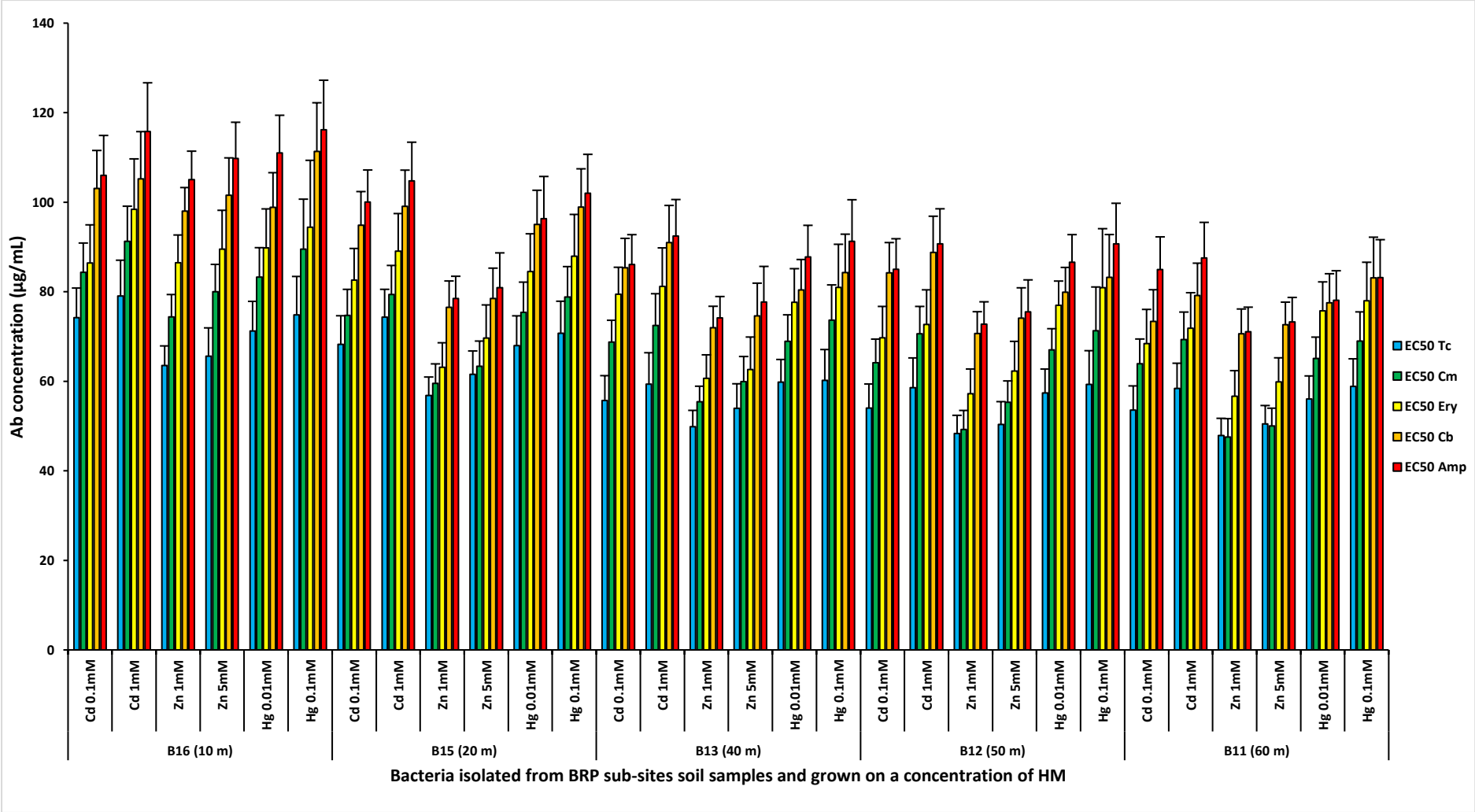

Fig. A2.49. Mean MIC values of BM assay with Tc, Cm, Ery, Cb and Amp for HMR isolates from BRP.

Appendix 3

Appendix 3 supplements Chapter 5 and presents the excess data not shown in that chapter.

A3.1. Physicochemical Properties of the Soil Sampling Sites.

Physicochemical properties of the soil samples collected from EW-13 and EW-24 sites were assessed and additional data over and above Table 5.1 is listed in Table A2.1.

Table A3.1. Continue of physicochemical properties of EW-13 and EW-24 soil samples.

Site No.	**EW-13**	**EW-24**
Anaerobically Mineralised N (mg kg^{-1})	118	281
Total porosity (%, v/v)	70.8	65.23
Bulk density t/m^3	0.68	0.85
F*	490	175
Al*	10800	32000
Sb*	0.06	0.05
As*	1.30	2.90
Ba*	46.0	38.0
Bi*	0.08	0.09
B*	<2	3.00
Cs*	0.81	0.96
Ca*	4600	1280
Cr*	2.40	28.0
Co*	0.98	2.00
Cu*	10.4	13.8
La*	4.10	1.81
Pb*	3.20	11.3
Li*	0.60	2.30
Mg*	430	560
Mn*	390	300
Mo*	0.33	0.68
Ni*	1.80	3.70
K*	350	250
Rb*	5.30	1.85
Se*	<2	<2
Ag*	0.04	0.04
Na*	200	76
Sr*	21.0	9.80
Ti*	0.08	0.09
Sn*	0.45	1.02
U*	0.58	1.03
V*	<10	138

*mg kg^{-1} of dry soil

A3.2. Resistance Bacterial Counts

A3.2.1. Microcosms Incubated for 6-weeks Microcosms (6WM)

Plates with Abs Additive

The ratio of AbR/total number of bacteria from 6WZnM at Time 0 increased from control microcosm to Zn 200 mM-spiked microcosm and then decreased in Zn 300 mM-spiked microcosms. There were significantly smaller AbR/total bacterial ratios for control microcosms compared to those from Zn-spiked microcosms ($p < 0.05$). The ratios of AbR/total bacteria in Zn-spiked microcosms at Time 0 were significantly less than those from the same microcosms after 6 weeks (Figure A3.1). The same trend was determined for AbR bacterial isolates from 6WHgM, although bacterial AbR/total ratio increased form control microcosm to 1 mM Hg-spiked one and then decreased in 5, 10 and 50 mM Hg-spiked microcosm (Figure A3.2).

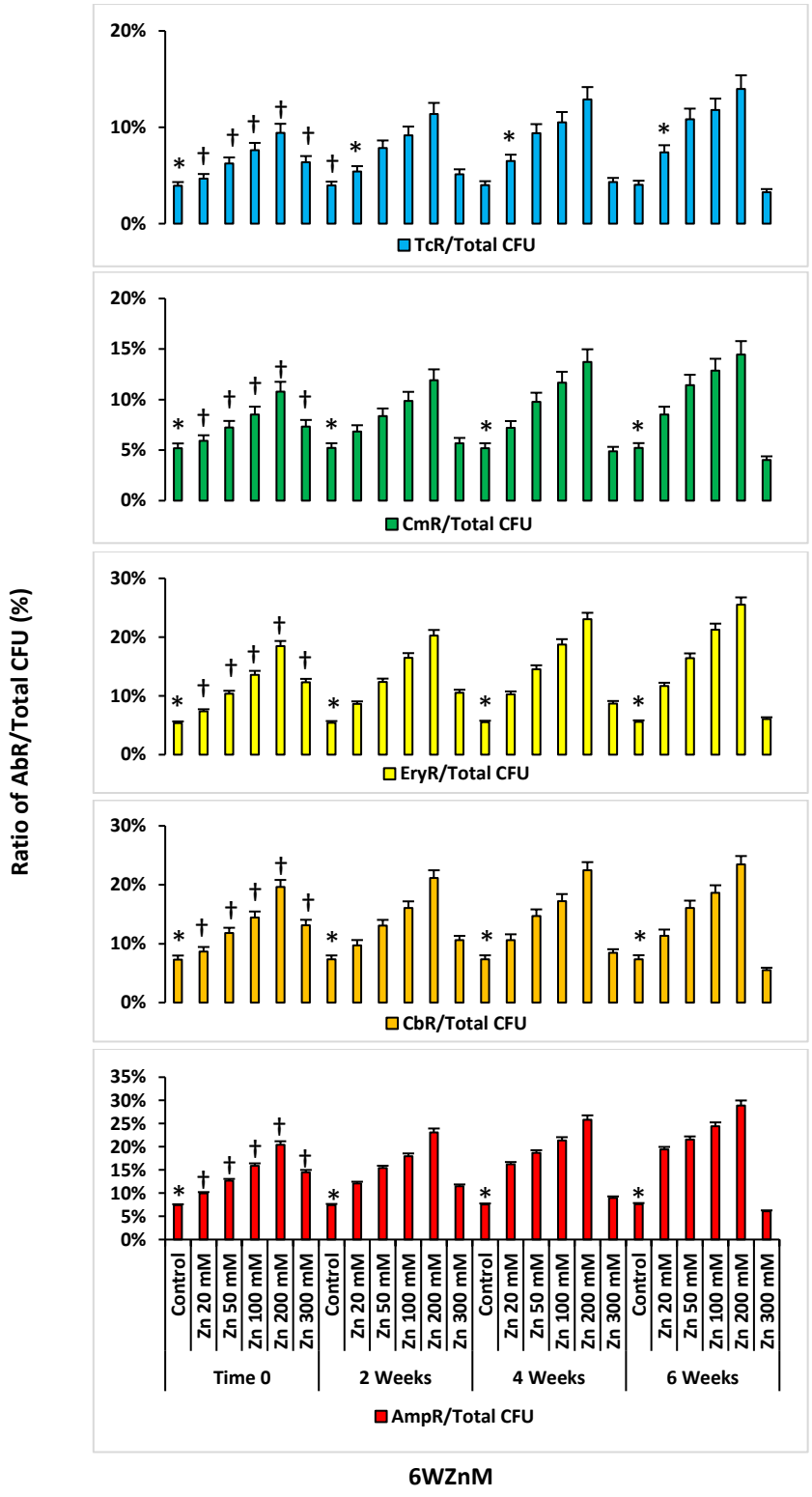

Fig. A3.1. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for 6WZnM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in 6WZnM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same 6WZnM at the 6-week interval.

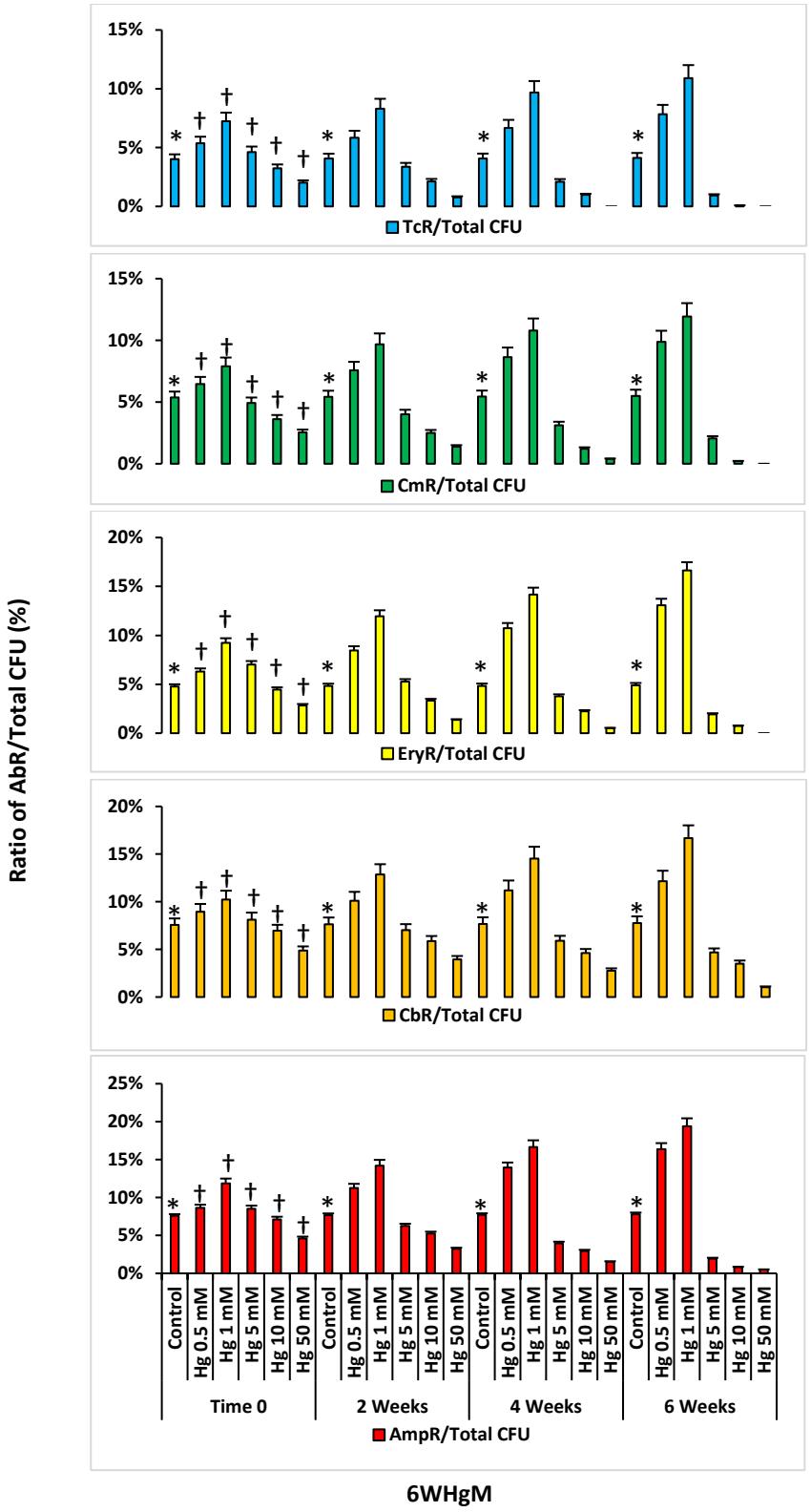

Fig. A3.2. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for 6WHgM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in 6WHgM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same 6WHgM at the 6-week interval.

A3.2.2. Background and Pasture soil-contained 6-month microcosms (B6MM/P6MM) Plates with Abs Additive

As discussed in Section 5.1.4, the ratio of AbR/total bacterial CFU ratios from B6MZnM and P6MZnM at Time 0 increased from control microcosms to Zn 200 mM-spiked microcosms and then decreased in Zn 300 mM-spiked microcosms. There were significantly smaller AbR/total bacterial ratios for control microcosms compared to those from Zn-spiked microcosms ($p < 0.05$). The ratios of AbR/total bacteria in Zn-spiked microcosms at Time 0 were significantly less than those from the same microcosms after 6 months (Figure A3.3). The same trend was determined for AbR bacterial isolates from B6MHgM and P6MHgM, although the bacterial AbR/total ratio increased form control microcosm to 1 mM Hg-spiked one and then decreased in the 5, 10 and 50 mM Hg-spiked microcosms (Figures A3.3-A3.6).

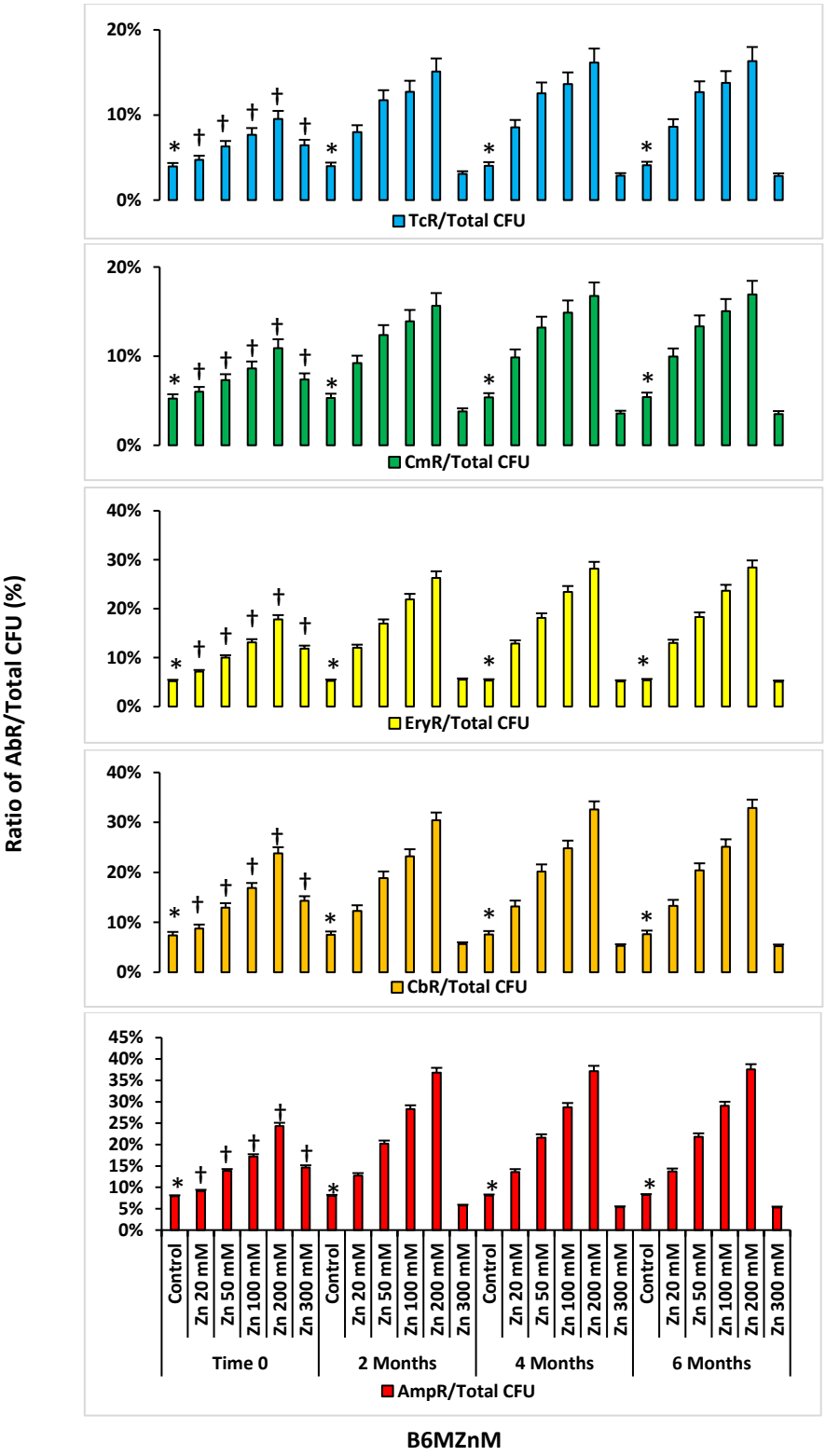

Fig. A3.3. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for B6MZnM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in B6MZnM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same B6MZnM at the 2-month interval.

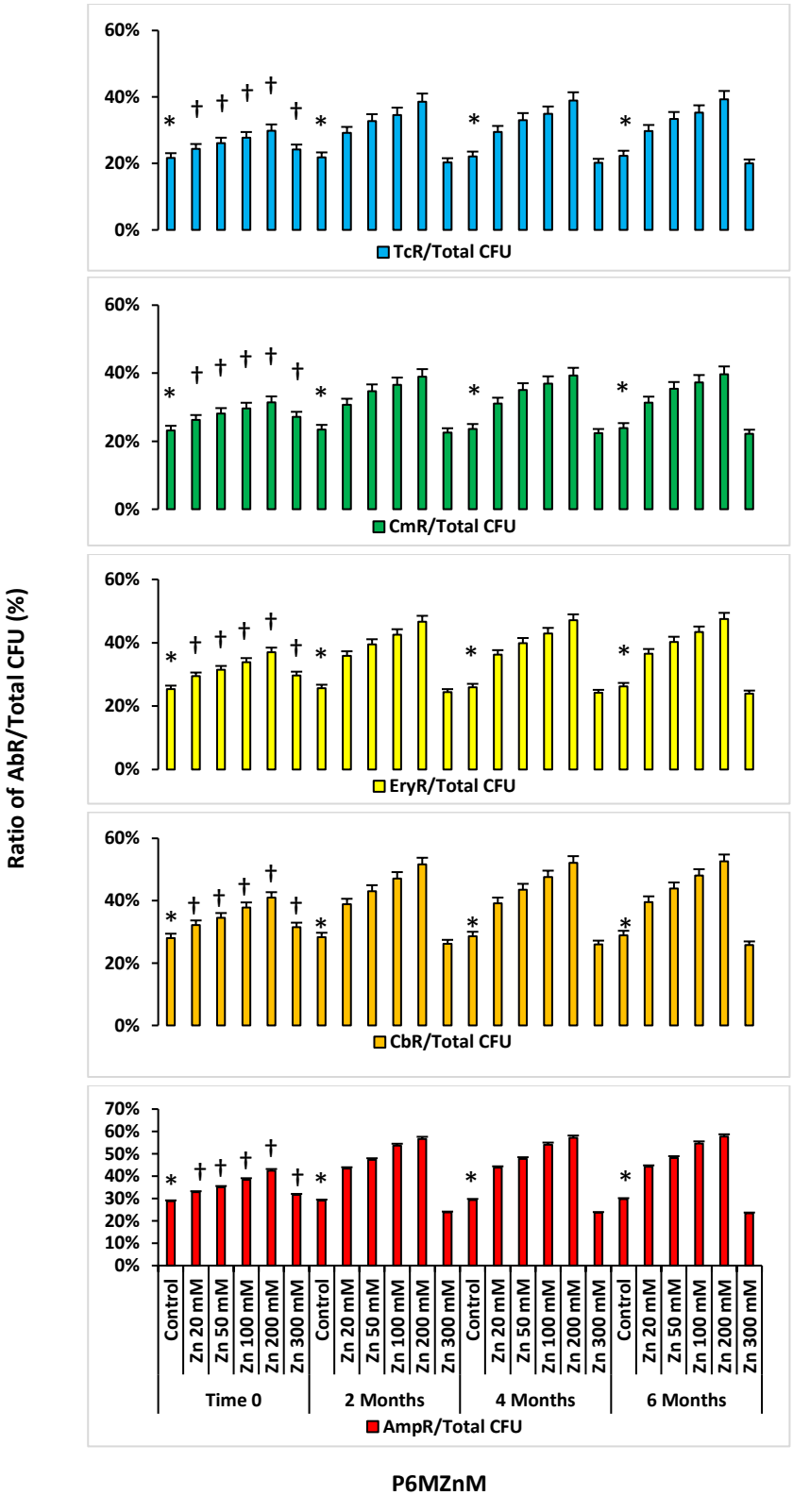

Fig. A3.4. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for P6MZnM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in P6MZnM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same P6MZnM at the 2-month interval.

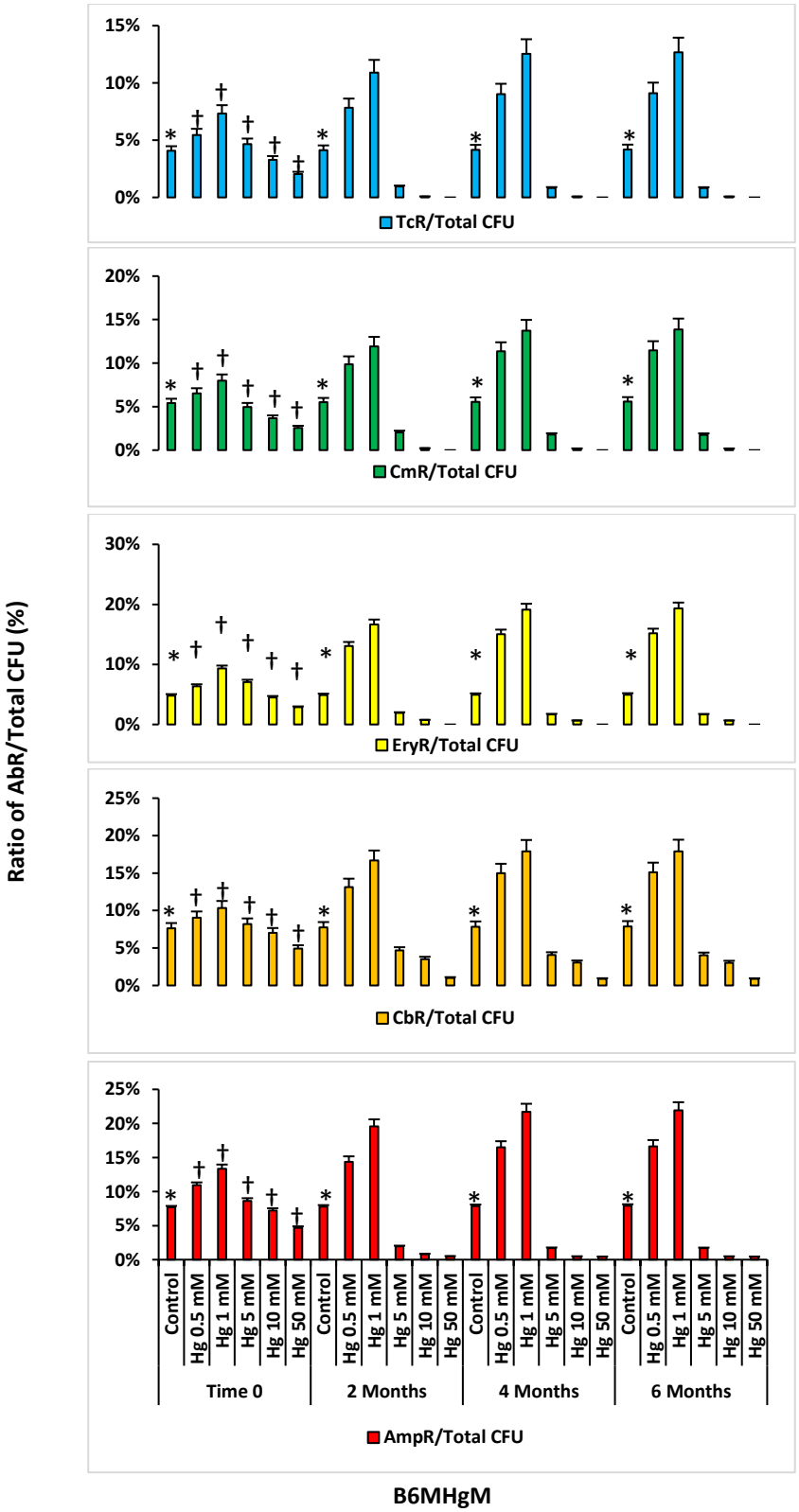

Fig. A3.5. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for B6MHgM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in B6MHgM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same B6MHgM at the 2-month interval.

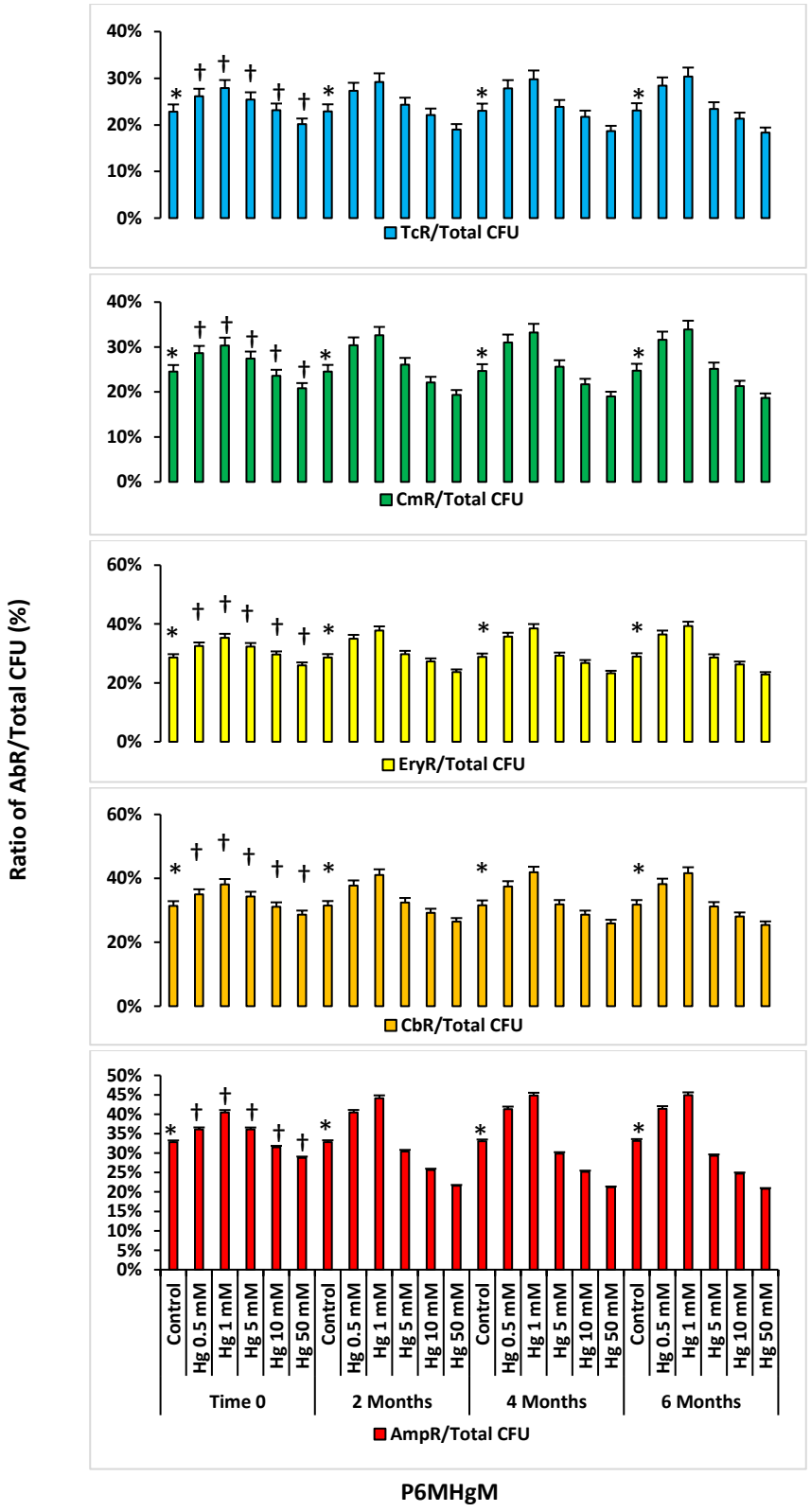

Fig. A3.6. Mean ratios of AbR/total bacterial CFU, selected on Abs (20 µg mL^{-1} of Cm, 100 µg mL^{-1} of Ery, 100 µg mL^{-1} of Cb and 200 µg mL^{-1} of Amp) for P6MHgM. *$p<0.05$ compared to the AbR/total bacterial CFU ratios in P6MHgM; †$p<0.05$ compared to the AbR/total bacterial CFU ratios in the same P6MHgM at the 2-month interval.

A3.3. Pollution Induced Community Tolerance (PICT) analysis

A3.3.1. 6-weeks microcosms (6WM)

Analysis Using HMs

There were higher HMs MIC and EC50 values for bacteria isolated from 6WZnM and 6WHgM compared to those from control microcosm. In addition, there were lower HMs MIC and EC50 values for bacteria from 6WZnM and 6WHgM at Time 0 compared to those from the same microcosms at 2, 4 and 6 weeks intervals (Figures A3.20-A3.25, and Tables A3.23-A3.28).

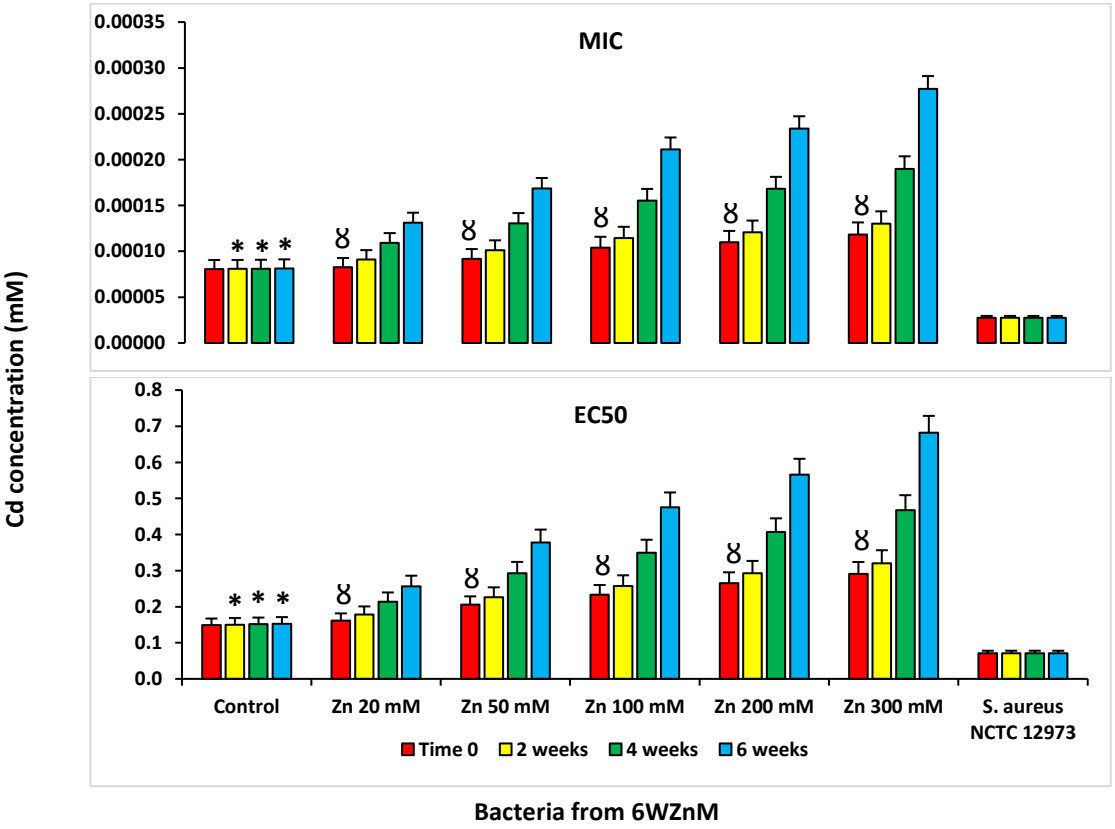

Fig. A3.7. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from 6WZnM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week.

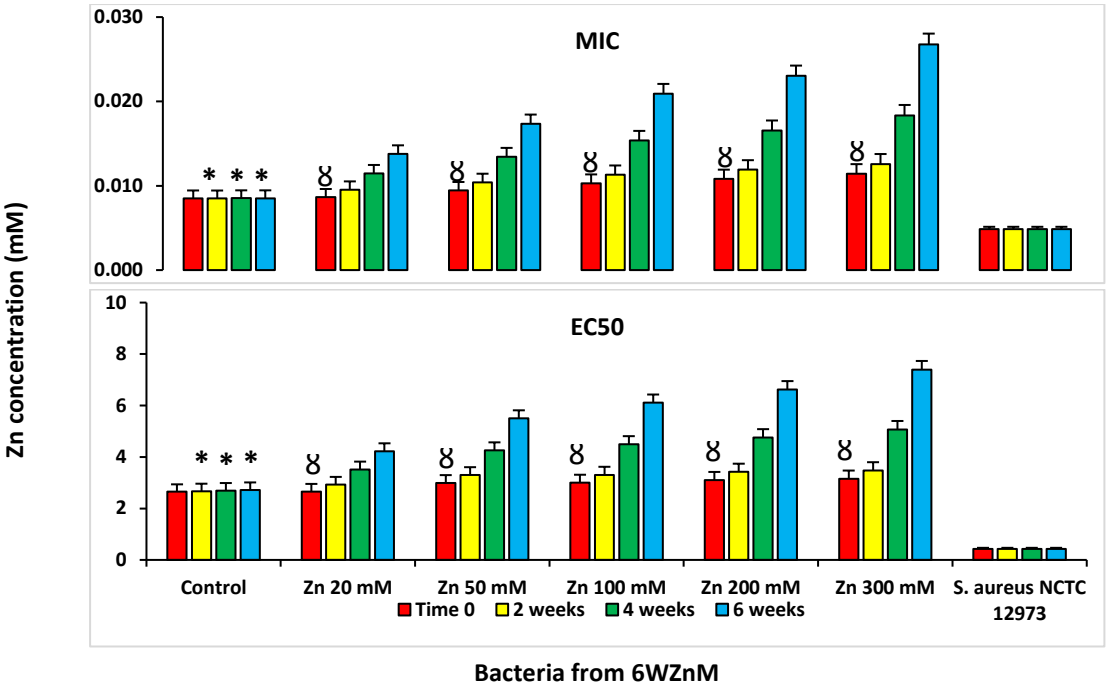

Fig. A3.8. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from 6WZnM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week.

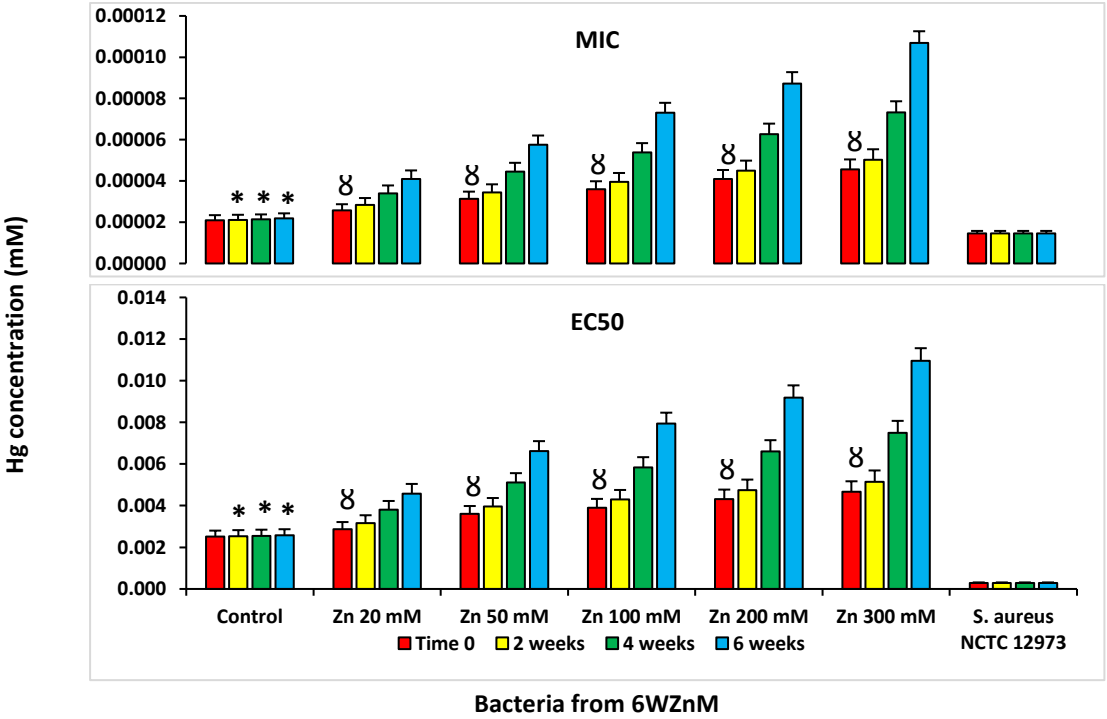

Fig. A3.9. Mean MIC and EC50 values of PICT analysis with Hg for bacteria from 6WZnM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week.

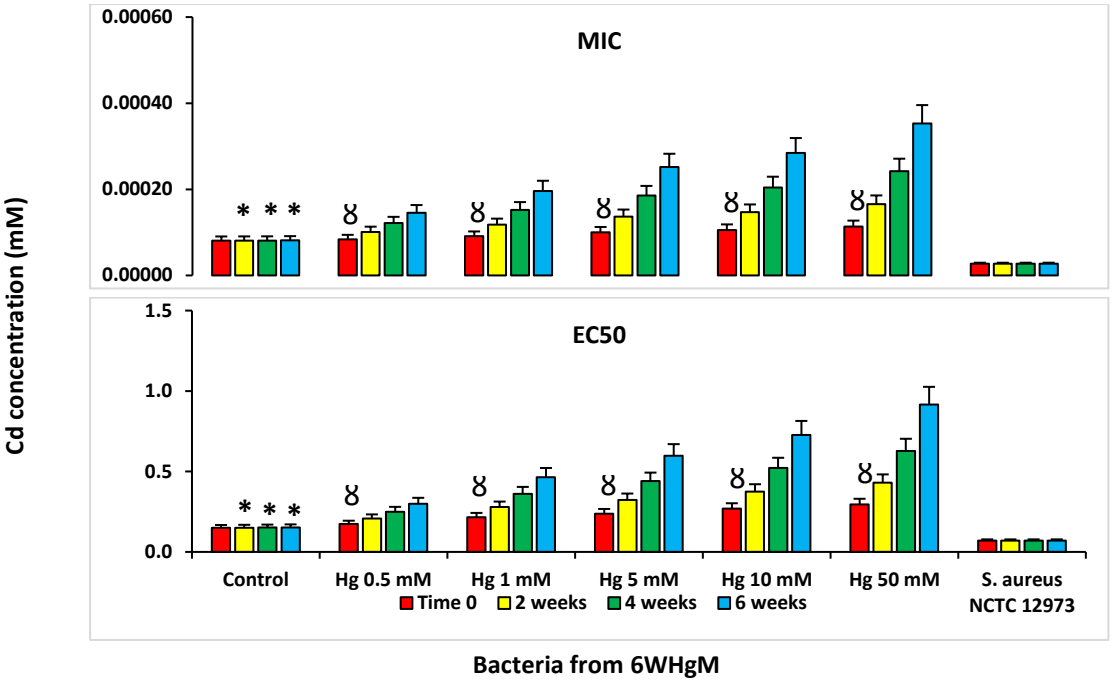

Fig. A3.10. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from 6WHgM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at 4 and 6 week.

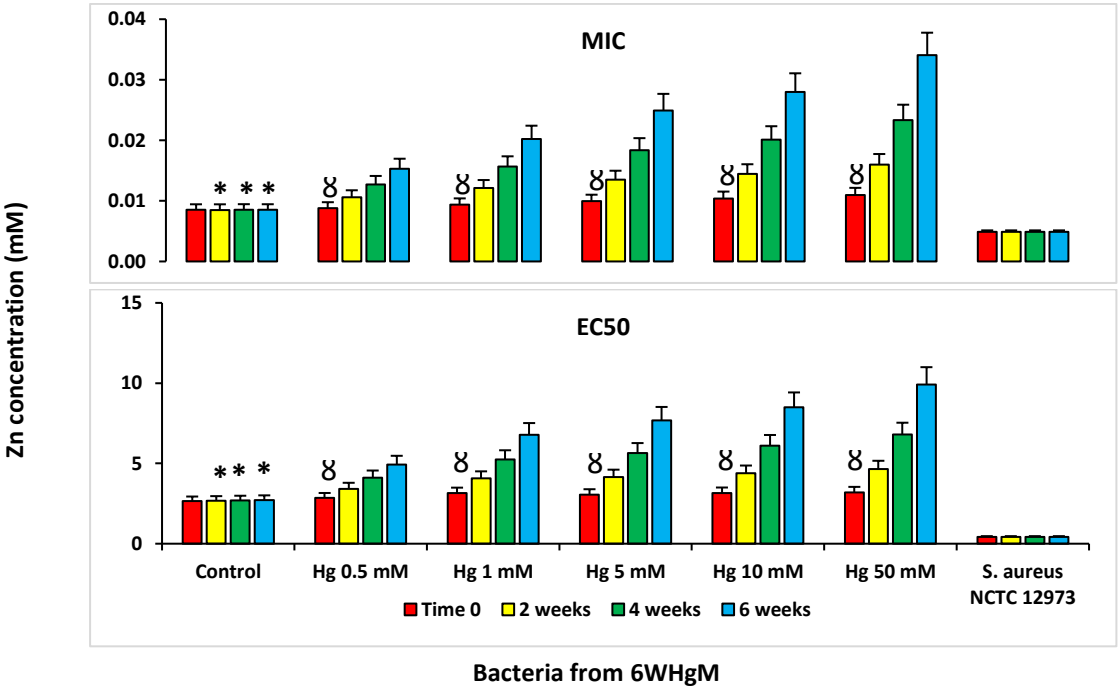

Fig. A3.11. MIC and EC50 values of PICT analysis with Zn for bacteria from 6WHgM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at 4 and 6 week.

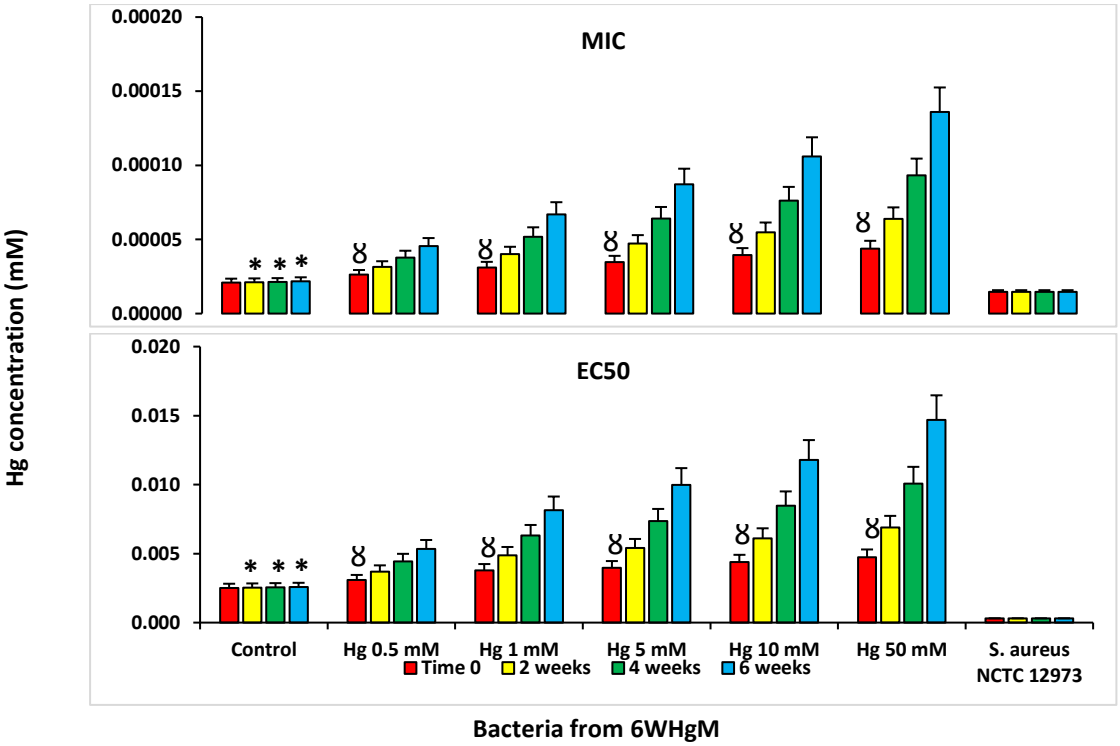

Fig. A3.12 Mean MIC and EC50 values of PICT analysis with Hg for bacteria from 6WHgM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at 4 and 6 week.

Analysis Using Abs

As discussed in Section 5.4.1 for bacteria from 6WCdM, there were significantly greater Abs MIC and EC50 values for bacteria from 6WZnM and 6WHgM compared to those from control microcosms ($p < 0.05$). Lower Abs MIC and EC50 values were determined for bacteria from 6WZnM and 6WHgM at Time 0 compared to those for bacteria at 2, 4 and 6 weeks intervals (Figures A3.30-A3.37 and Tables A3.32-A3.43).

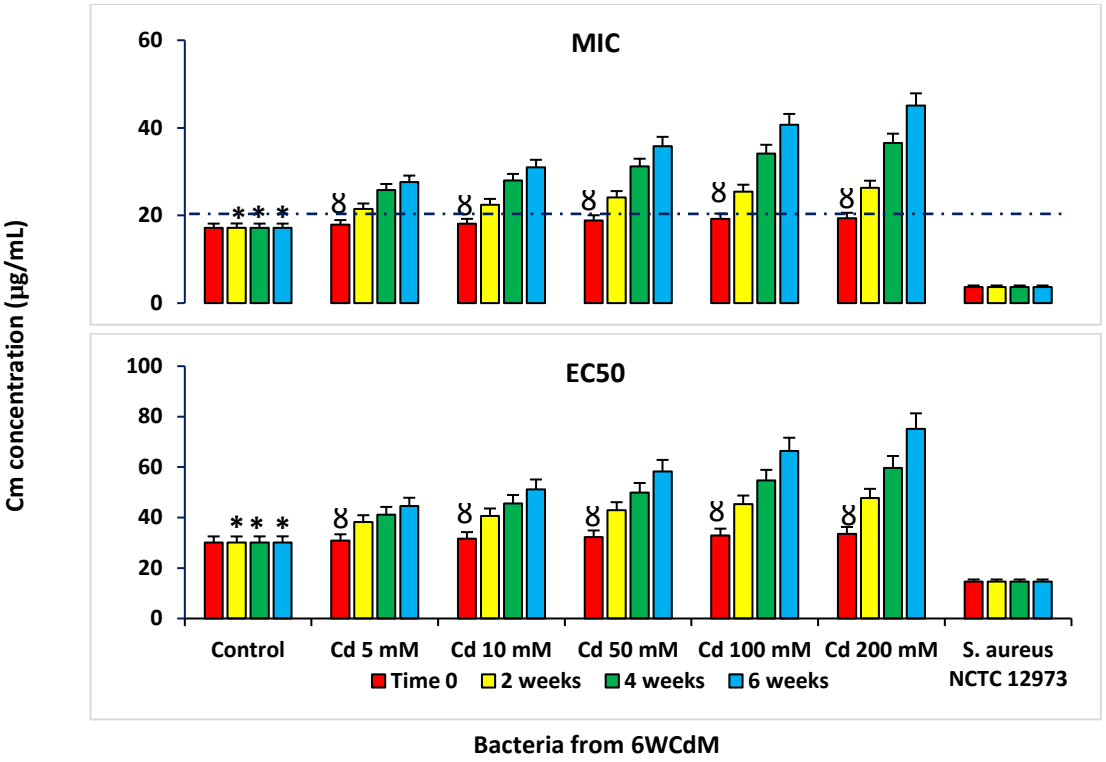

Fig. A3.13. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from 6WCdM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

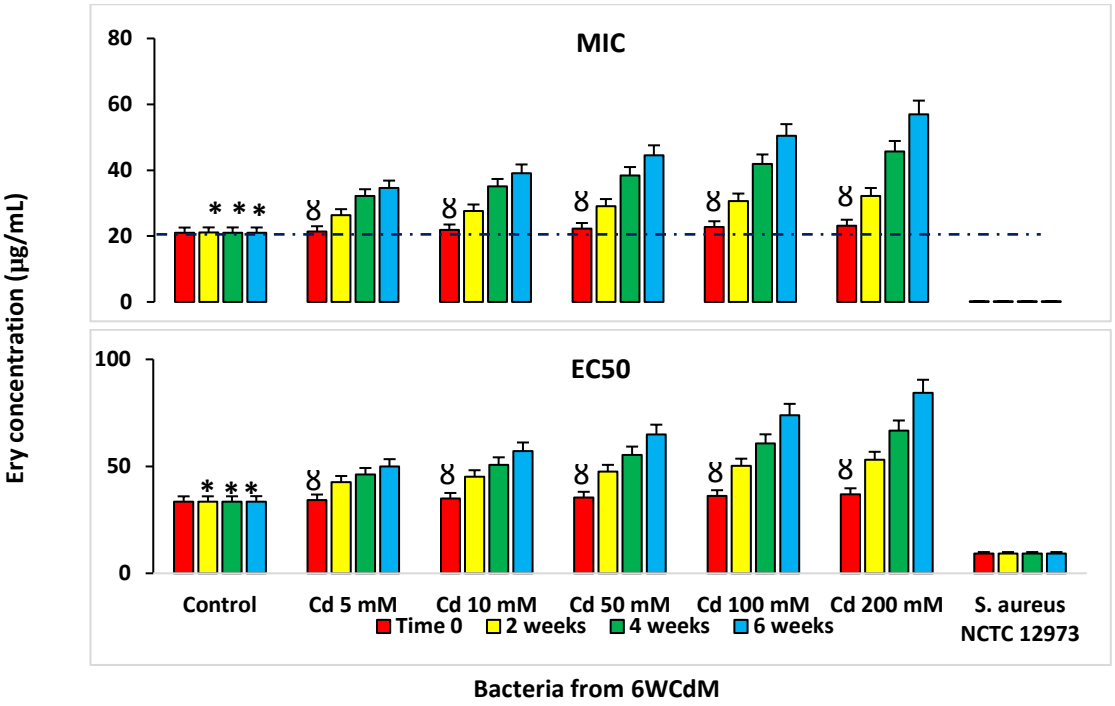

Fig. A3.14. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from 6WCdM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

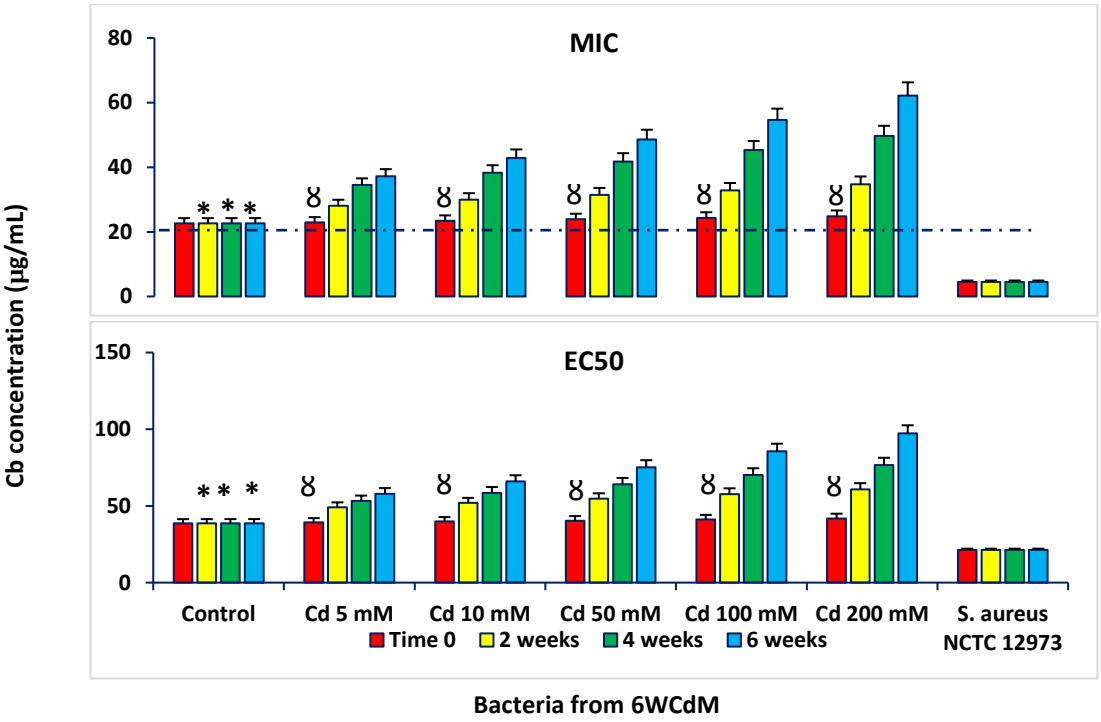

Fig. A3.15. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from 6WCdM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

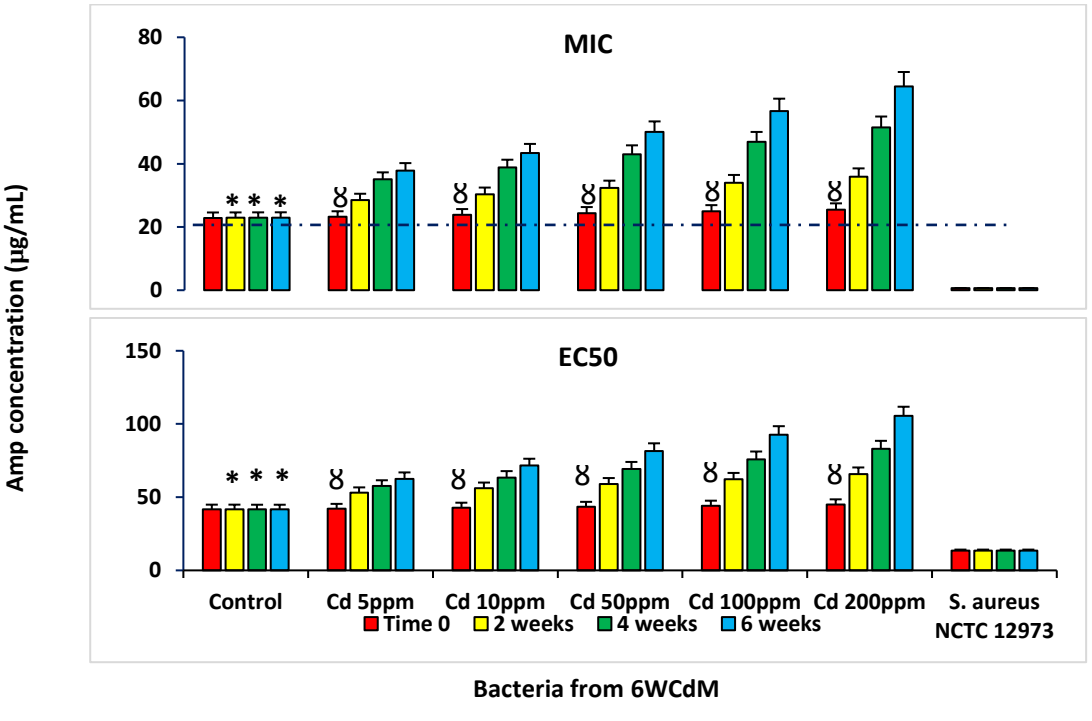

Fig. A3.16. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from 6WCdM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

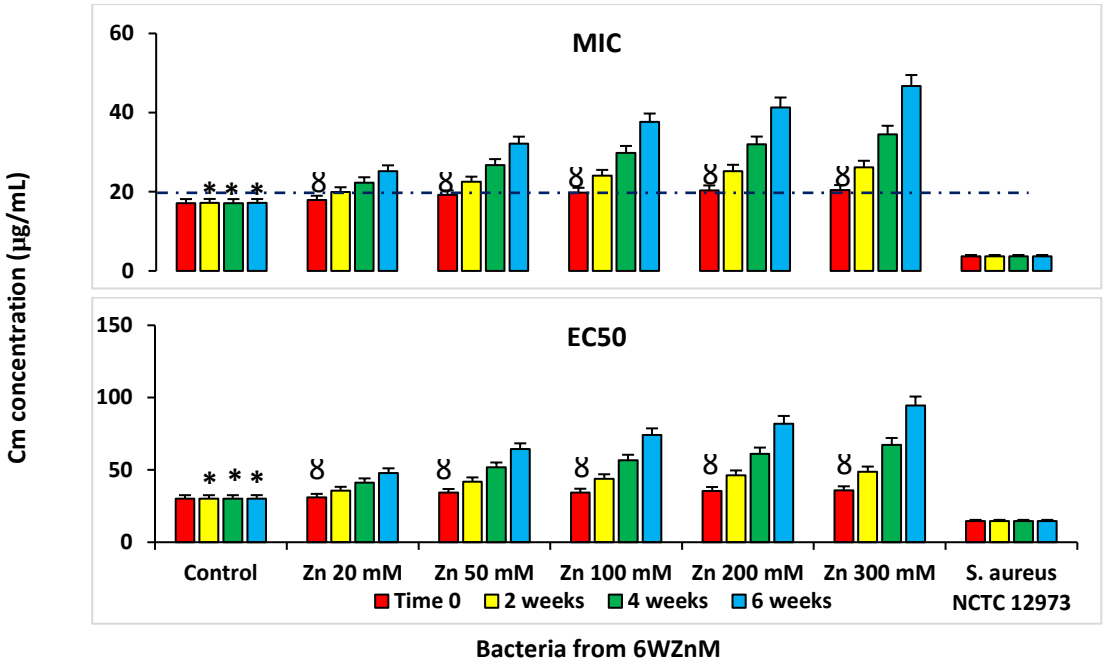

Fig. A3.17. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from 6WZnM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week. The dash line defines AbR level of soil bacteria.

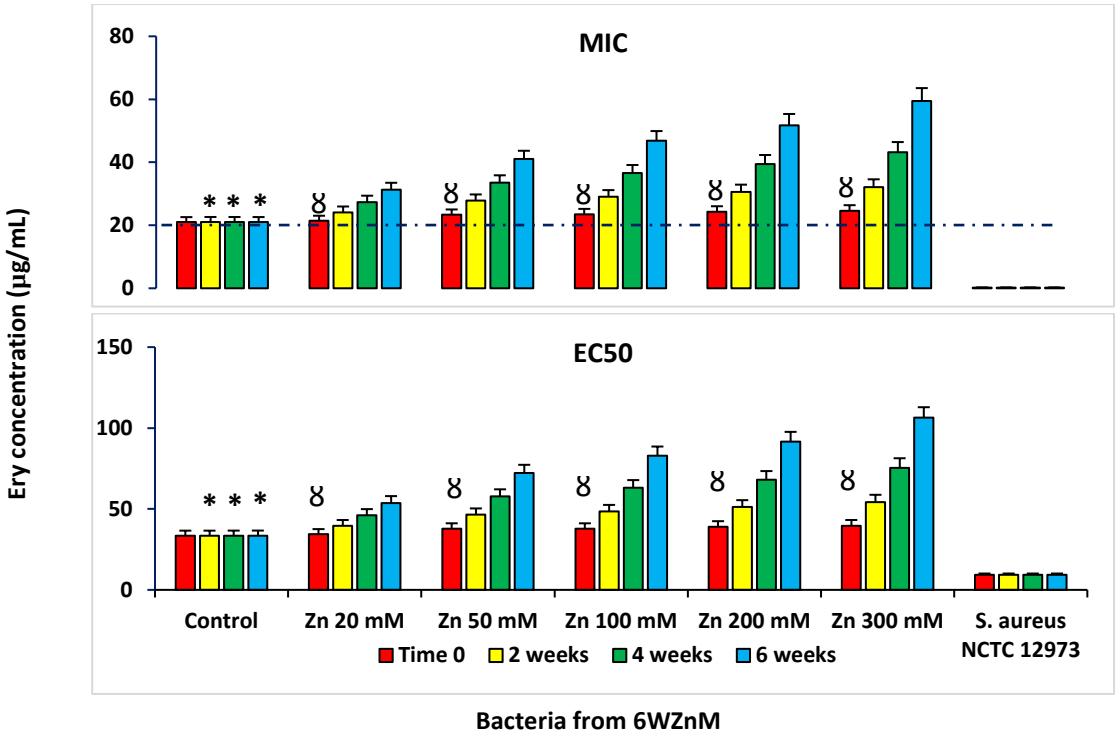

Fig. A3.18. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from 6WZnM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week. The dash line defines AbR level of soil bacteria.

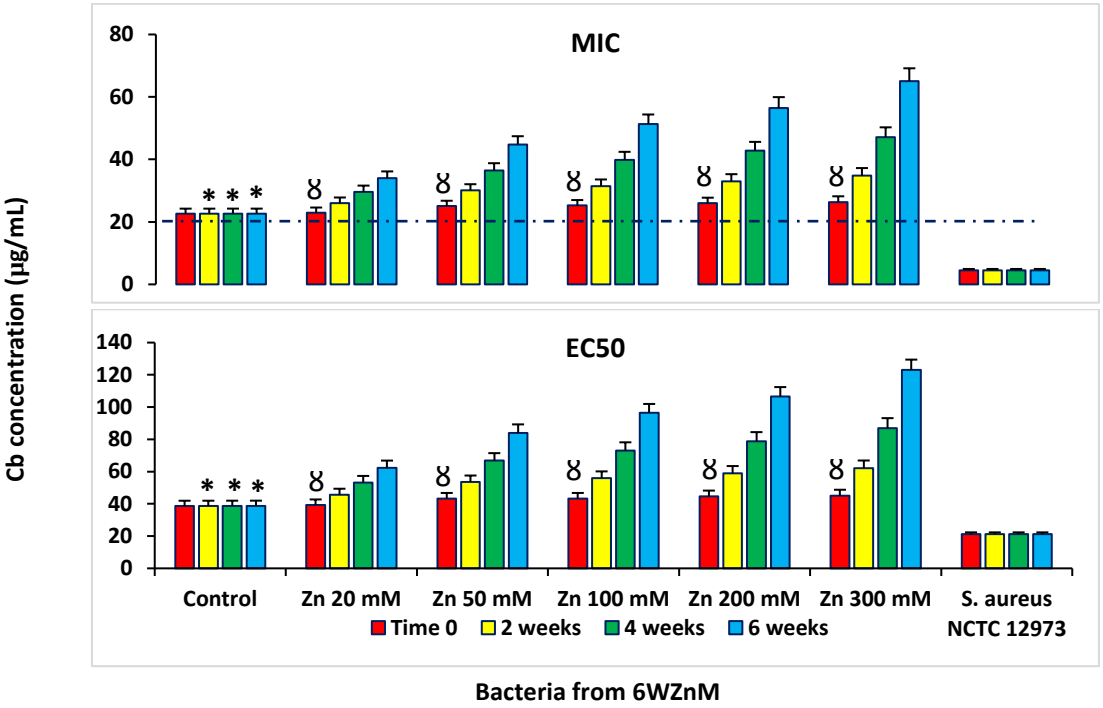

Fig. A3.19. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from 6WZnM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week. The dash line defines AbR level of soil bacteria.

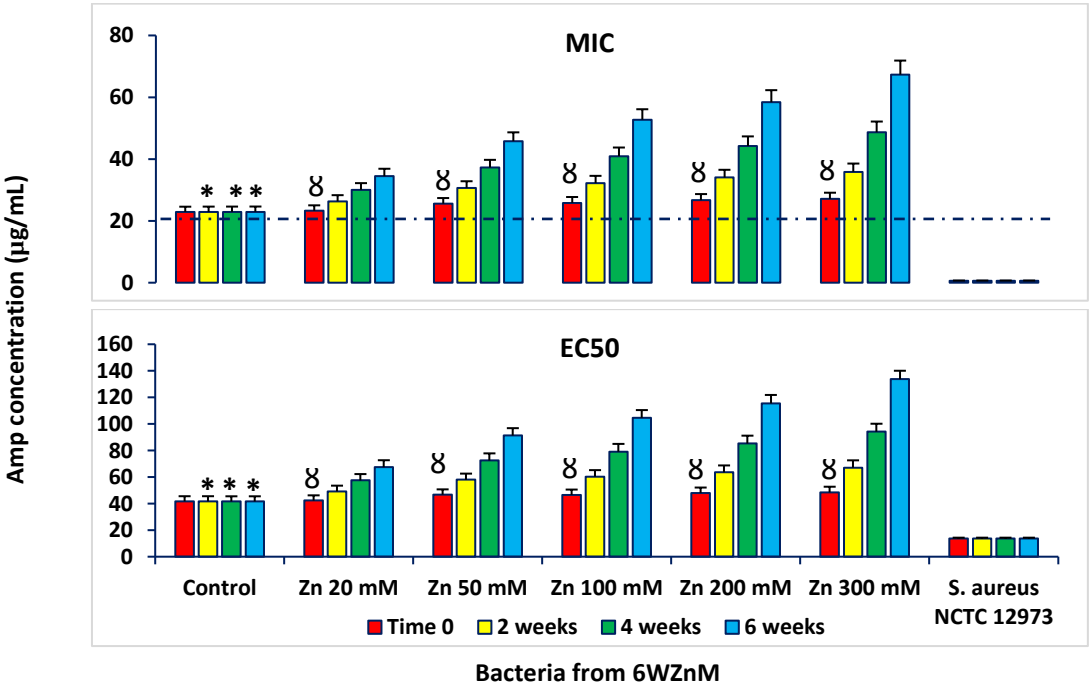

Fig. A3.20. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from 6WZnM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at 4 and 6 week. The dash line defines AbR level of soil bacteria.

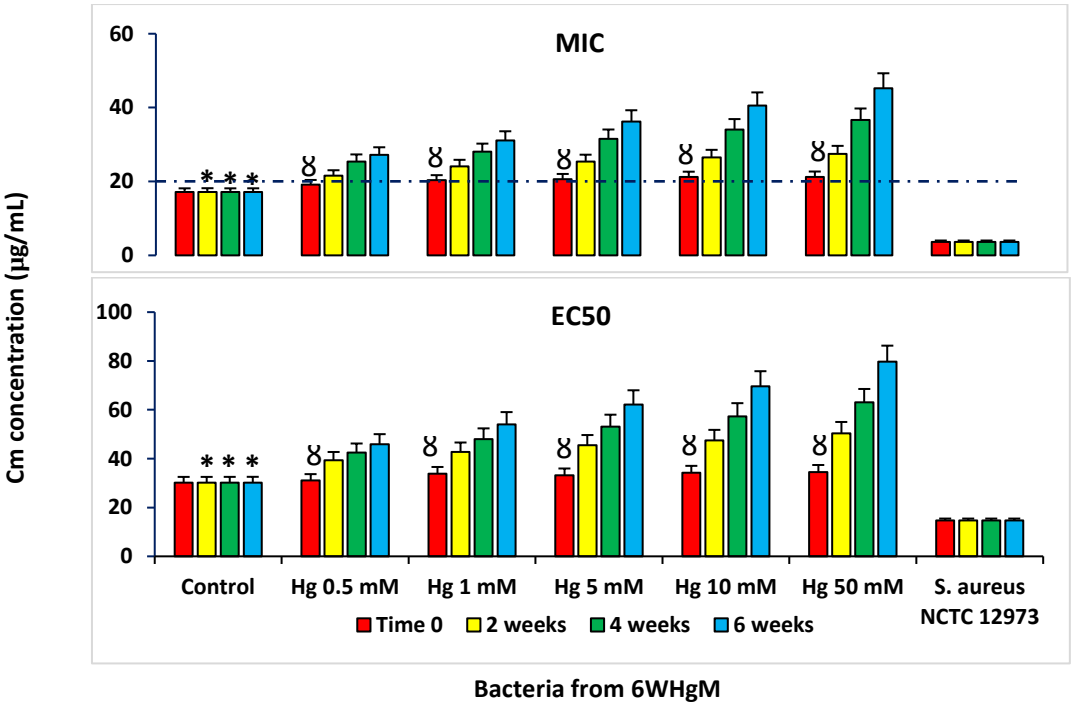

Fig. A3.21. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from 6WHgM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϐ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

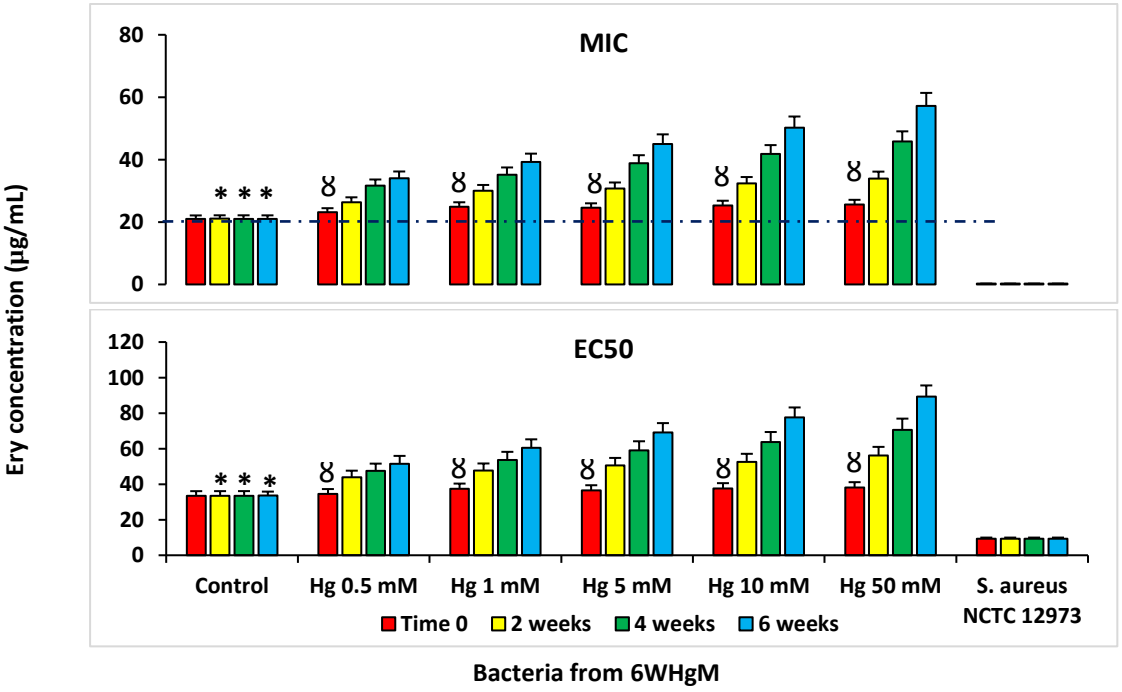

Fig. A3.22. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from 6WHgM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϐ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

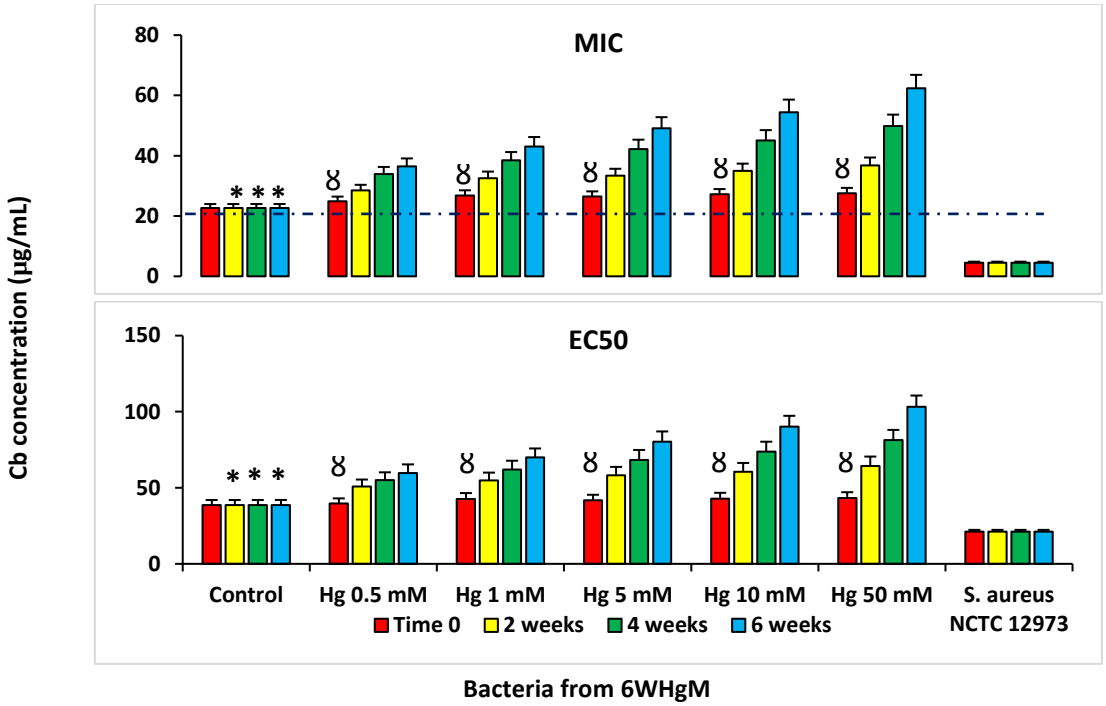

Fig. A3.23. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from 6WHgM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

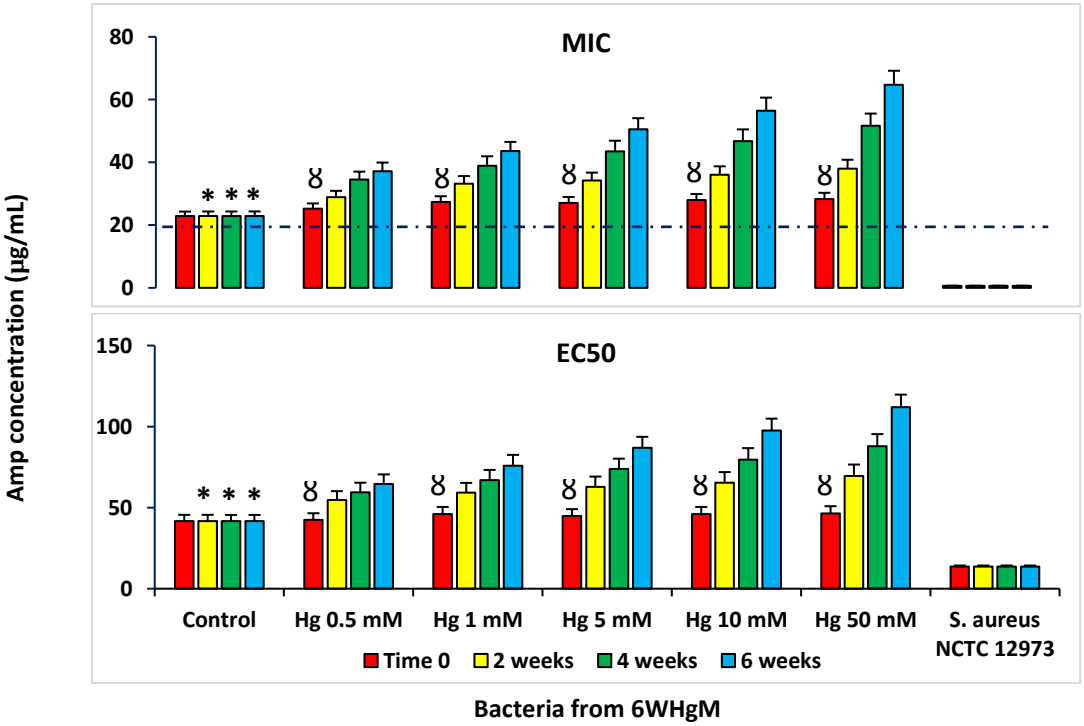

Fig. A3.24. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from 6WHgM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 week. The dash line defines AbR level of soil bacteria.

A3.3.2. Background and Pasture soil-contained 6-month microcosms (B6MM and P6MM)

Analysis Using HMs

There were significantly greater HMs MIC and EC50 values for bacterial communities from Zn or Hg-spiked microcosms compared to those for bacteria from control microcosms ($p < 0.05$). In addition, there were lower HMs MIC and EC50 values for bacteria from Zn or Hg-spiked microcosms at Time 0 compared to those for bacteria from the same microcosms at 2, 4 and 6 months intervals (Figures A3.25-A3.36).

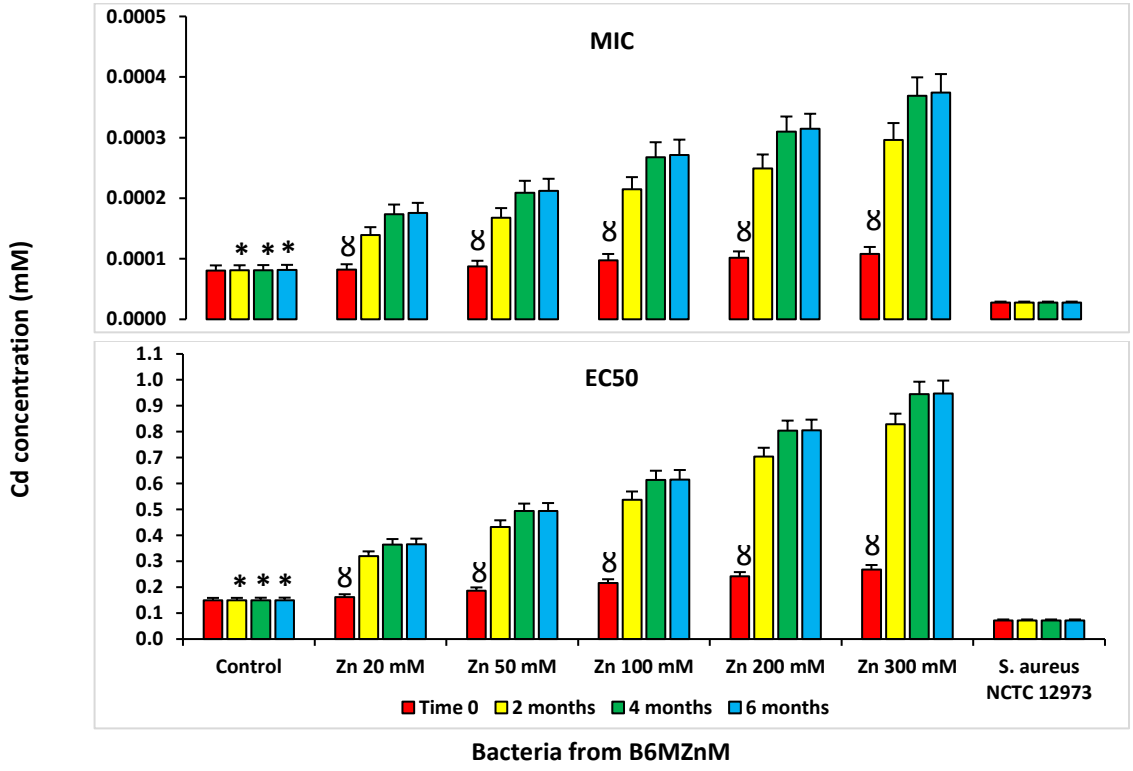

Fig. A3.25. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from B6MZnM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

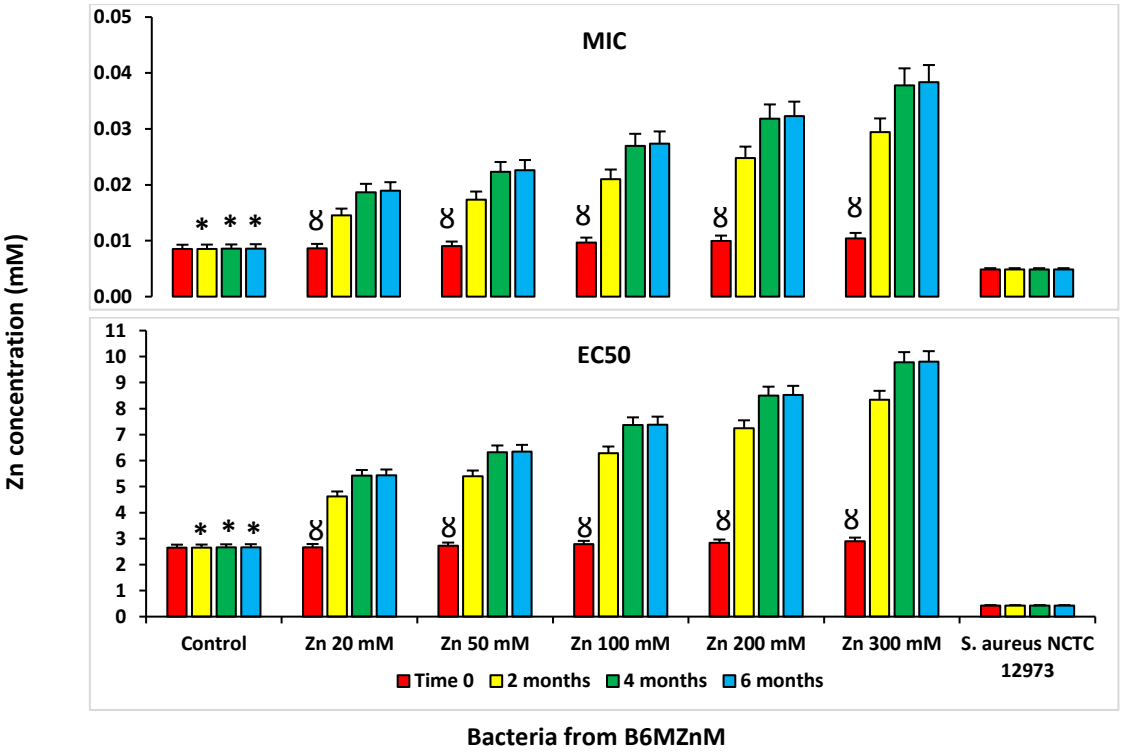

Fig. A3.26. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from B6MZnM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

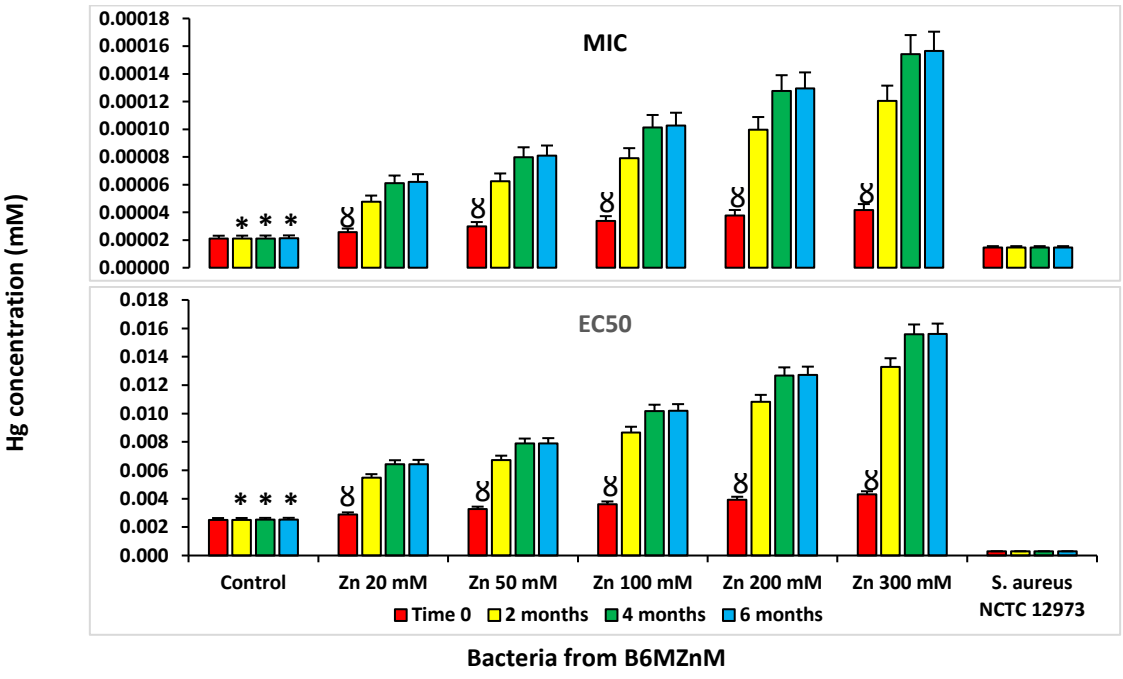

Fig. A3.27. Mean MIC and EC50 values of PICT analysis with Hg for bacteria from B6MZnM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

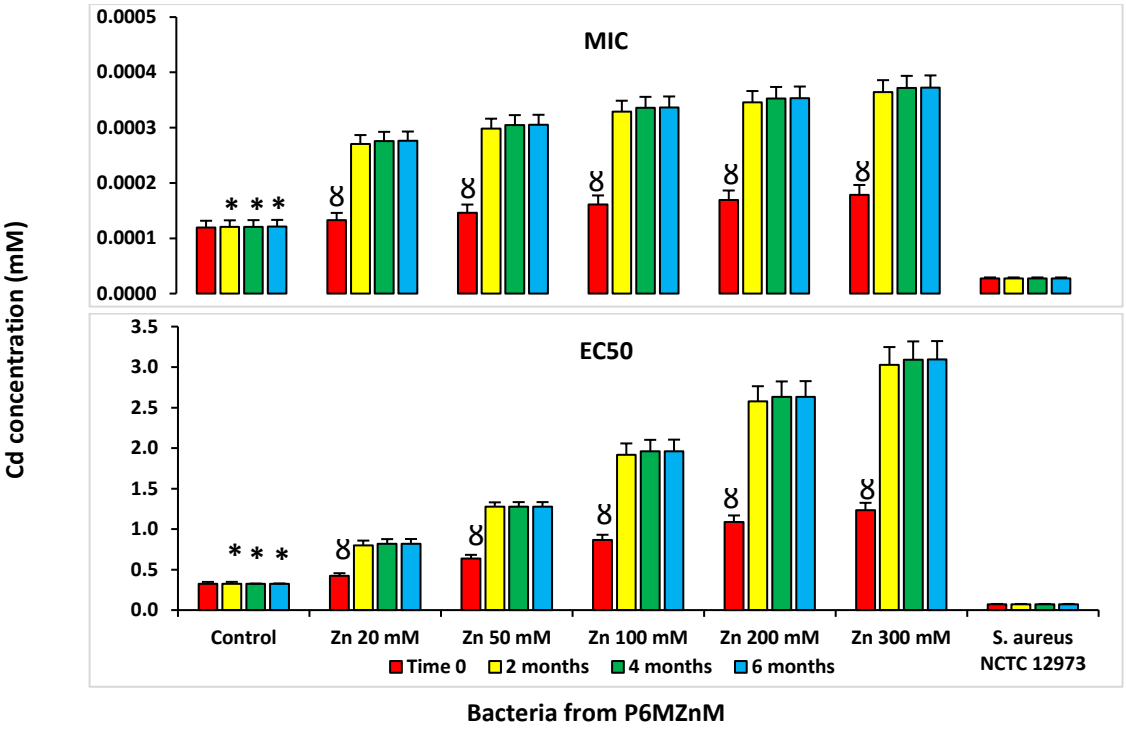

Fig. A3.28. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from P6MZnM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

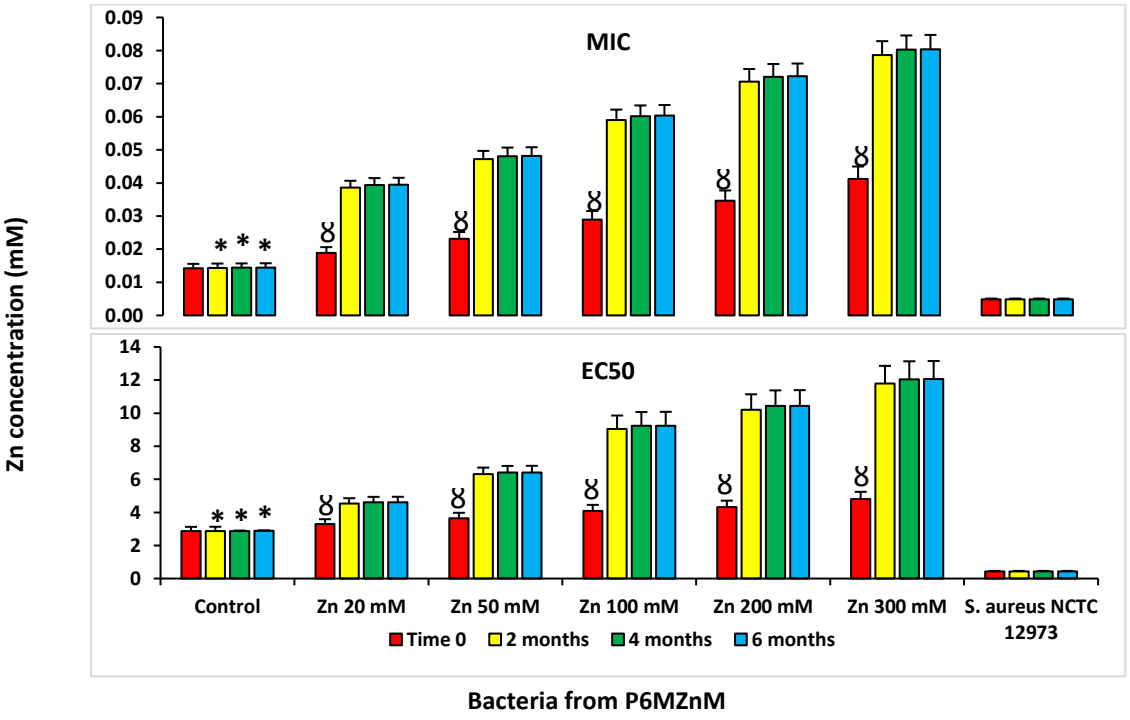

Fig. A3.29. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from P6MZnM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

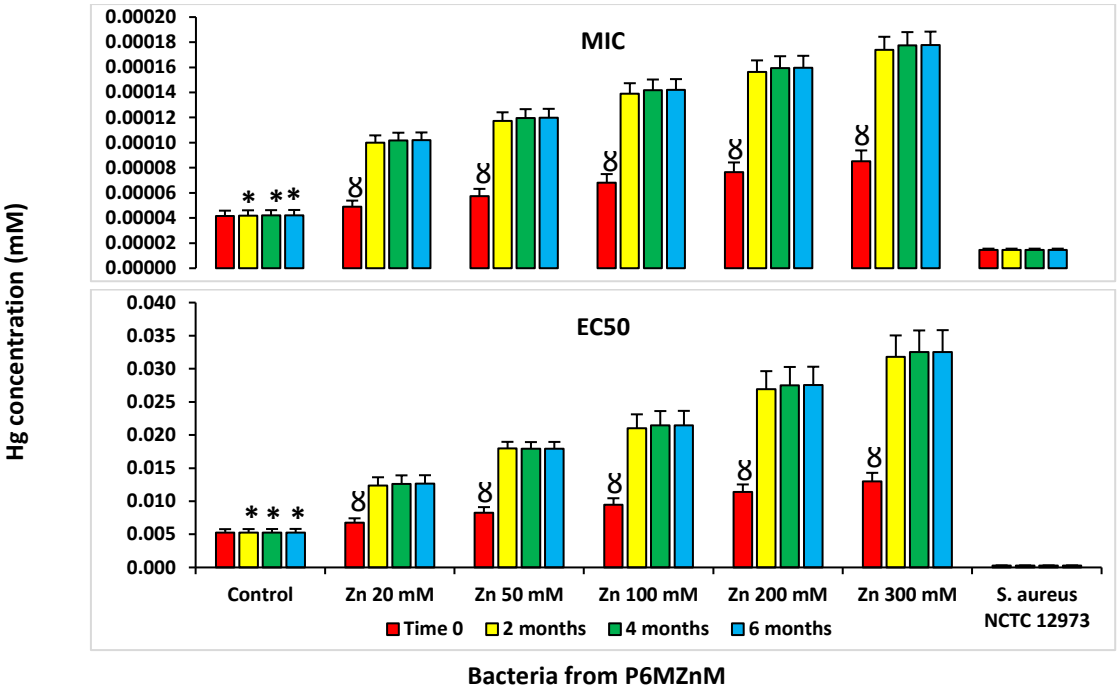

Fig. A3.30. Mean MIC and EC50 values of PICT analysis with Hg for bacteria from P6MZnM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month.

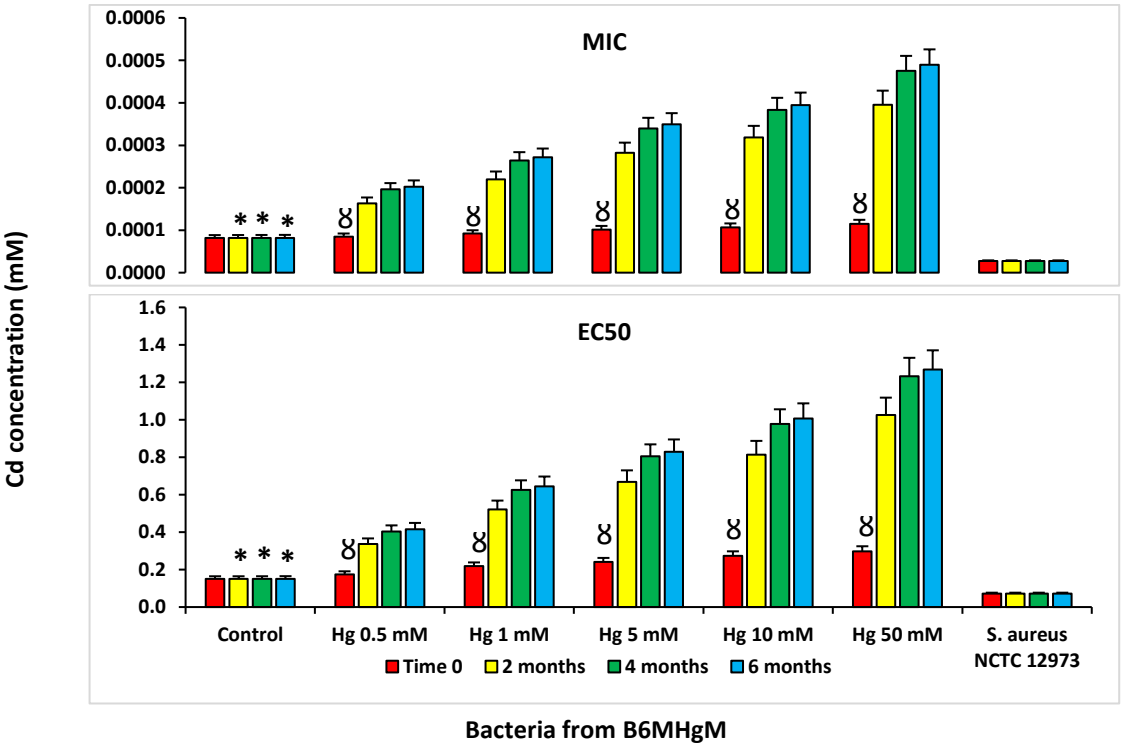

Fig. A3.31. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from B6MHgM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

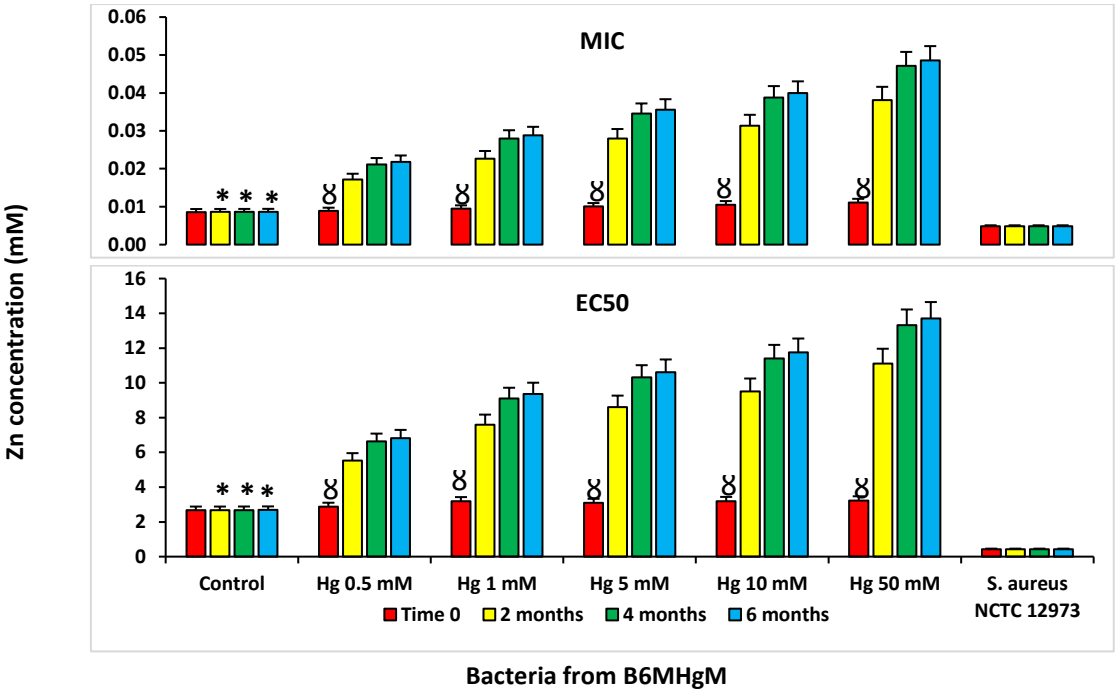

Fig. A3.32. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from B6MHgM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

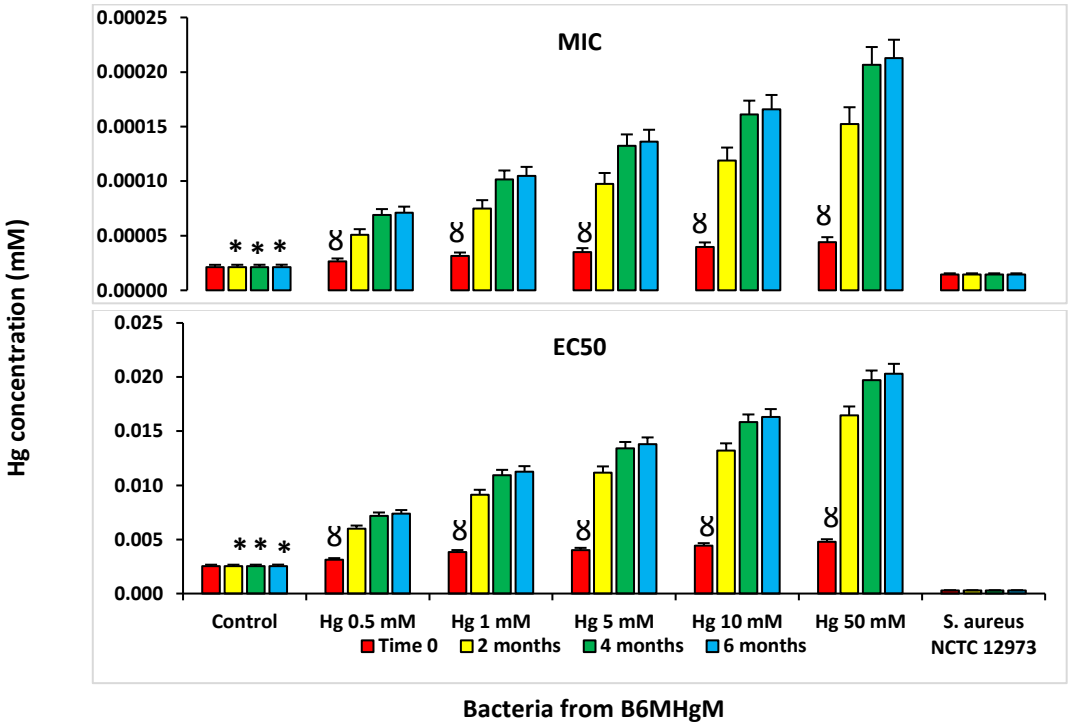

Fig. A3.33. Mean MIC and EC50 values of PICT analysis with Hg for bacteria B6MHgM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

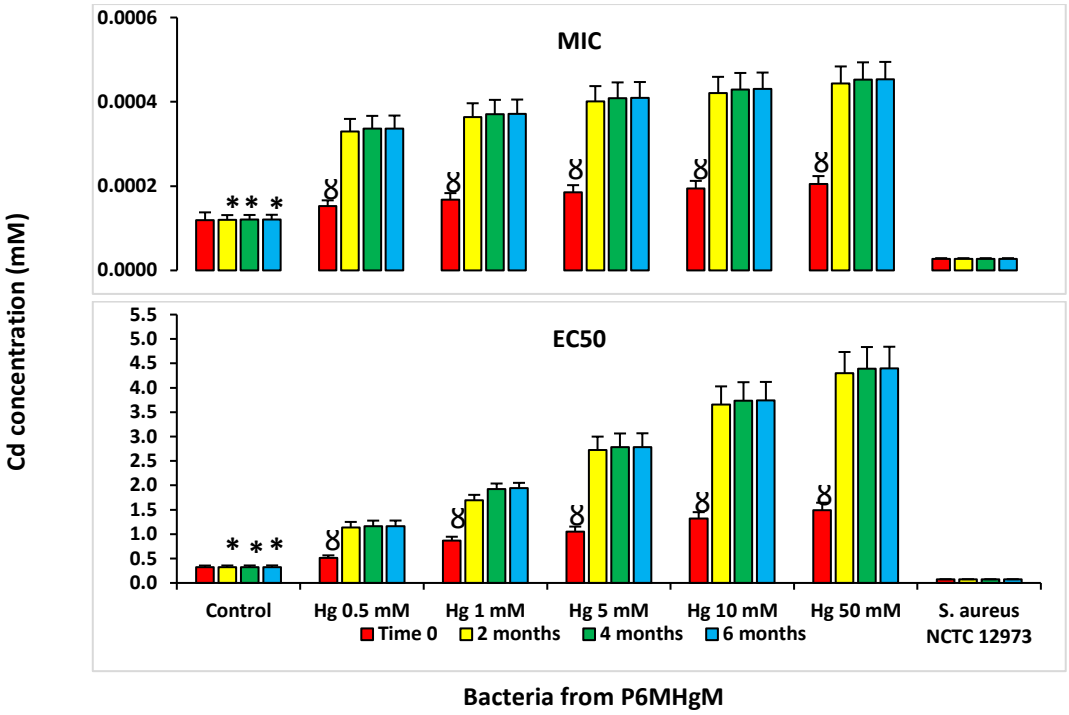

Fig. A3.34. Mean MIC and EC50 values of PICT analysis with Cd for bacteria from P6MHgM. *$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cd MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

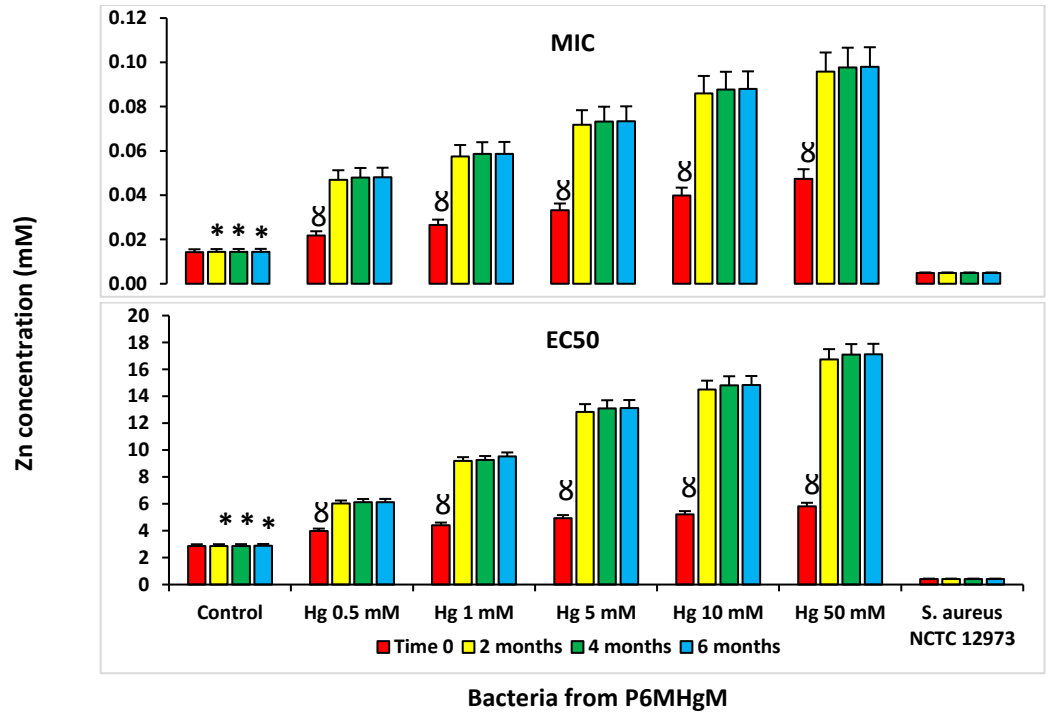

Fig. A3.35. Mean MIC and EC50 values of PICT analysis with Zn for bacteria from P6MHgM. *$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Zn MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

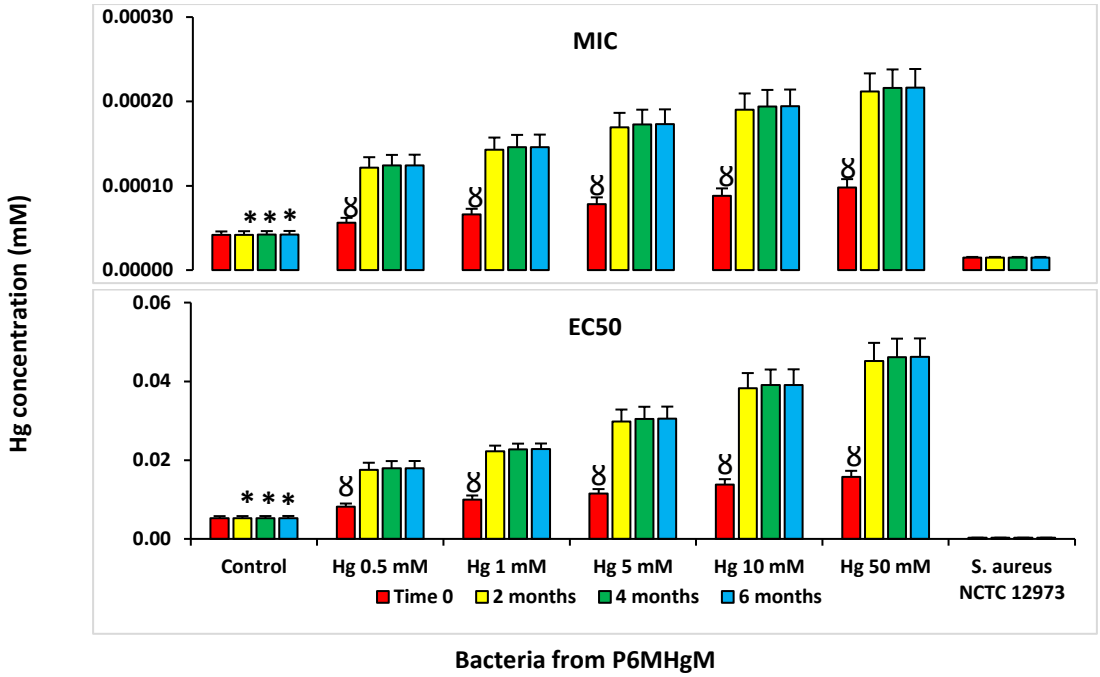

Fig. A3.36. Mean MIC and EC50 values of PICT analysis with Hg for bacteria from P6MHgM. *$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϐ$p < 0.05$ compared to Hg MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month.

Analysis Using Abs

There were significantly higher Abs MIC and EC50 values for bacteria from B6MZnM, B6MHgM, P6MZnM and P6MHgM compared to those for bacteria from control microcosm. There were lower Abs MIC and EC50 values for bacteria from B6MZnM, B6MHgM, P6MZnM and P6MHgM at Time 0 compared to those for bacteria from the same microcosms at 2 , 4 and 6 months intervals. According to EUCAST ECOFF recommendation, the MIC values determined for Cm, Ery, Cb and Amp for bacterial consortia isolates from B6MCdM, P6MCdM, B6MZnM, P6MZnM, B6MHgM and P6MHgM were greater than 20 µg mL^{-1} at timepoints 2, 4 and 6 months of incubation, although they were greater than 20 µg mL^{-1} at for pastoral soil-containing microcosms at Time 0 too (Figures 5.37, 5.38, 5.40 and 5.41; and Figures A2.60-A2.69 and A2.74-A2.83).

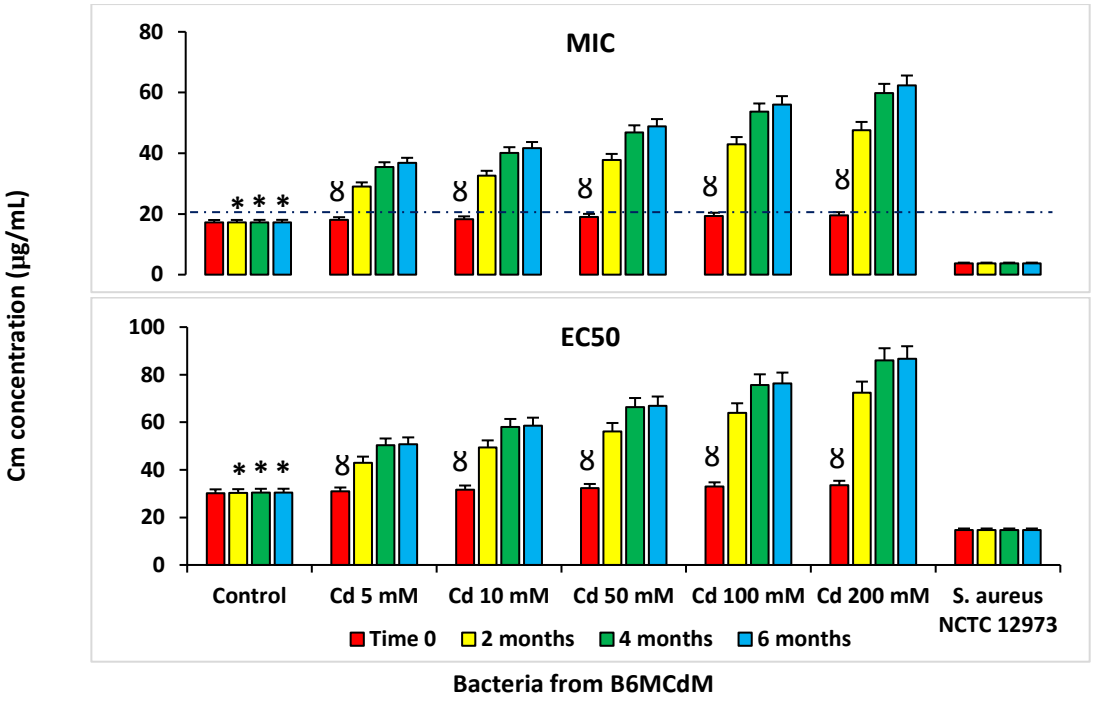

Fig. A3.37. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from B6MCdM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; 8$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

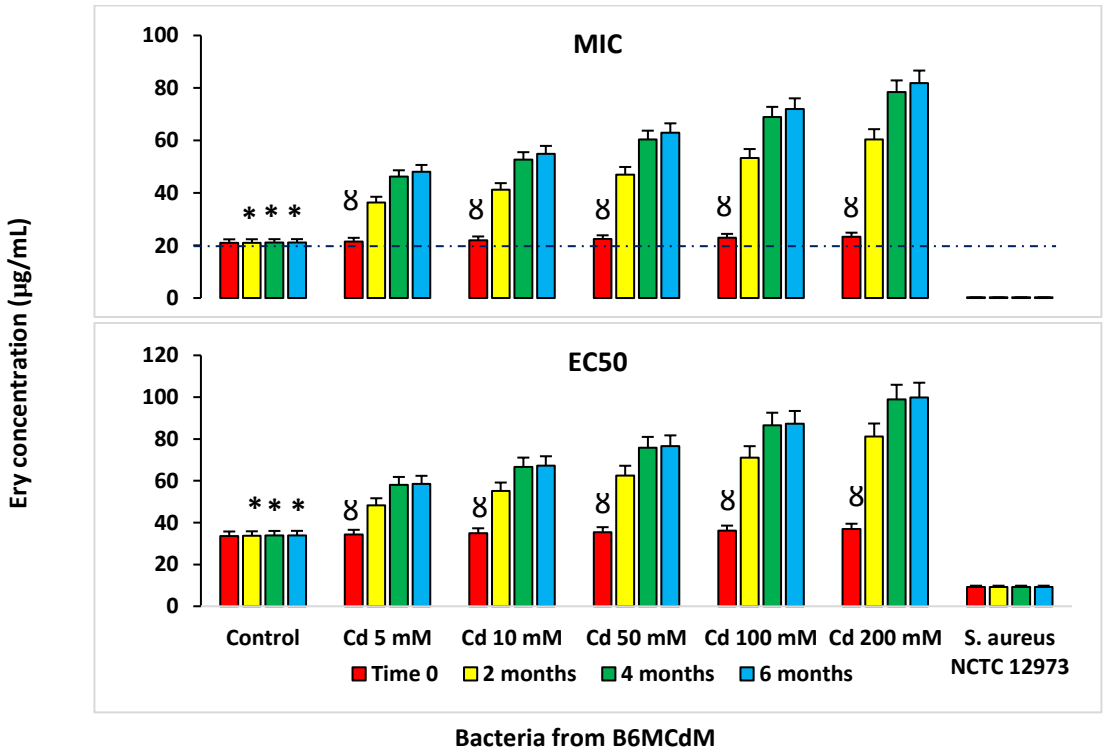

Fig. A3.38. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from B6MCdM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; 8$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

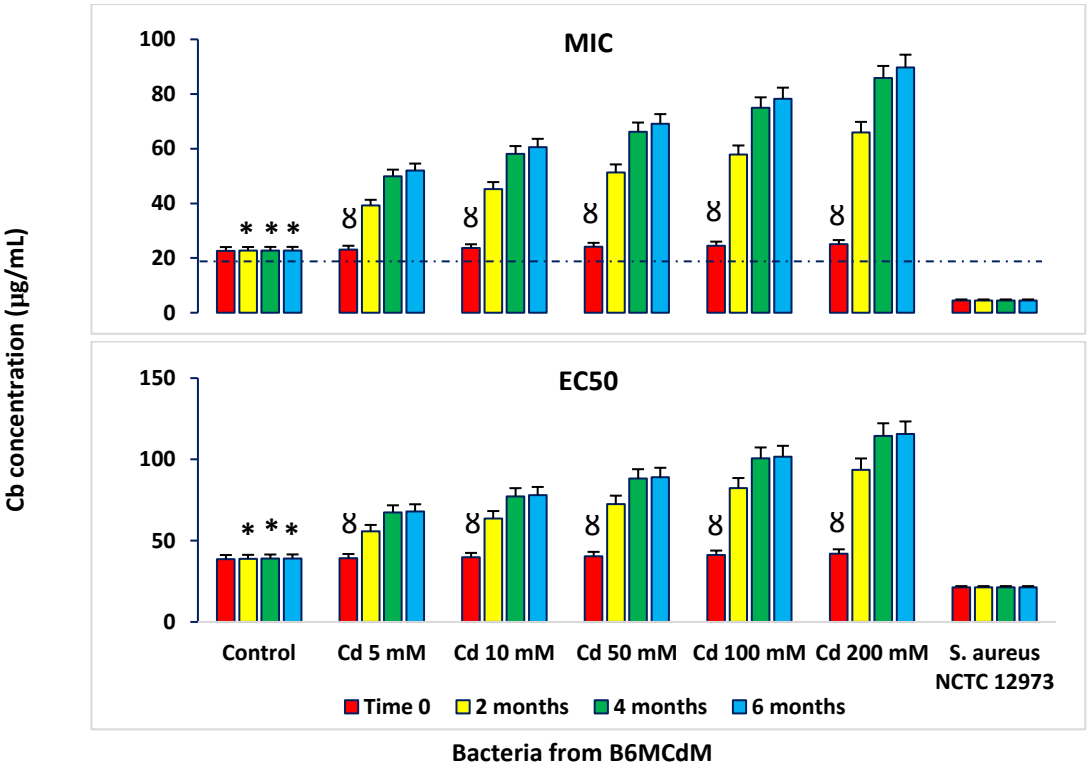

Fig. A3.39. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from B6MCdM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosmsat the same timepoint; 8$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

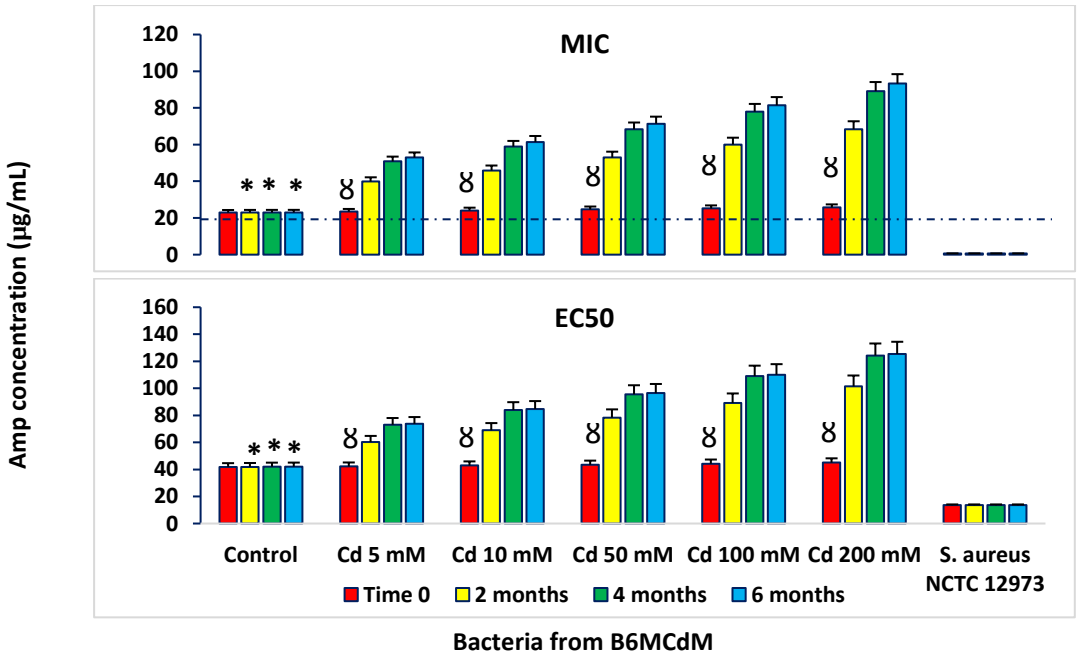

Fig. A3.40. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from B6MCdM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; 8$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

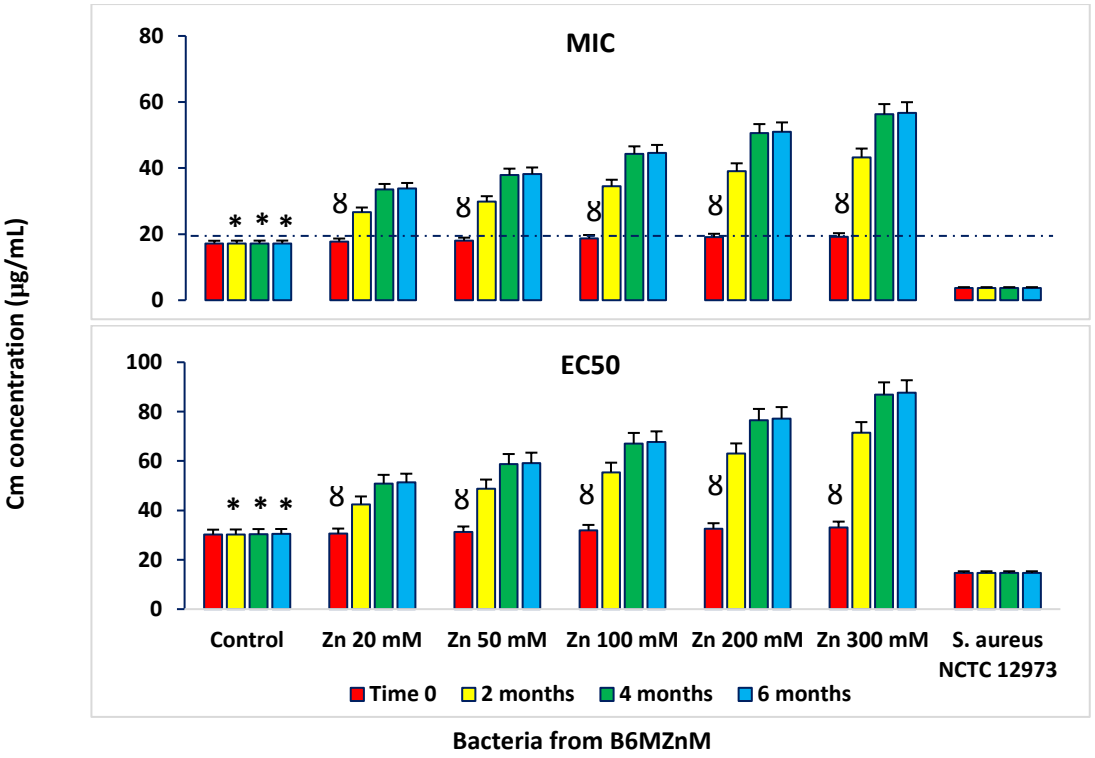

Fig. A3.41. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from B6MZnM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

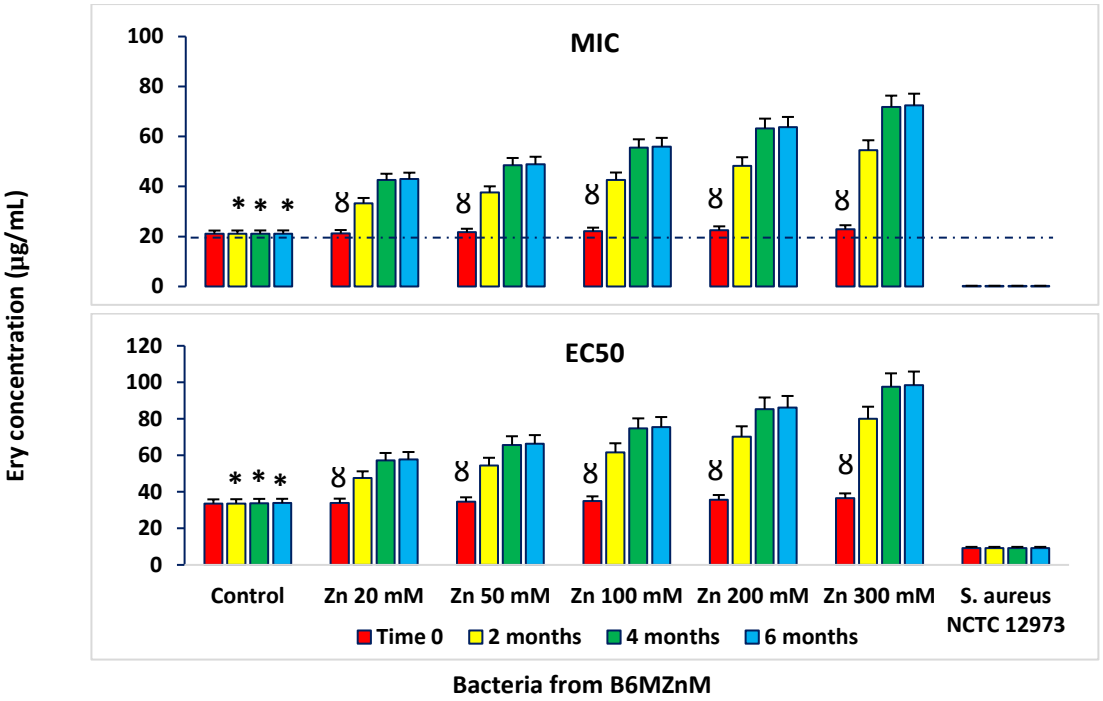

Fig. A3.42. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from B6MZnM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

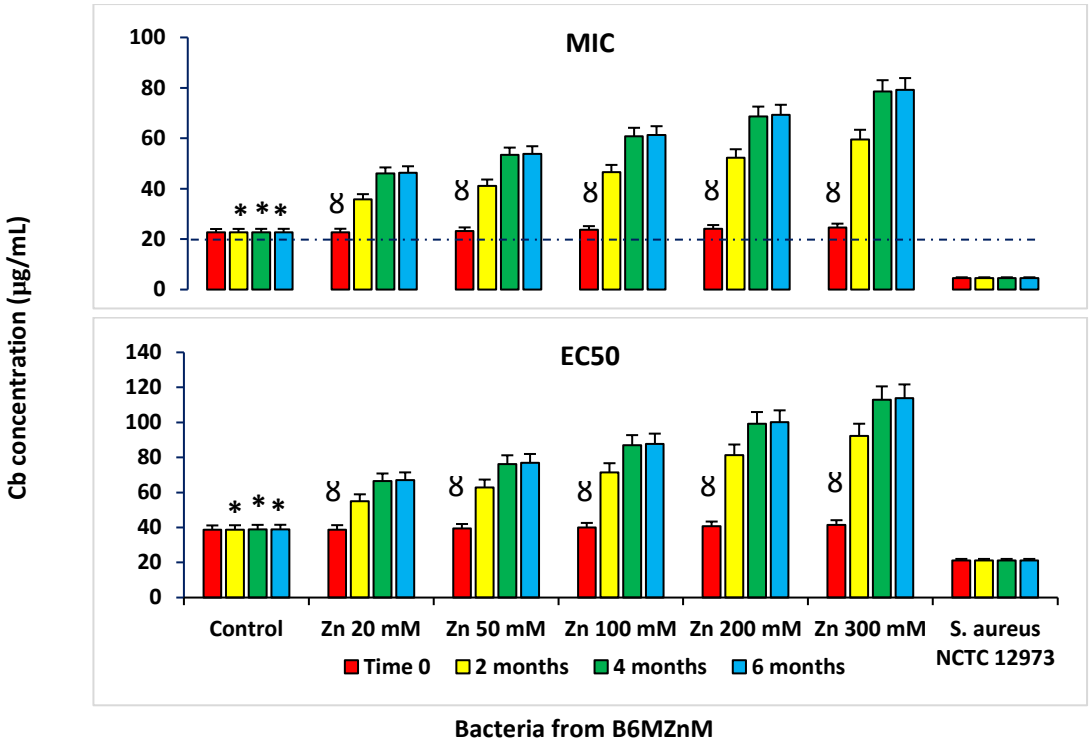

Fig. A3.43. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from B6MZnM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

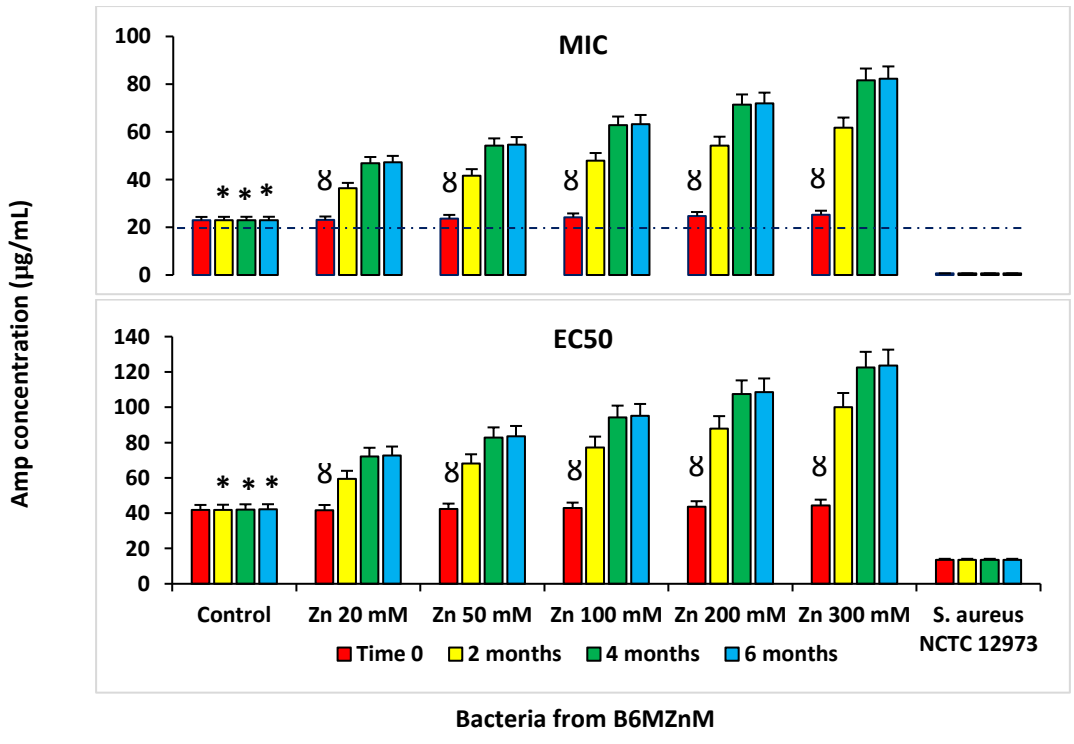

Fig. A3.44. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from B6MZnM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

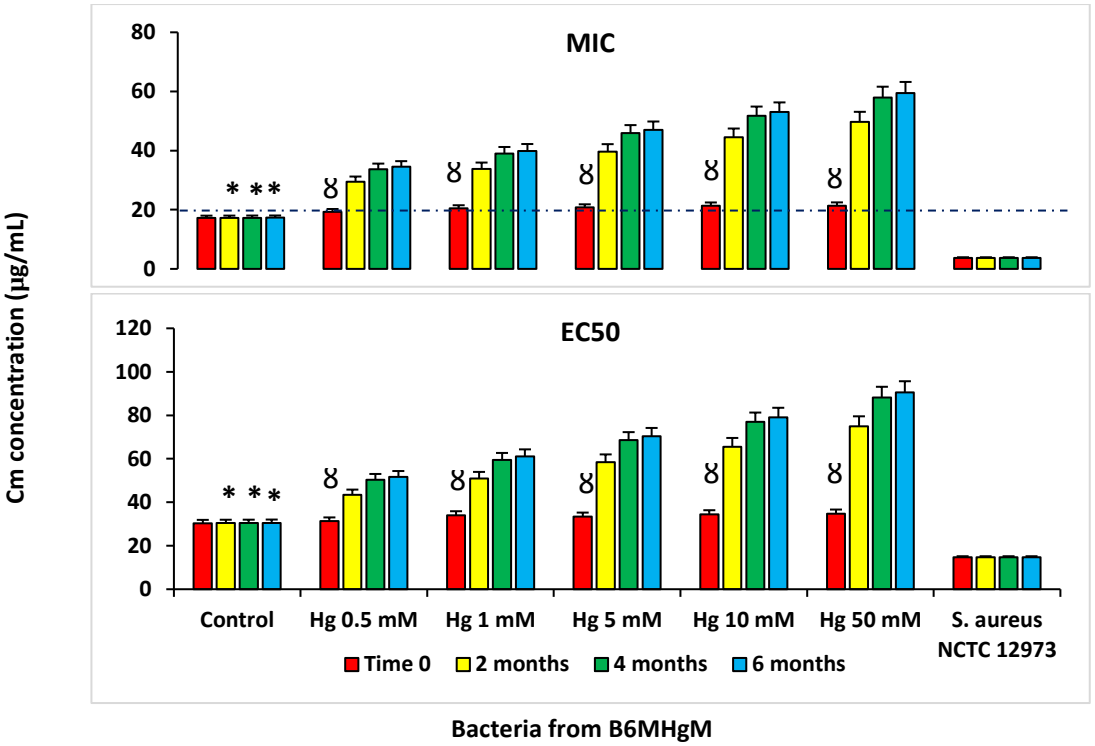

Fig. A3.45. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from B6MHgM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

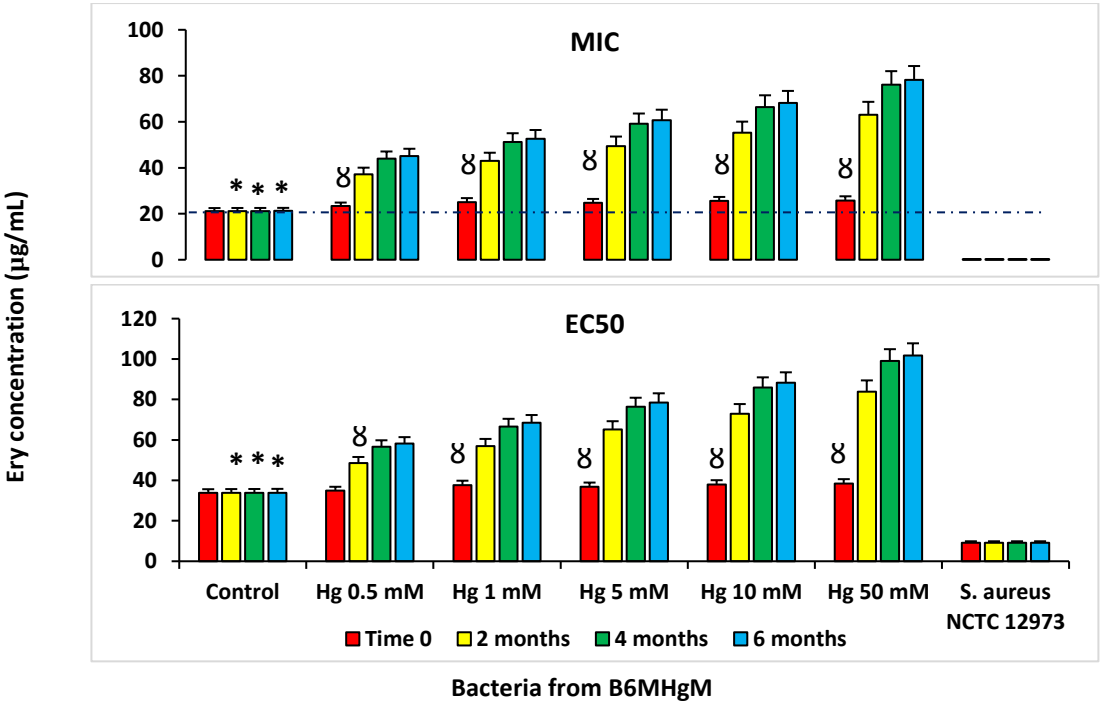

Fig. A3.46. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from B6MHgM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

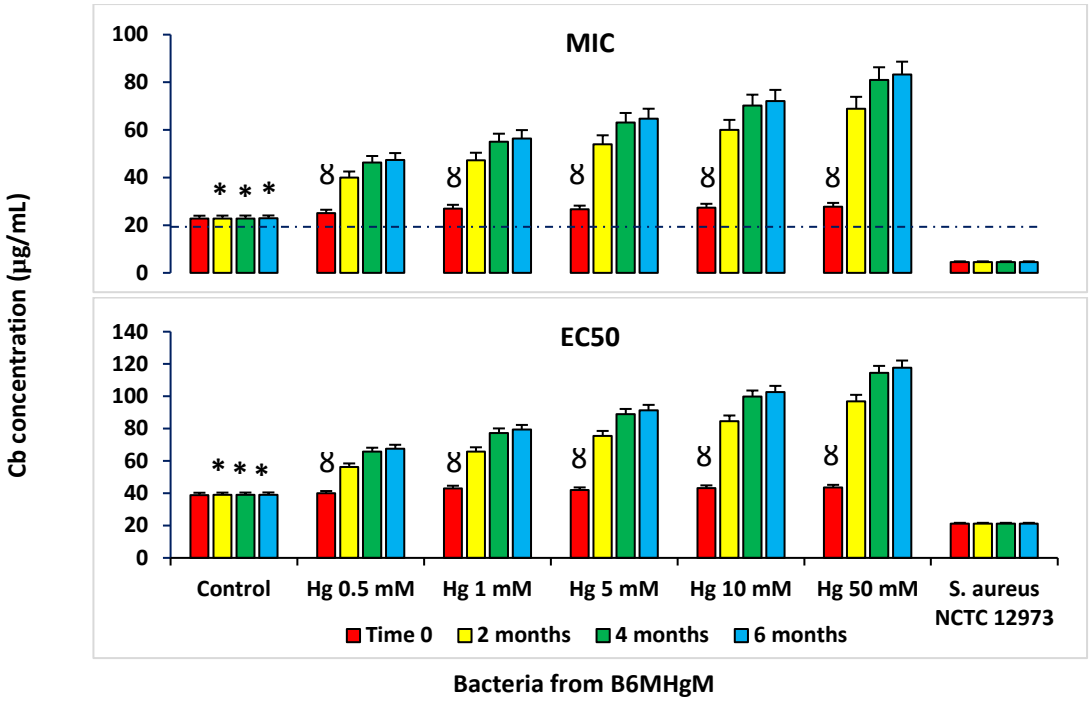

Fig. A3.47. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from B6MHgM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

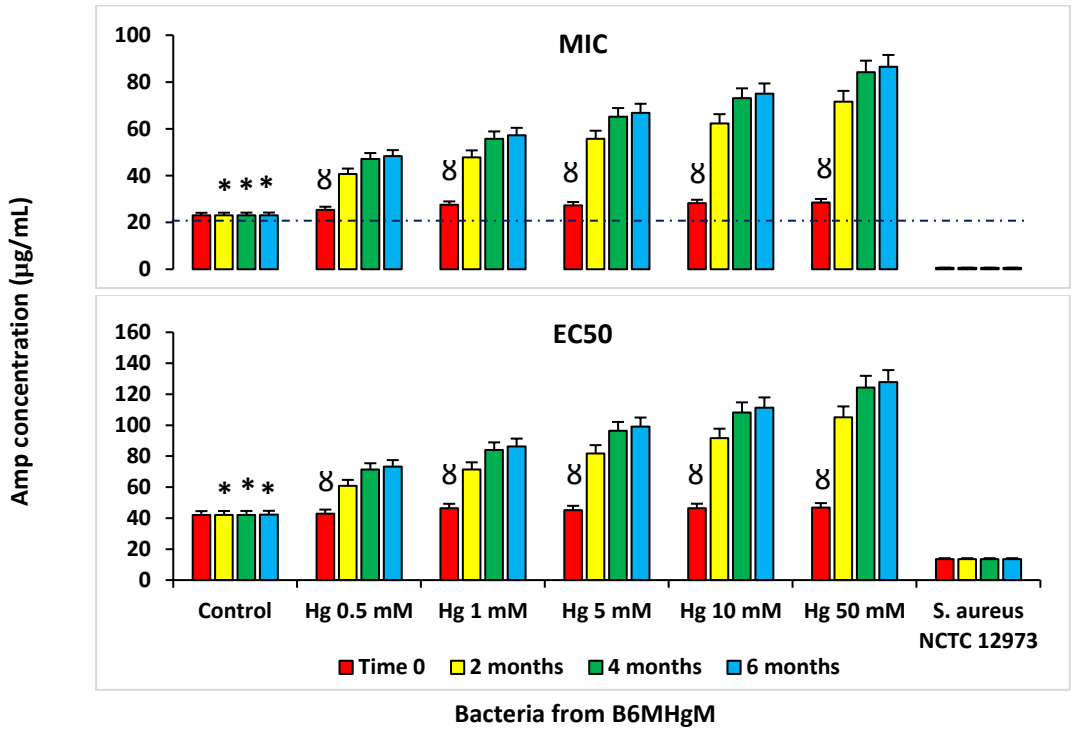

Fig. A3.48. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from B6MHgM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

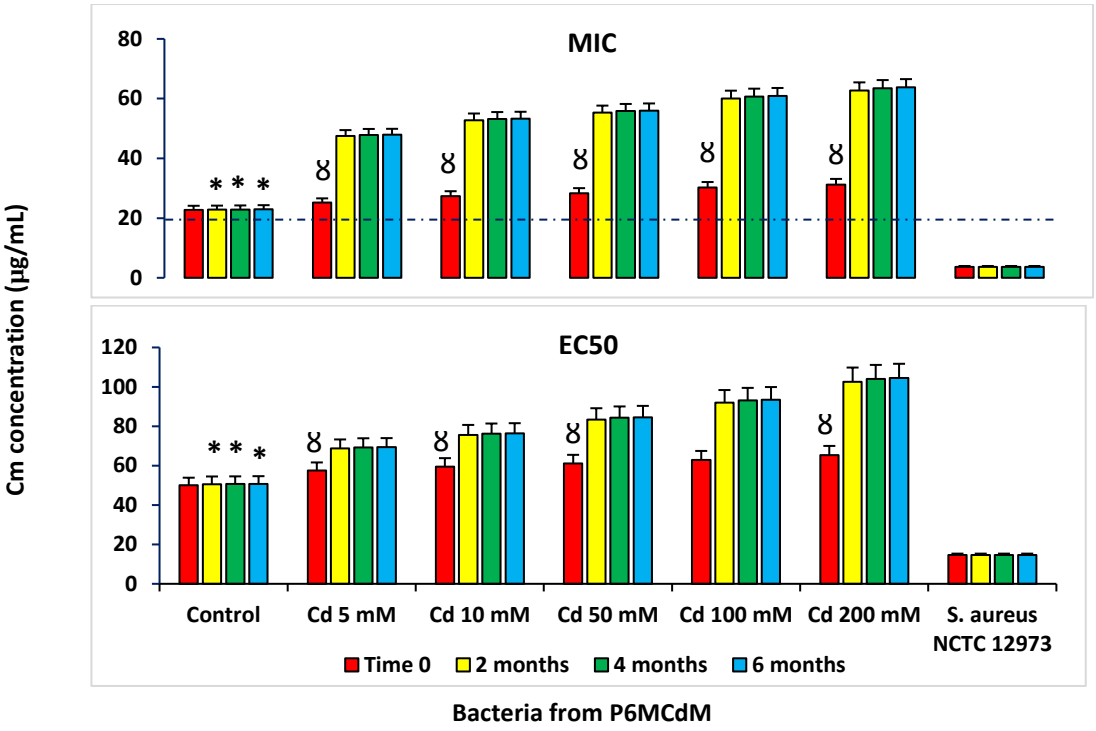

Fig. A3.49. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from P6MCdM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

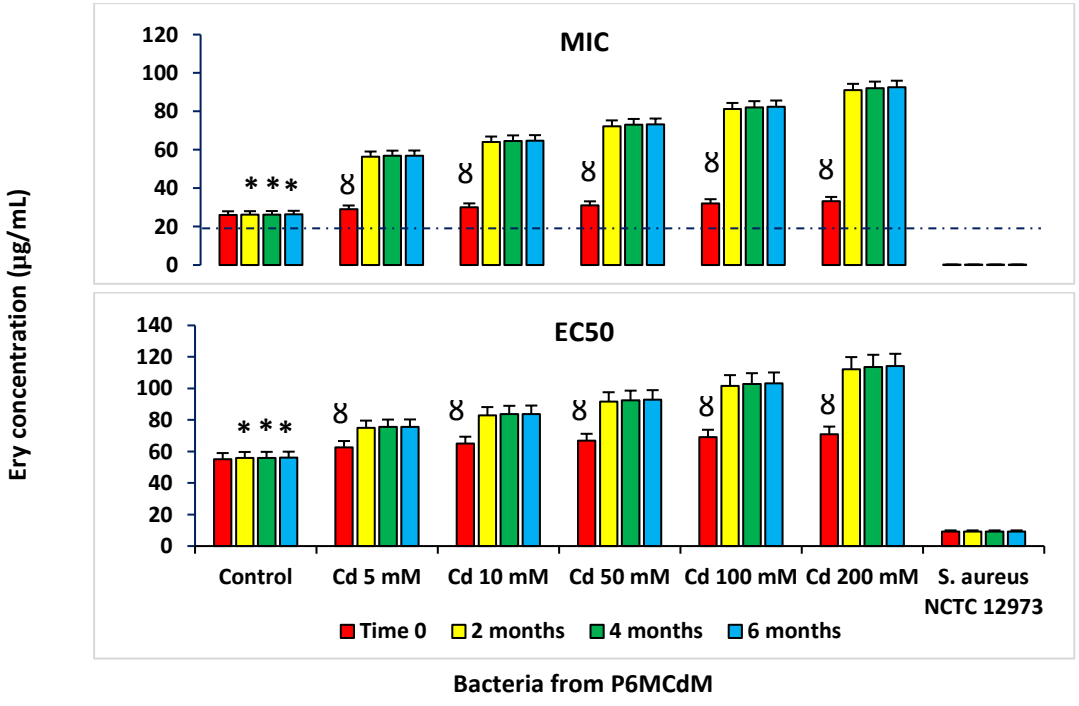

Fig. A3.50. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from P6MCdM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

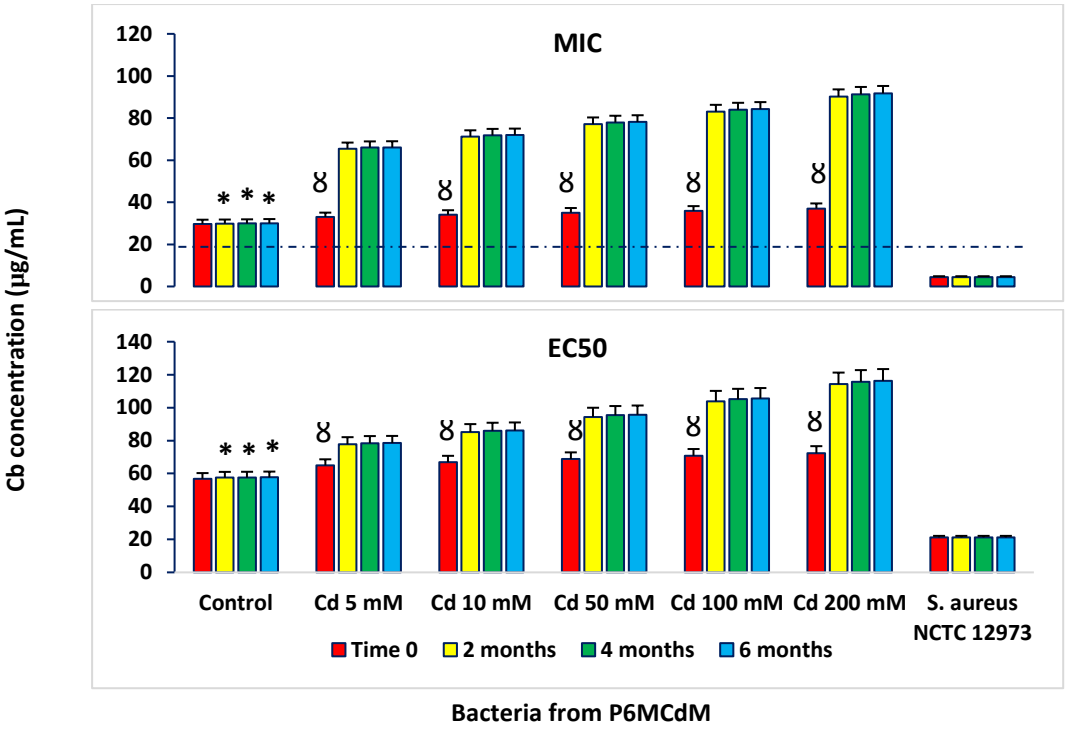

Fig. A3.51. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from P6MCdM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

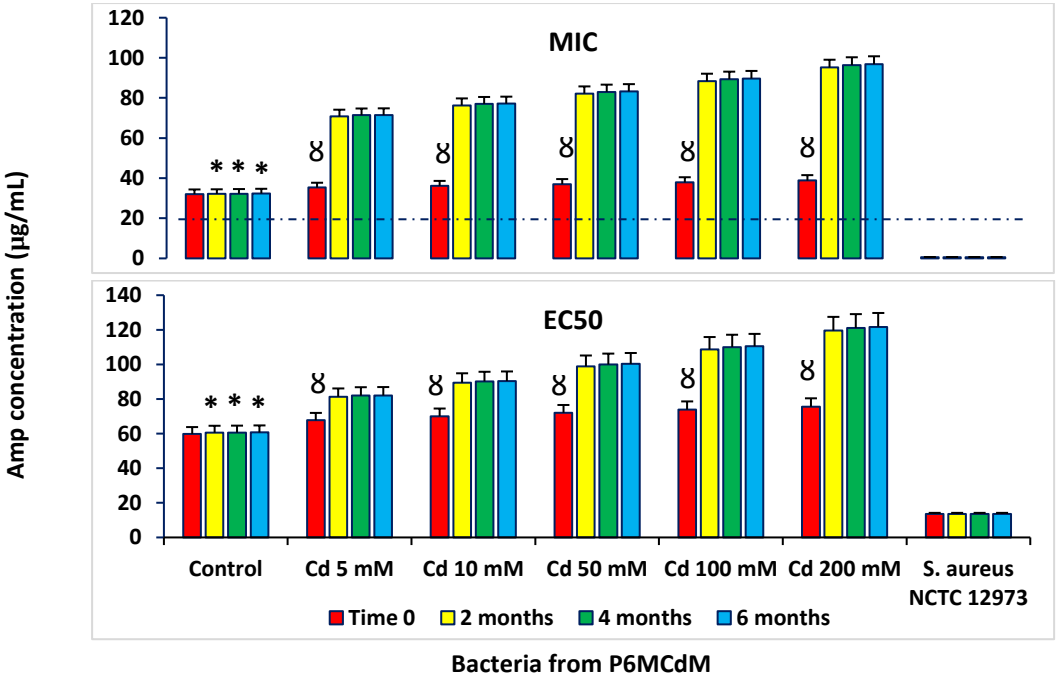

Fig. A3.52. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from P6MCdM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

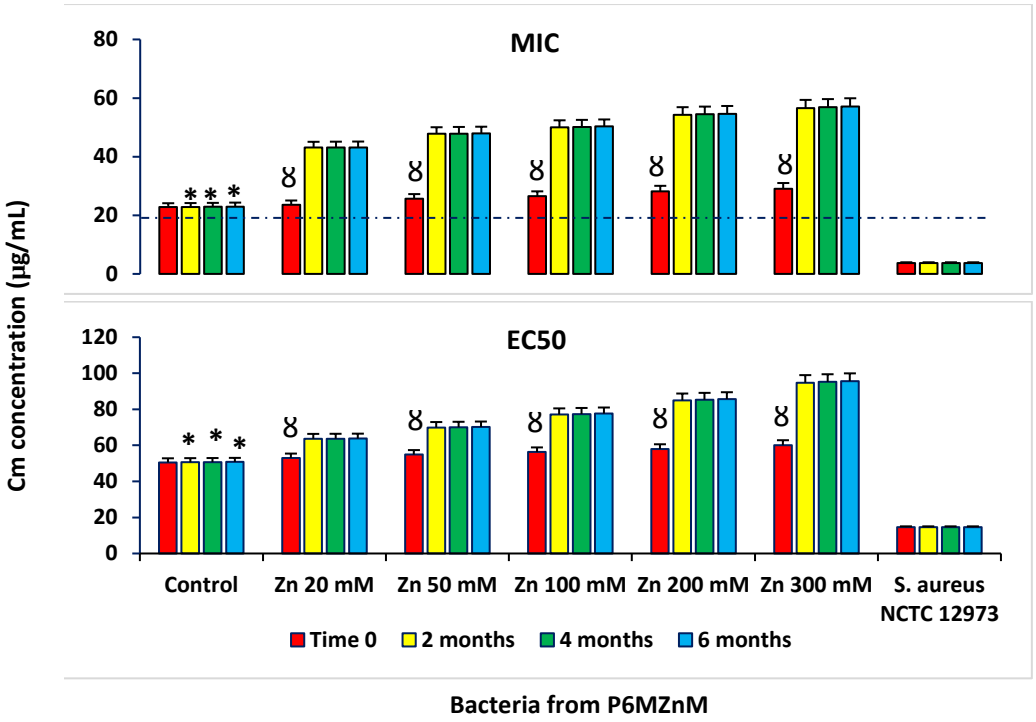

Fig. A3.53. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from P6MZnM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

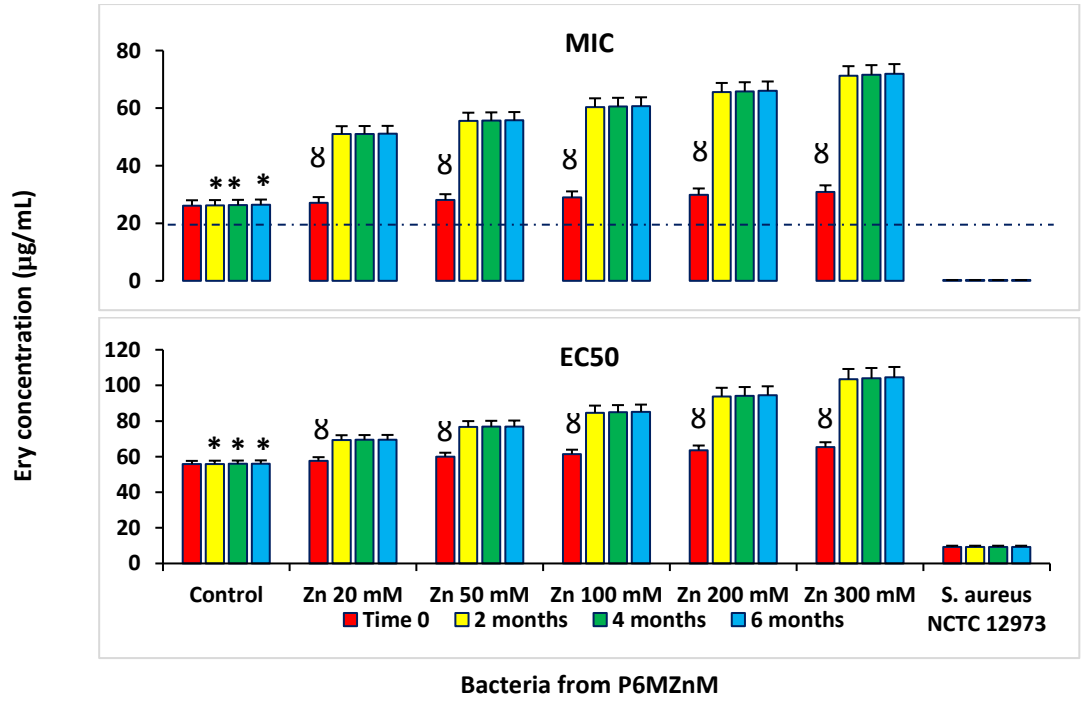

Fig. A3.54. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from P6MZnM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

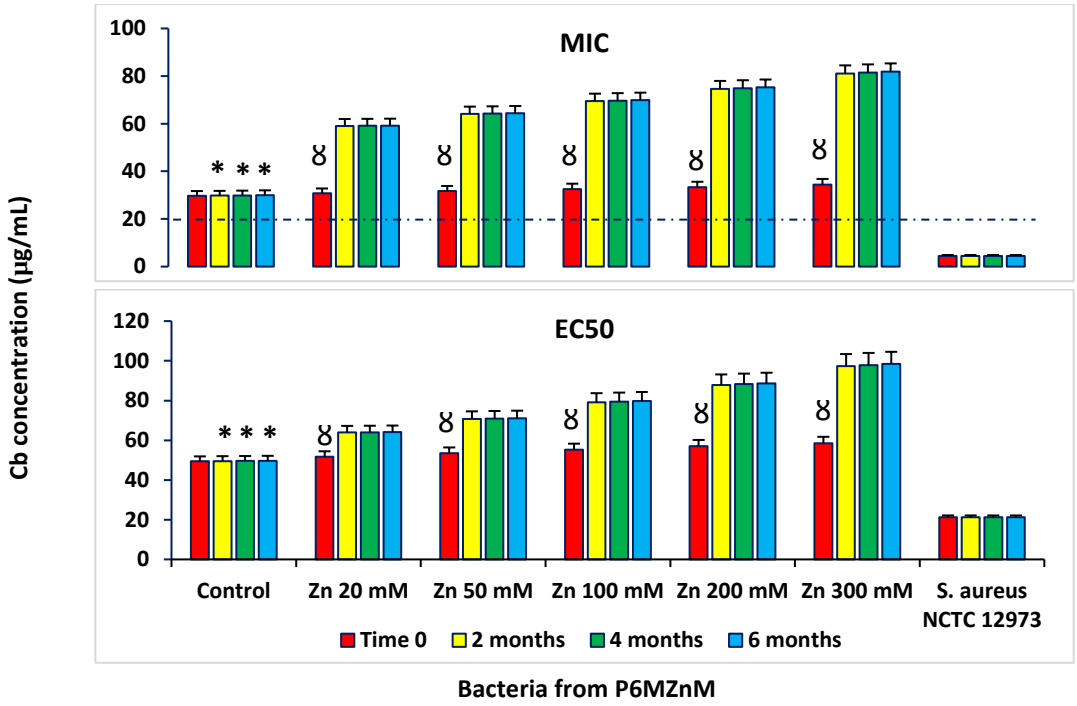

Fig. A3.55. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from P6MZnM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

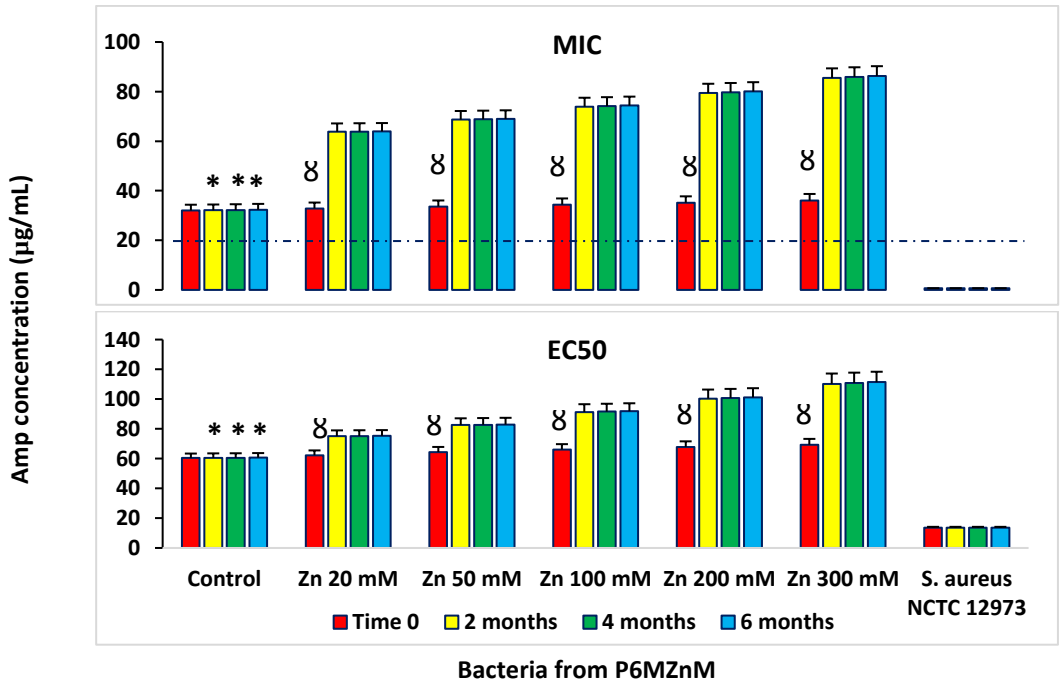

Fig. A3.56. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from P6MZnM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Zn-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

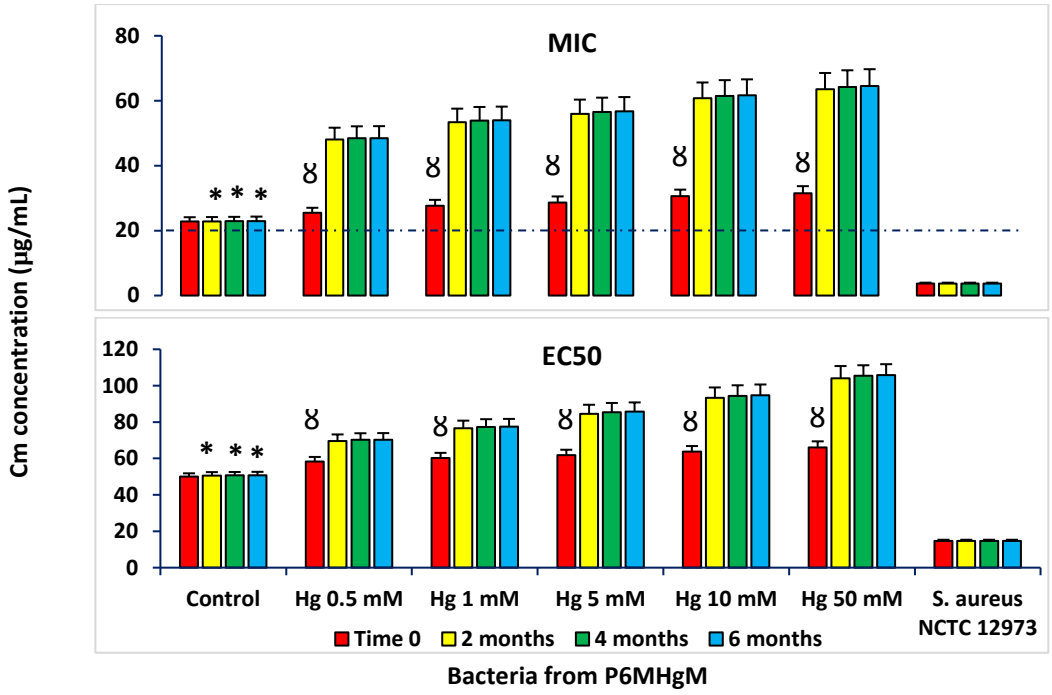

Fig. A3.57. Mean MIC and EC50 values of PICT analysis with Cm for bacteria from P6MHgM. *$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms; ϑ$p < 0.05$ compared to Cm MIC and EC50 values for bacteria from Cd-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

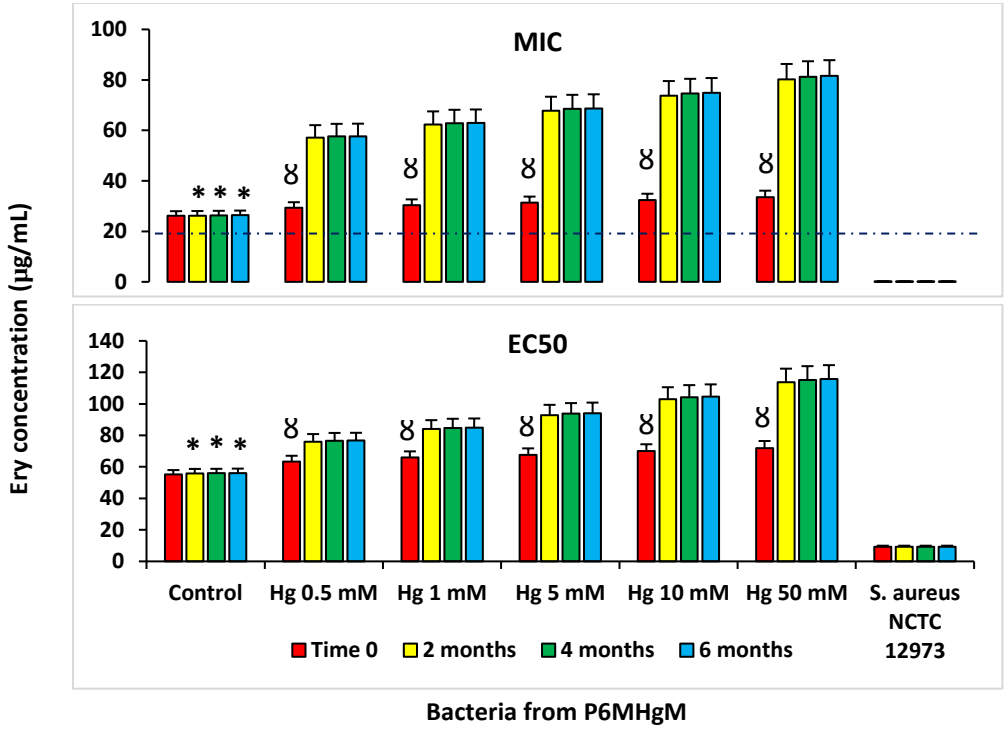

Fig. A3.58. Mean MIC and EC50 values of PICT analysis with Ery for bacteria from P6MHgM. *$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Ery MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

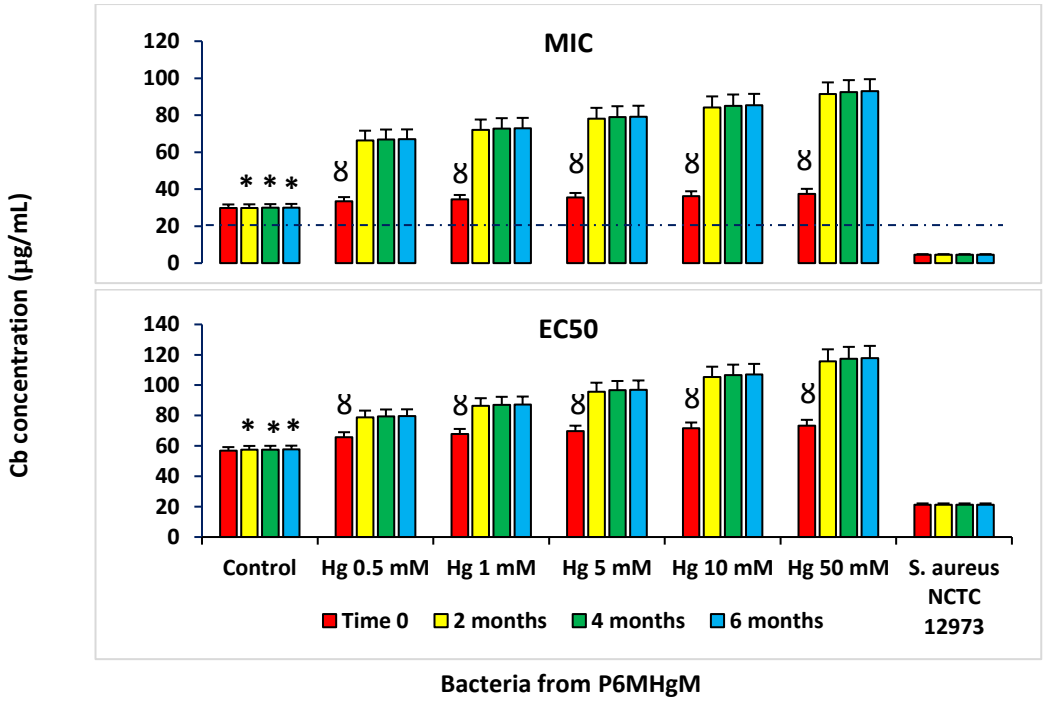

Fig. A3.59. Mean MIC and EC50 values of PICT analysis with Cb for bacteria from P6MHgM. *$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Cb MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

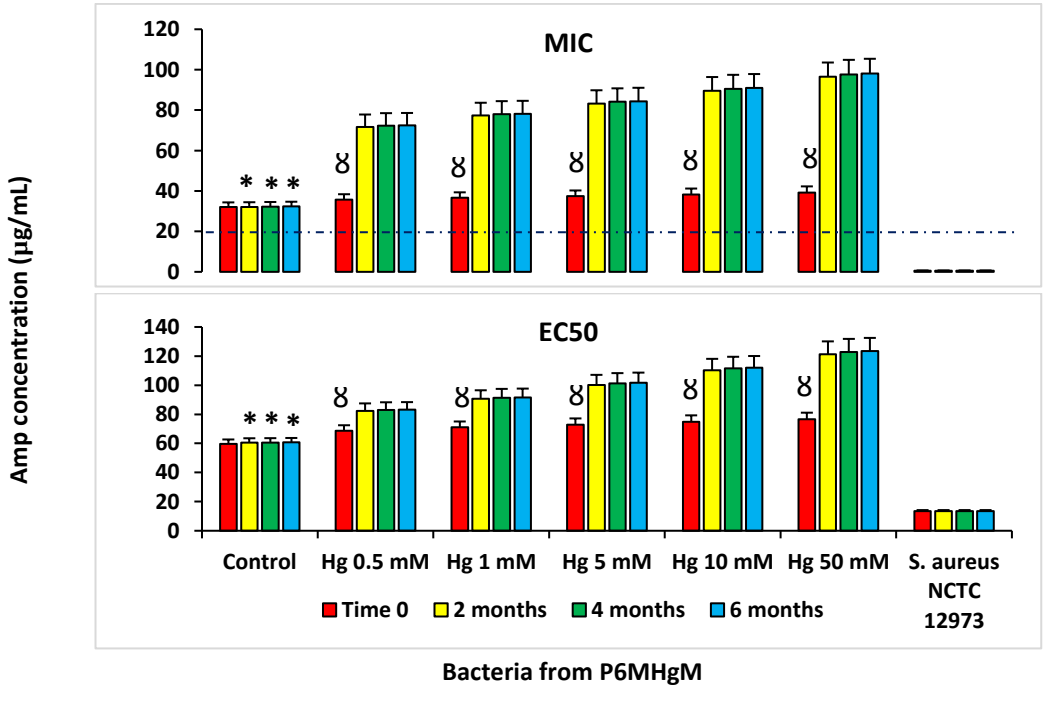

Fig. A3.60. Mean MIC and EC50 values of PICT analysis with Amp for bacteria from P6MHgM. *$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at the same timepoint; ϑ$p < 0.05$ compared to Amp MIC and EC50 values for bacteria from Hg-spiked microcosms at 2, 4 and 6 month. The dash line defines AbR level of soil bacteria.

A3.4. Broth Microdilution (BM)

A3.4.1. 6-weeks microcosms (6WM)

Analysis with Abs

As discussed in Section 5.1.6 for Tc MIC and EC50 for bacterial isolates from HMs-spiked 6WM, there were lower Abs MIC and EC50 values for Cm, Ery, Cb and Amp for the HM-sensitive bacterial isolates from HMs-spiked microcosms 6WM compared to those HMR isolates from the same microcosms. In addition, there were significantly higher Abs MIC and EC50 values for the HMR bacterial isolates from the HMs-spiked 6WM compared to those for the HMR bacterial isolates from the control microcosm ($p < 0.05$). Lower Abs MIC and EC50 values were determined for the HMR bacterial isolates from HMs-spiked 6WM at Time 0 compared to those for the bacteria from the same microcosms and after 6 weeks of incubation (Figures A3.61-A3.64).

A3.4.2. Background/Pasture soil-contained 6-month microcosms (B6MM/P6MM)

BM Analysis with HMs

As explained in Section 5.1.6 for Cd MIC and EC50 for bacterial isolates from HMs-spiked B6MM and P6MM, there were significantly lower MIC and EC50 of Zn and Hg for HM-sensitive bacterial isolates from HMs-spiked microcosms compared to those for the HMR isolates from the same microcosms ($p < 0.05$). There were lower MIC and EC50 values of Zn and Hg for the HMR isolates from control microcosms compared to those for HMR isolates from HMs-spiked microcosms. Result showed there were lower MIC and EC50 values of Zn and Hg for the HMR bacteria from HMs-spiked microcosms at Time 0 compared to those for the bacteria from the same microcosms and after 6 months of incubation (Figures A3.65-A3.68).

BM Analysis with Abs

Similar to Tc MIC and EC50 (Section 5.1.6), result showed there were significantly lower MIC and EC50 values of Cm, Ery, Cb and Amp for the HM-sensitive bacterial isolates from HMs-spiked

B6MM/P6MM compared to those for the HMR isolates from the same microcosms ($p < 0.05$). There were lower Abs MIC and EC50 values for HMR bacteria from control microcosms compared to those for the HMR bacteria from HMs-spiked microcosms. Lower Abs MIC and EC50 values for HMR bacteria from HM-spiked B6MM/P6MM at Time 0 compared to those for the HMR isolates from the same microcosms after 6 months of incubation were determined (Figures A3.69-A3.76).

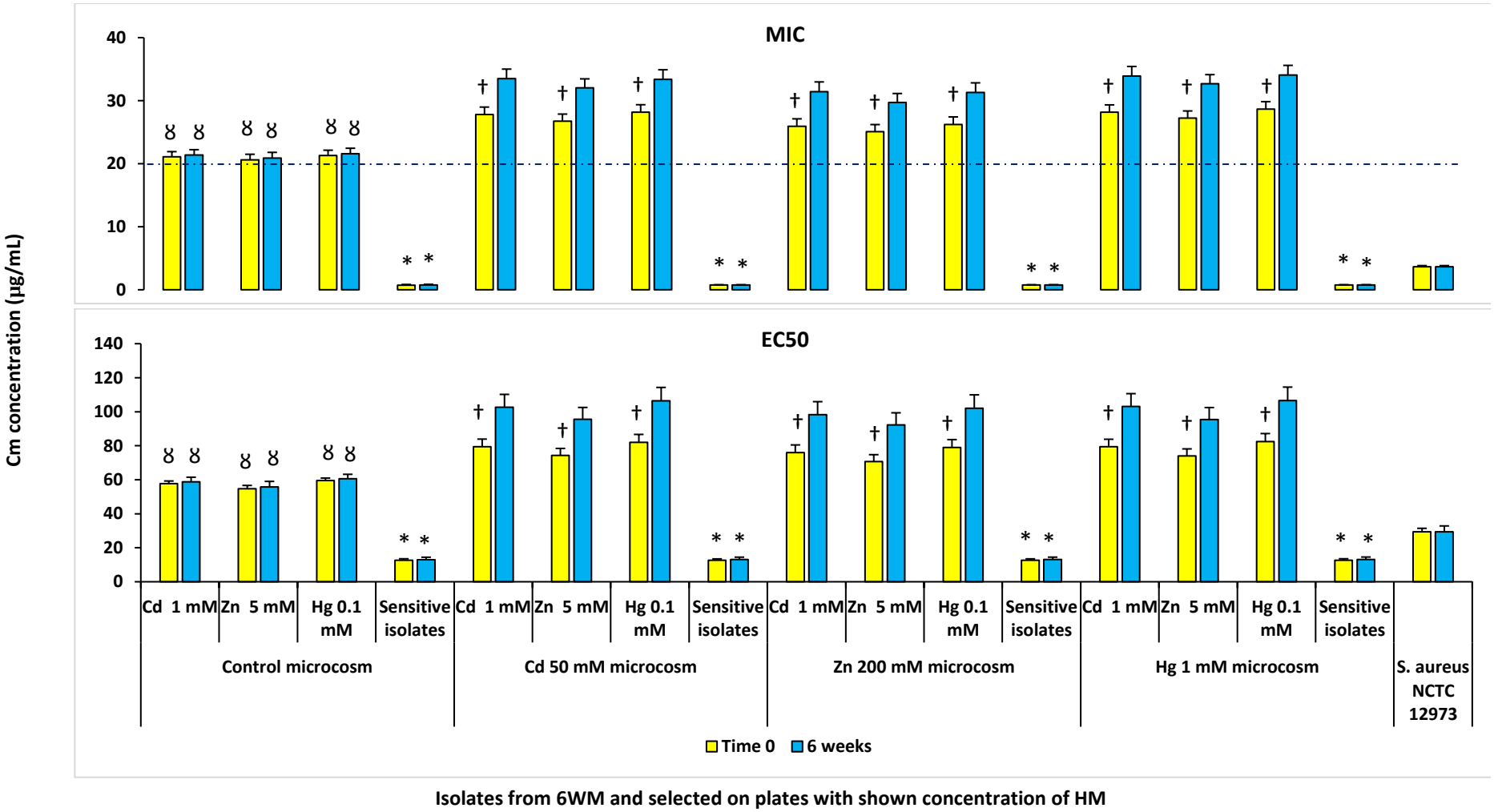

Fig. A3.61. Mean MIC and EC50 values of BM analysis with Cm for HMR isolates from 6WM. *$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation. The dash line defines AbR level of soil bacteria.

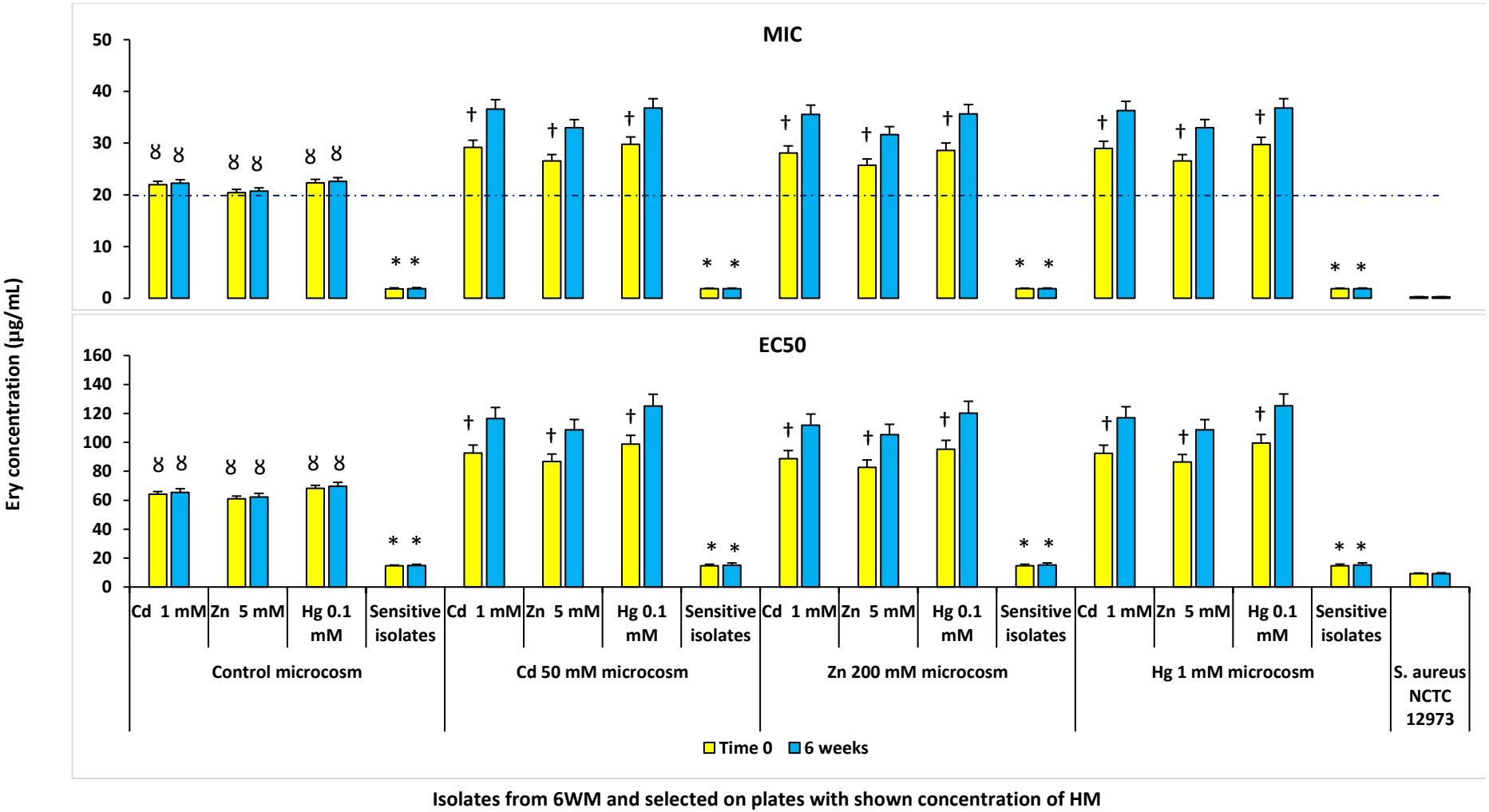

Fig. A3.62. Mean MIC and EC50 values of BM analysis with Ery for HMR isolates from 6WM. *$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation. The dash line defines AbR level of soil bacteria.

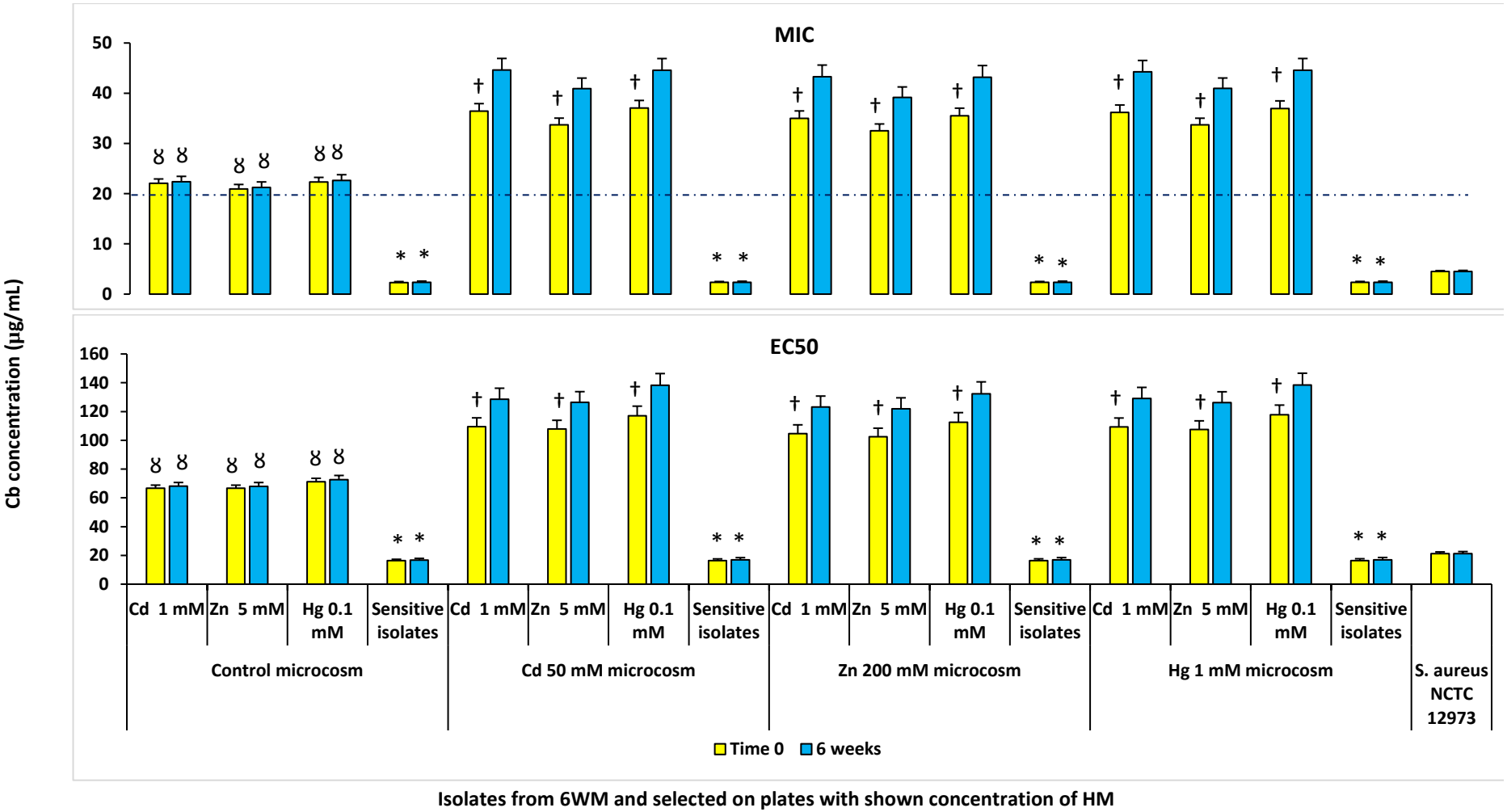

Fig. A3.63. Mean MIC and EC50 values of BM analysis with Cb for HMR isolates from 6WM. *$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation. The dash line defines AbR level of soil bacteria.

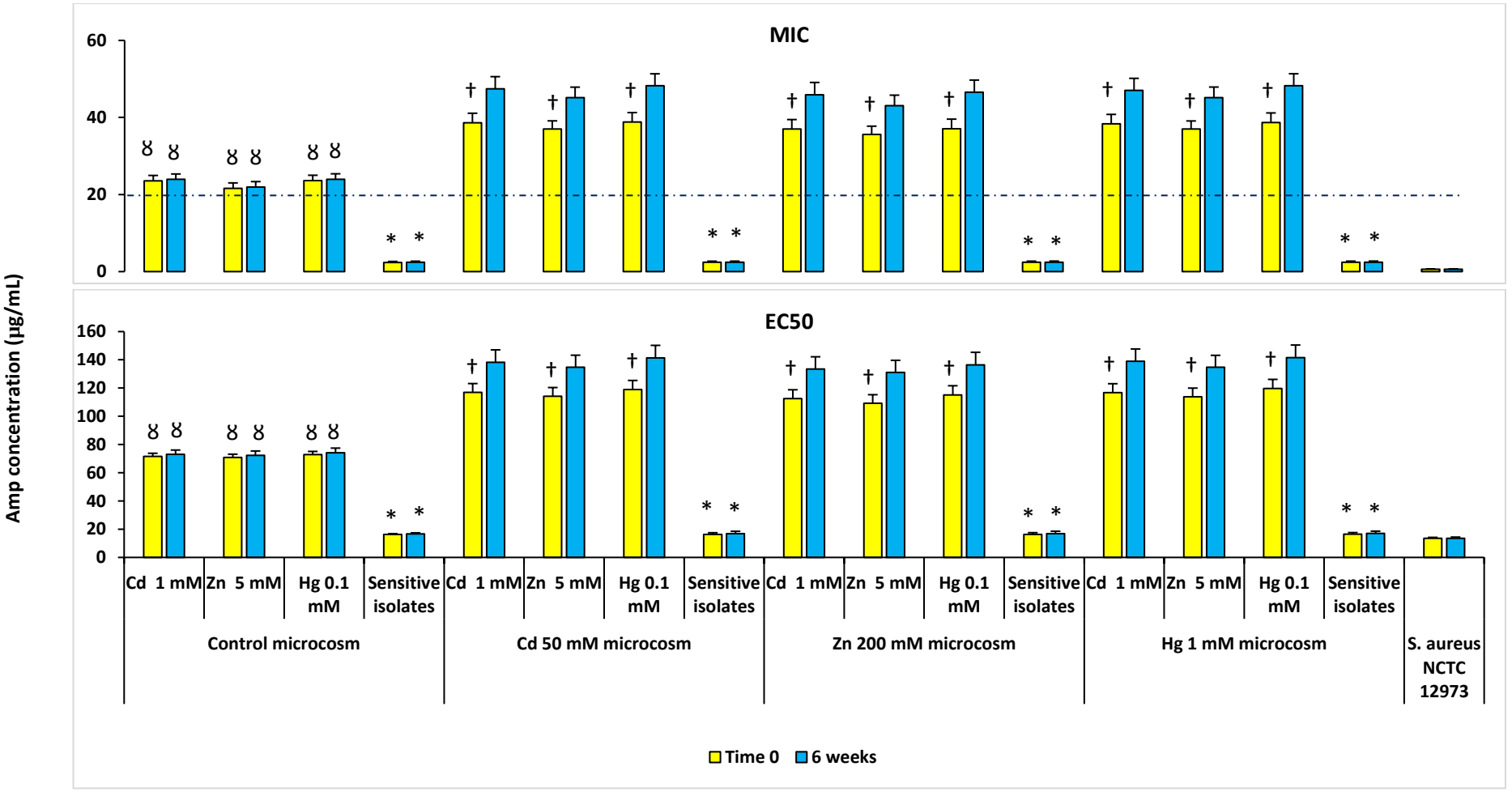

Fig. A3.64. Mean MIC and EC50 values of BM analysis with Amp for HMR isolates from 6WM. *$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm's soil; ȣ$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm after 6 weeks of incubation. The dash line defines AbR level of soil bacteria.

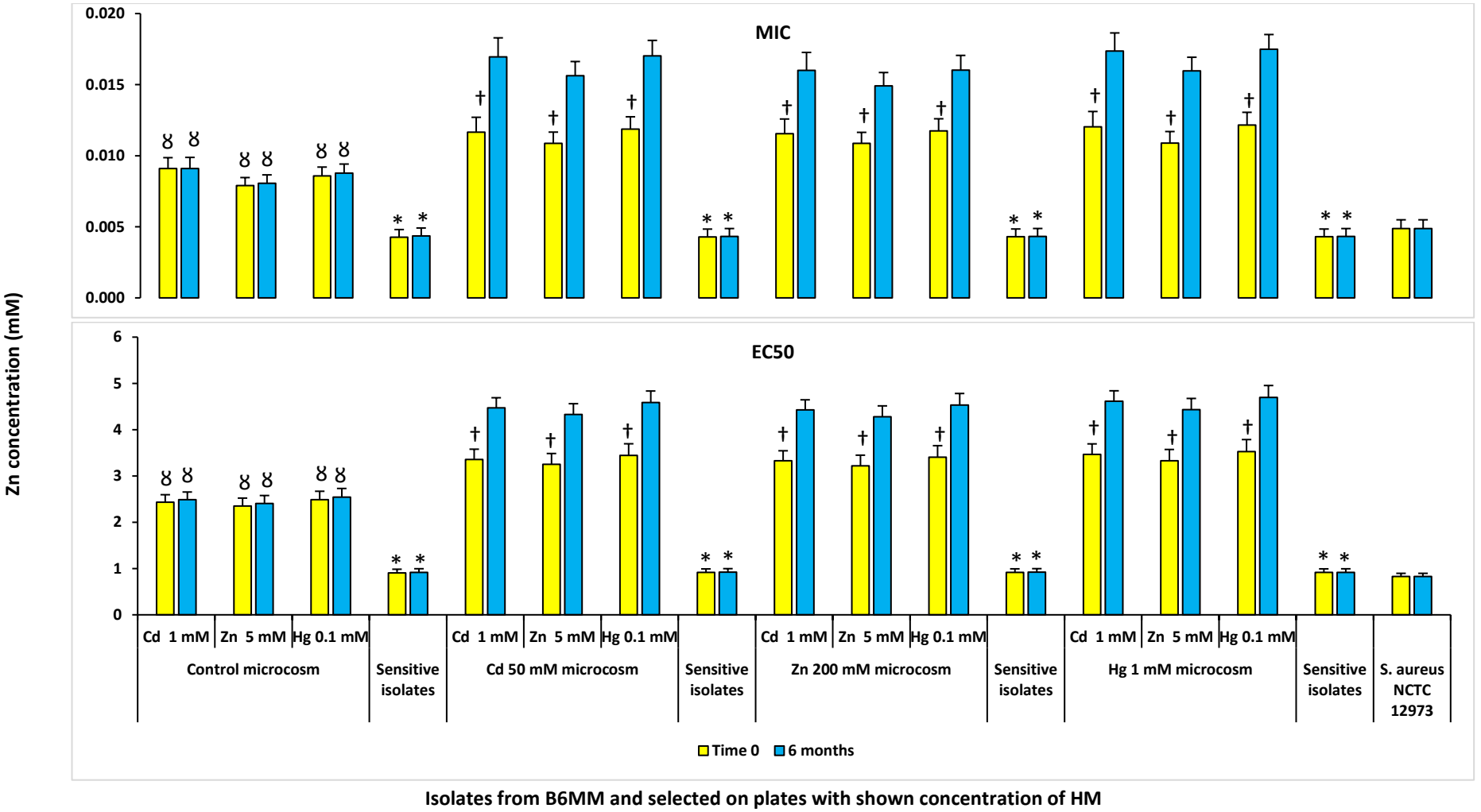

Fig. A3.65. Mean MIC and EC50 values of BM analysis with Zn for HMR isolates from B6MM. *$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

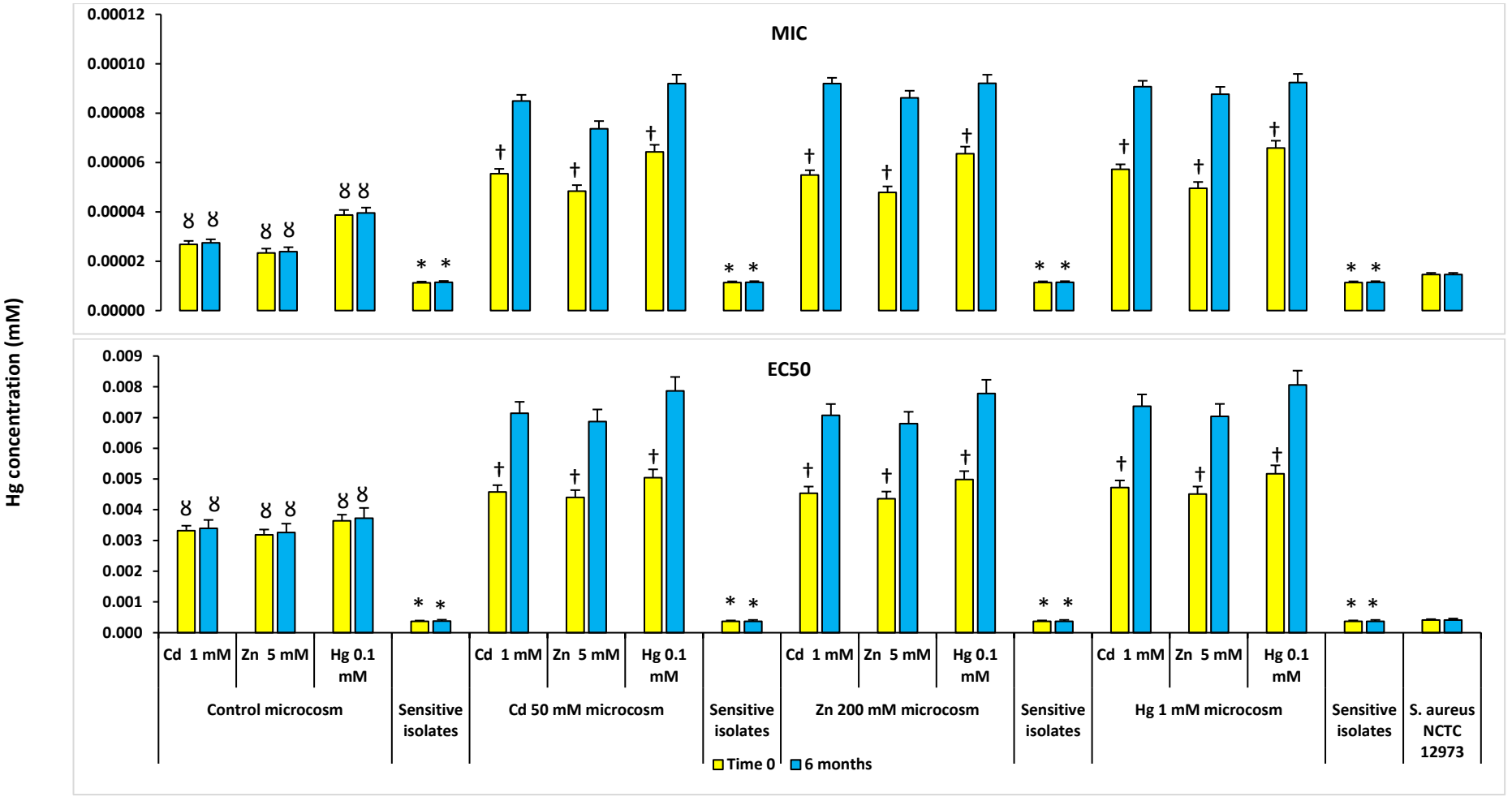

Fig. A3.66. Mean MIC and EC50 values of BM analysis with Hg for HMR isolates from B6MM. *$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

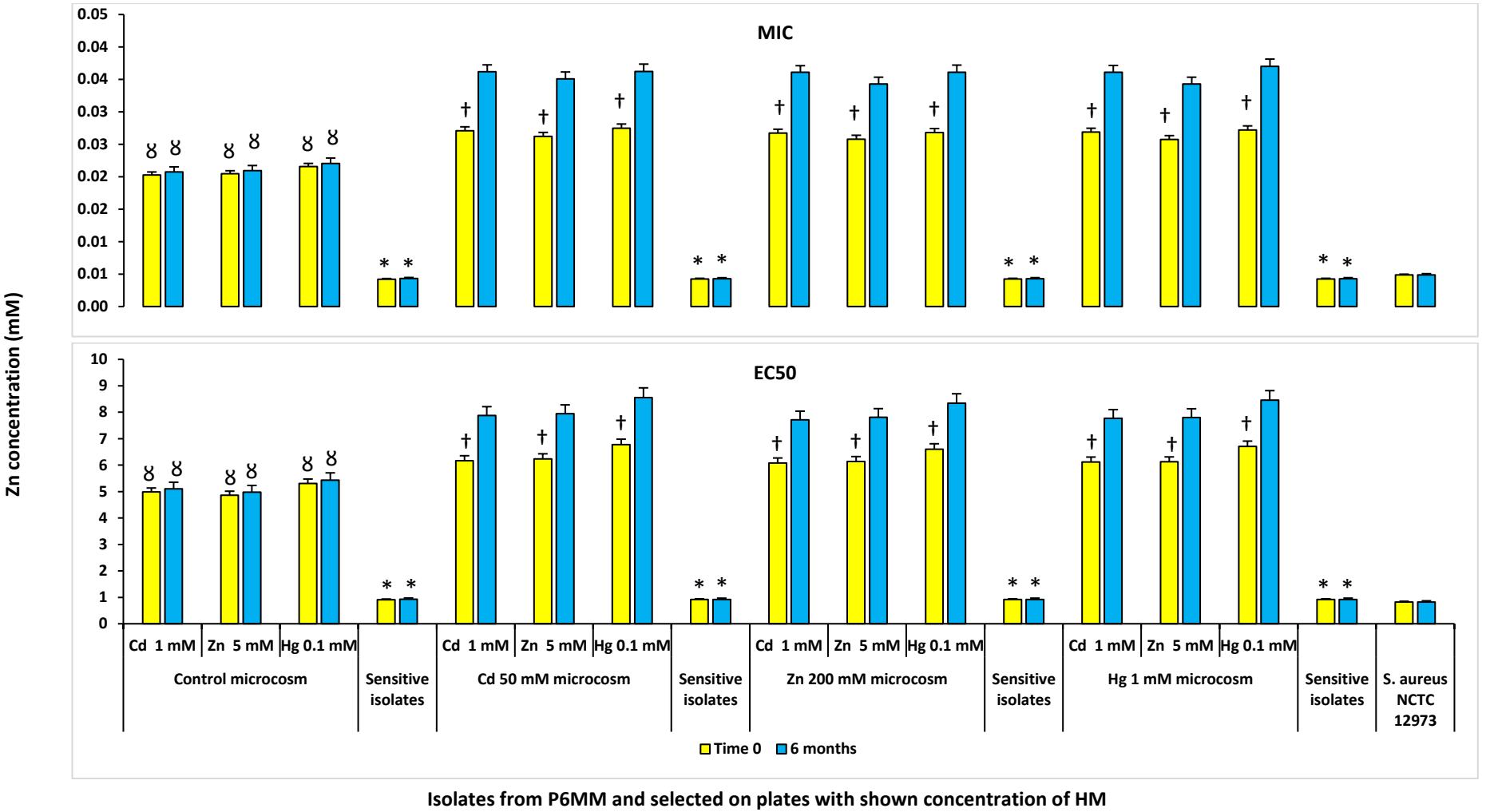

Fig. A3.67. Mean MIC and EC50 values of BM analysis with Zn for HMR isolates from P6MM. *$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Zn MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

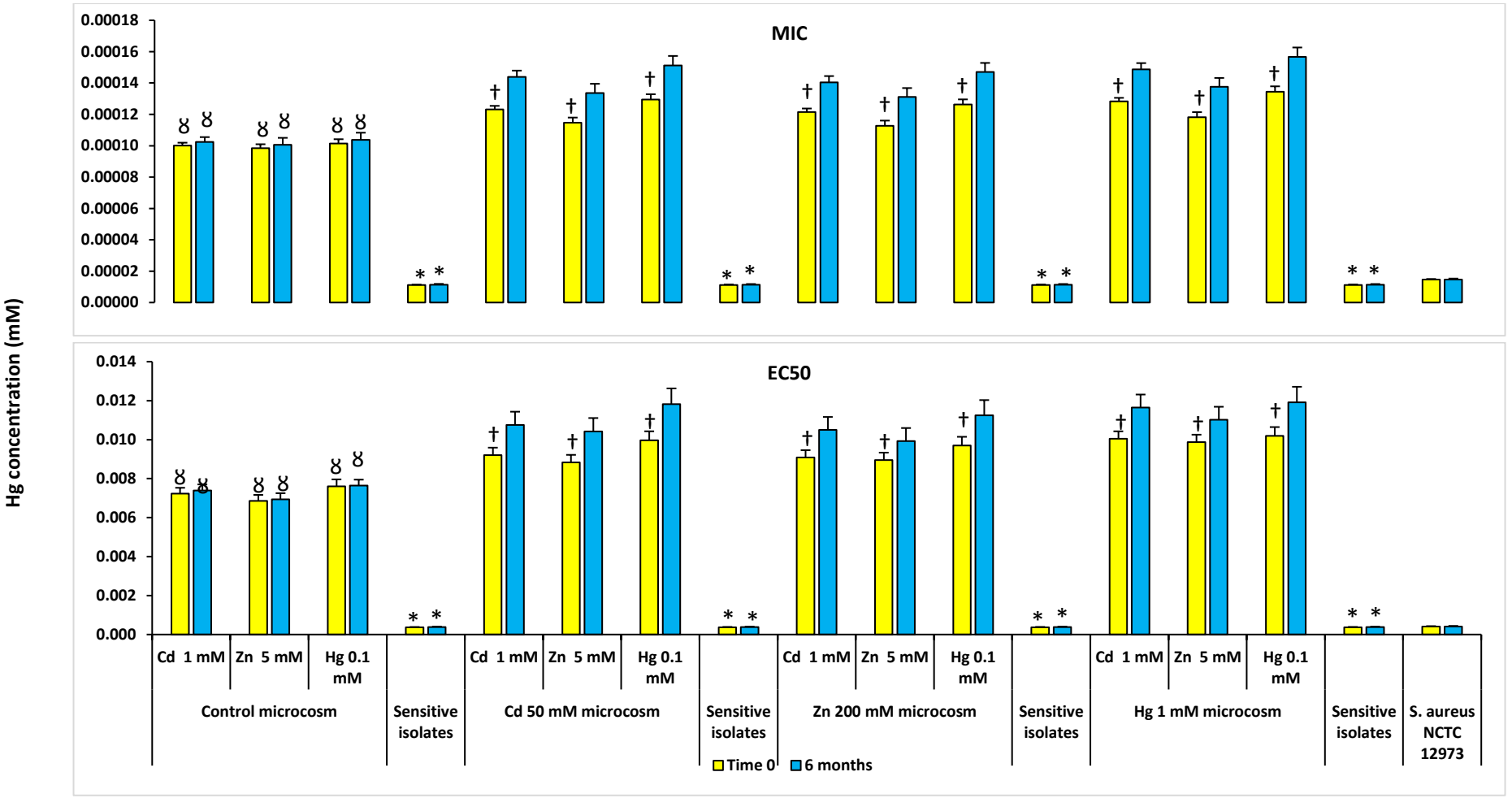

Fig. A3.68. Mean MIC and EC50 values of BM analysis with Hg for HMR isolates from P6MM. *$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Hg MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation.

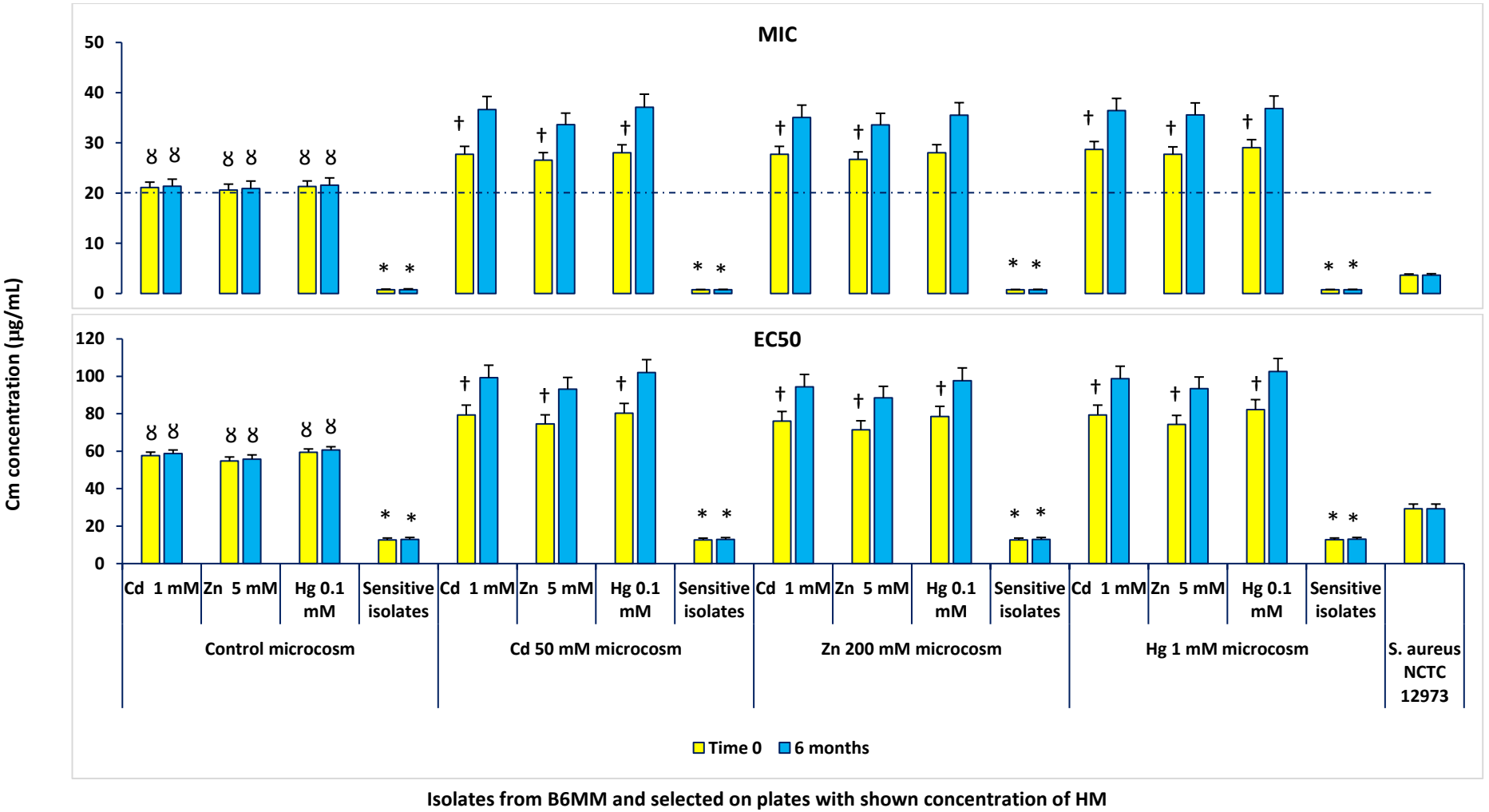

Fig. A3.69. Mean MIC and EC50 values of BM analysis with Cm for HMR isolates from B6MM. *$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

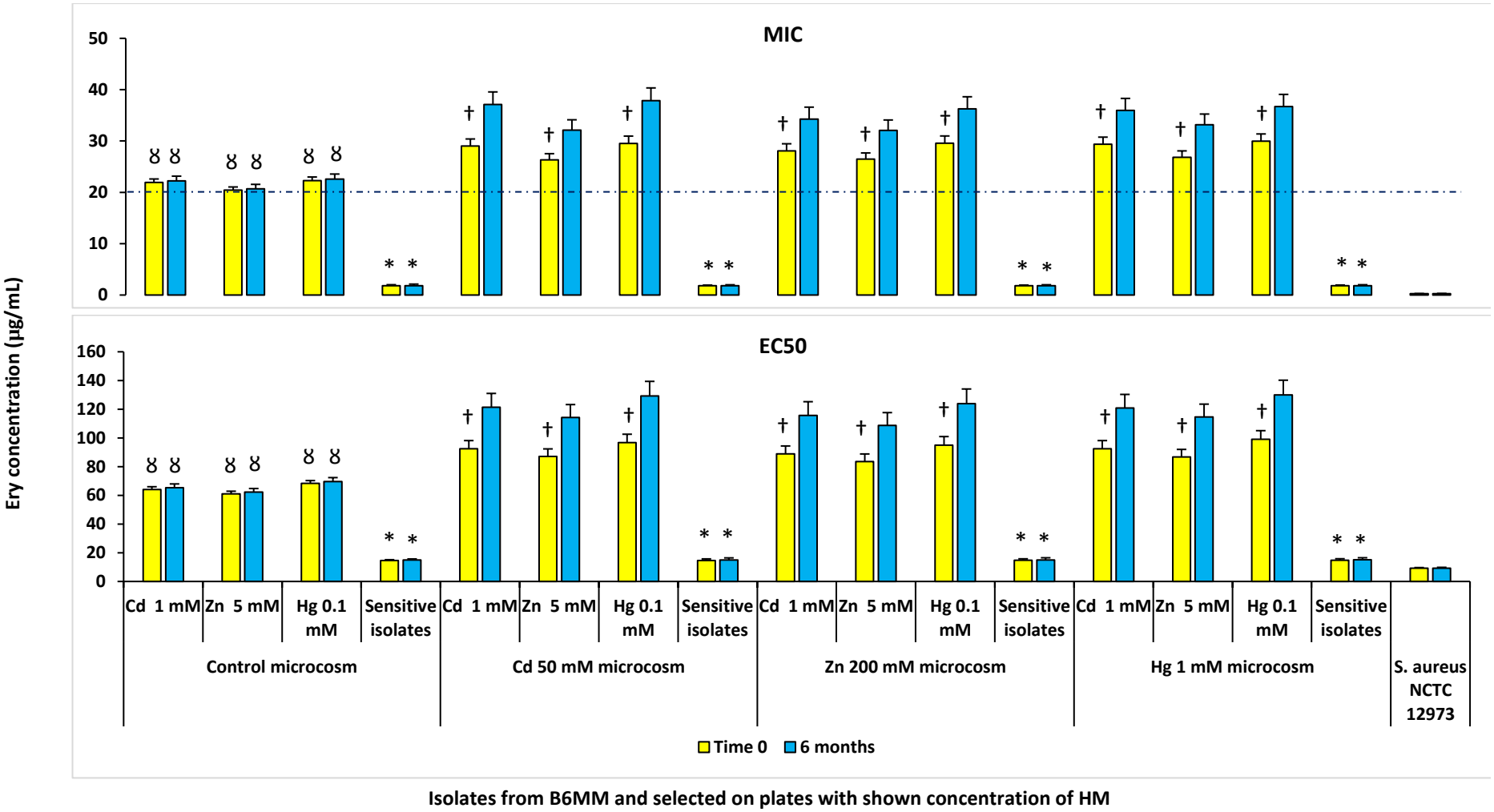

Fig. A3.70. Mean MIC and EC50 values of BM analysis with Ery for HMR isolates from B6MM. *$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm's soil; ȣ$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

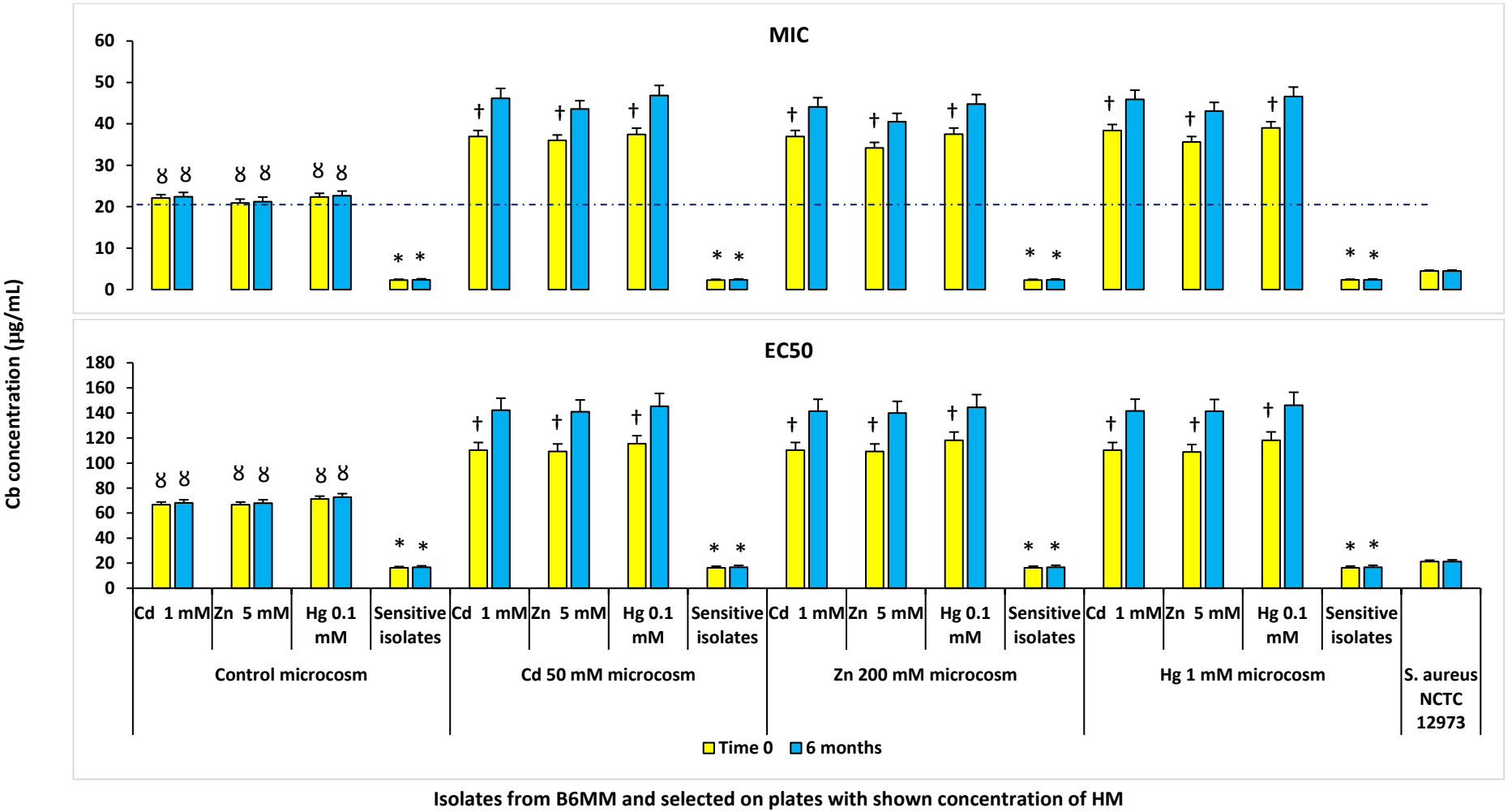

Fig. A3.71. Mean MIC and EC50 values of BM analysis with Cb for HMR isolates from B6MM. *$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

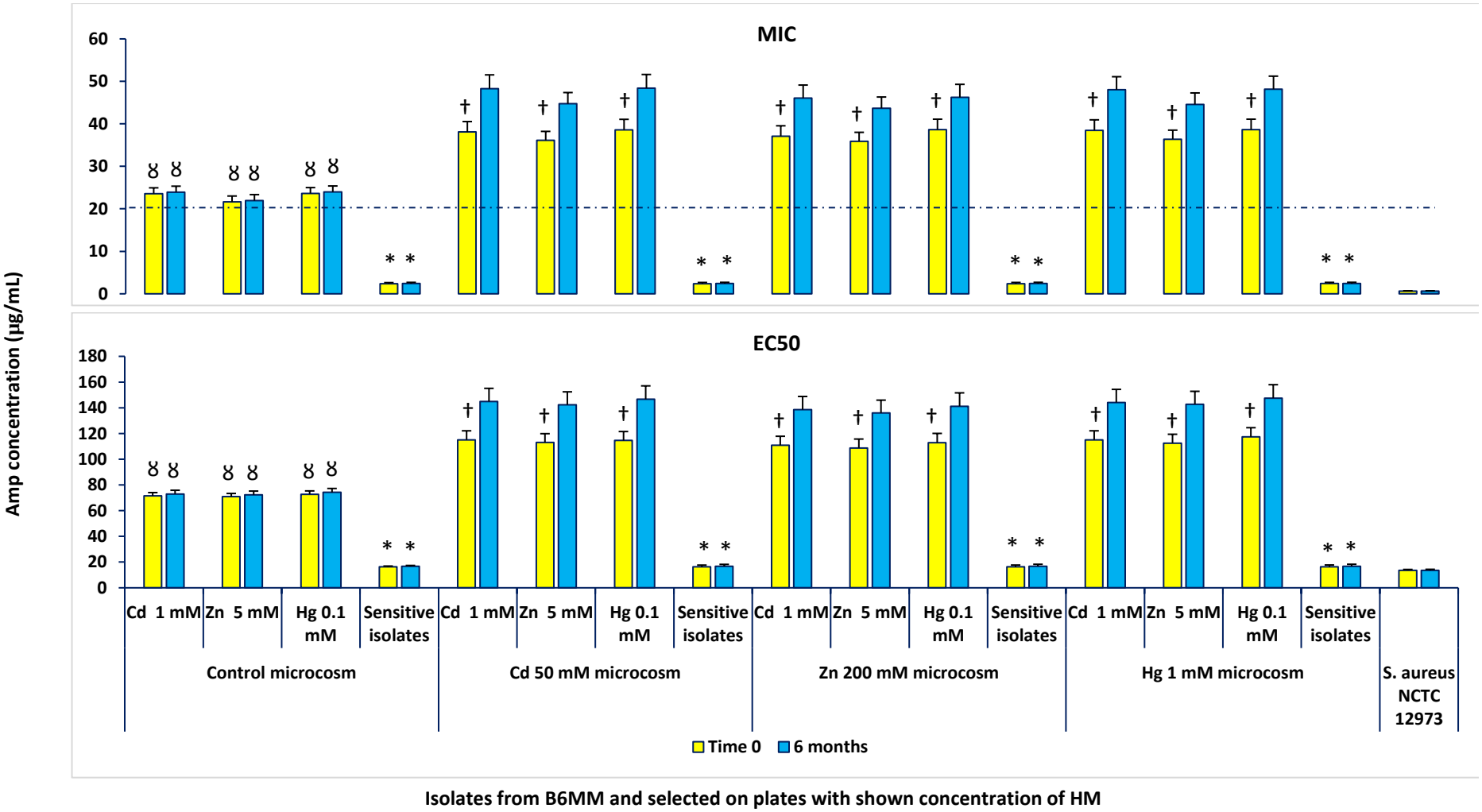

Fig. A3.72. Mean MIC and EC50 values of BM analysis with Amp for HMR isolates from B6MM. *$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

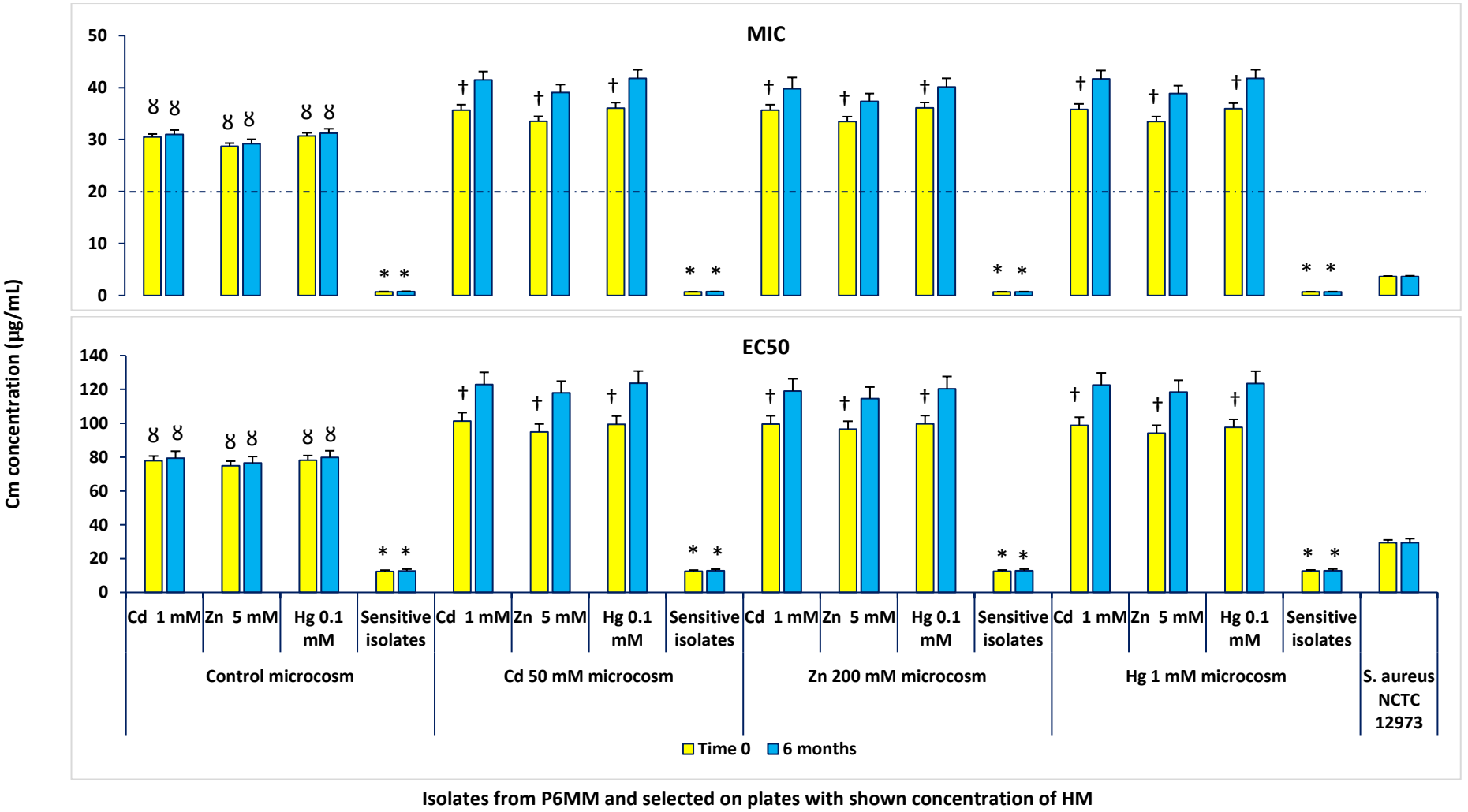

Fig. A3.73. Mean MIC and EC50 values of BM analysis with Cm for HMR isolates from P6MM. *$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm's soil; ȣ$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cm MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

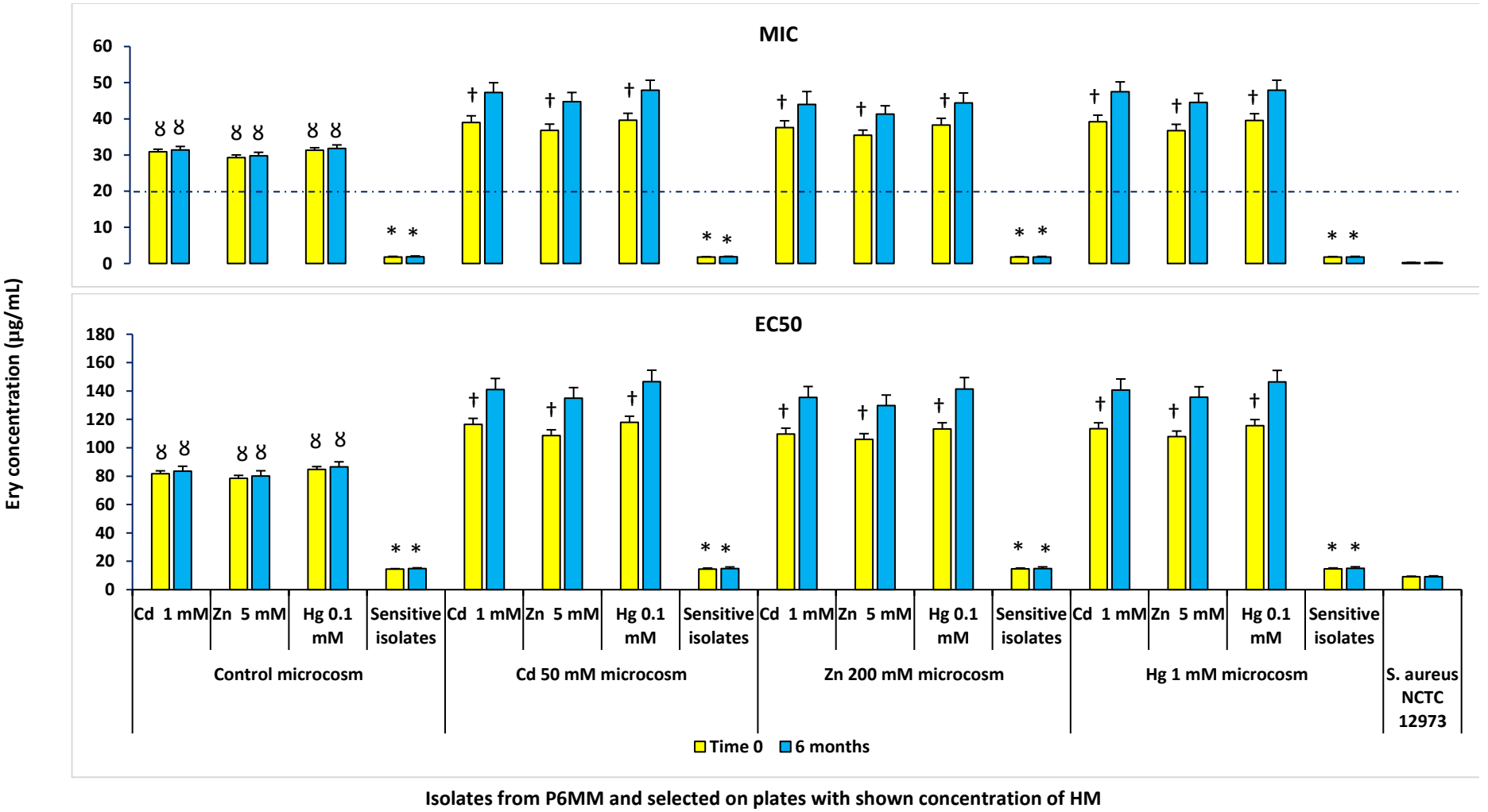

Fig. A3.74. Mean MIC and EC50 values of BM analysis with Ery for HMR isolates from P6MM. *$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm's soil; 8$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Ery MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

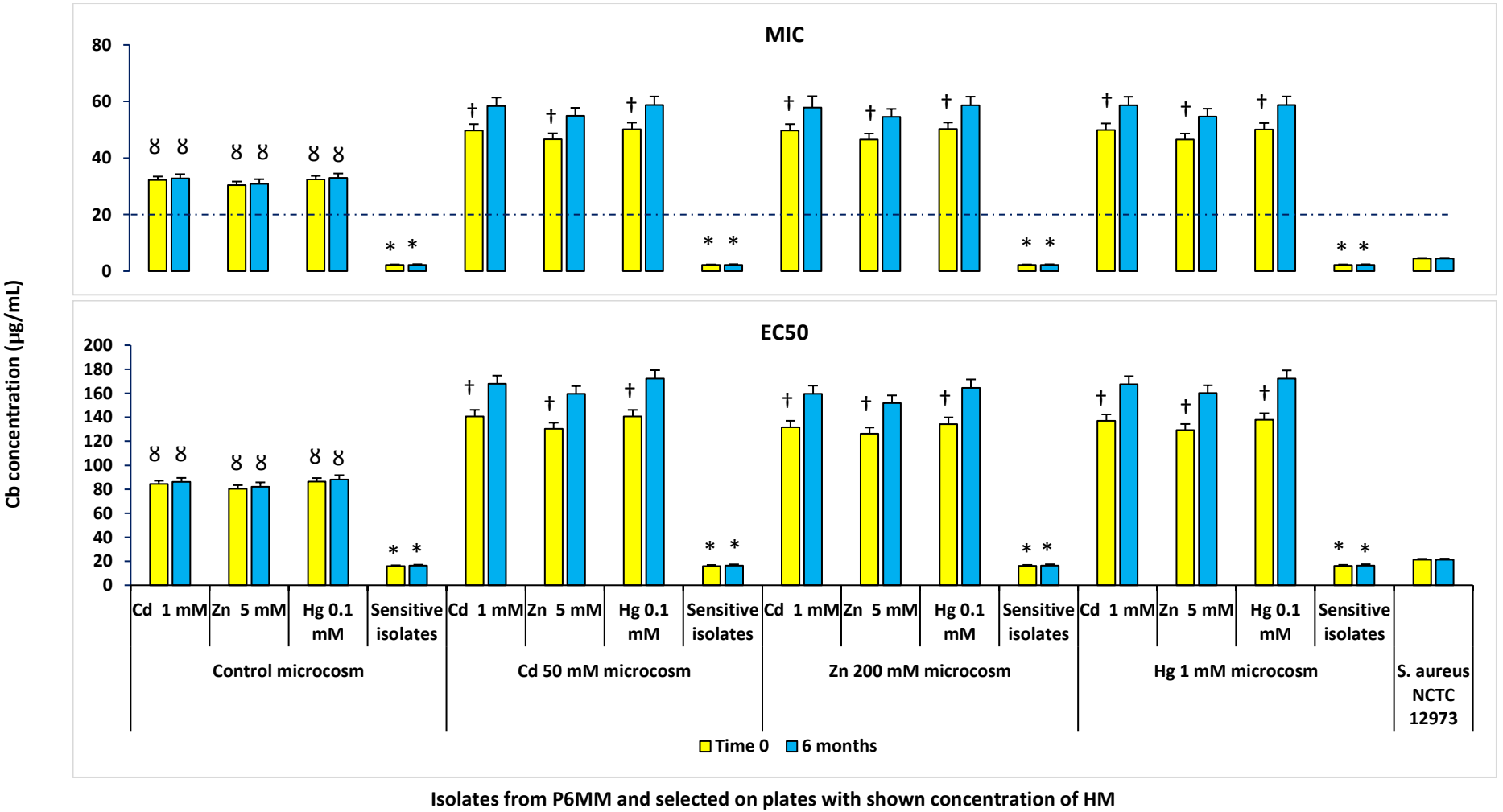

Fig. A3.75. Mean MIC and EC50 values of BM analysis with Cb for HMR isolates from P6MM. *$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm's soil; ϑ$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Cb MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.

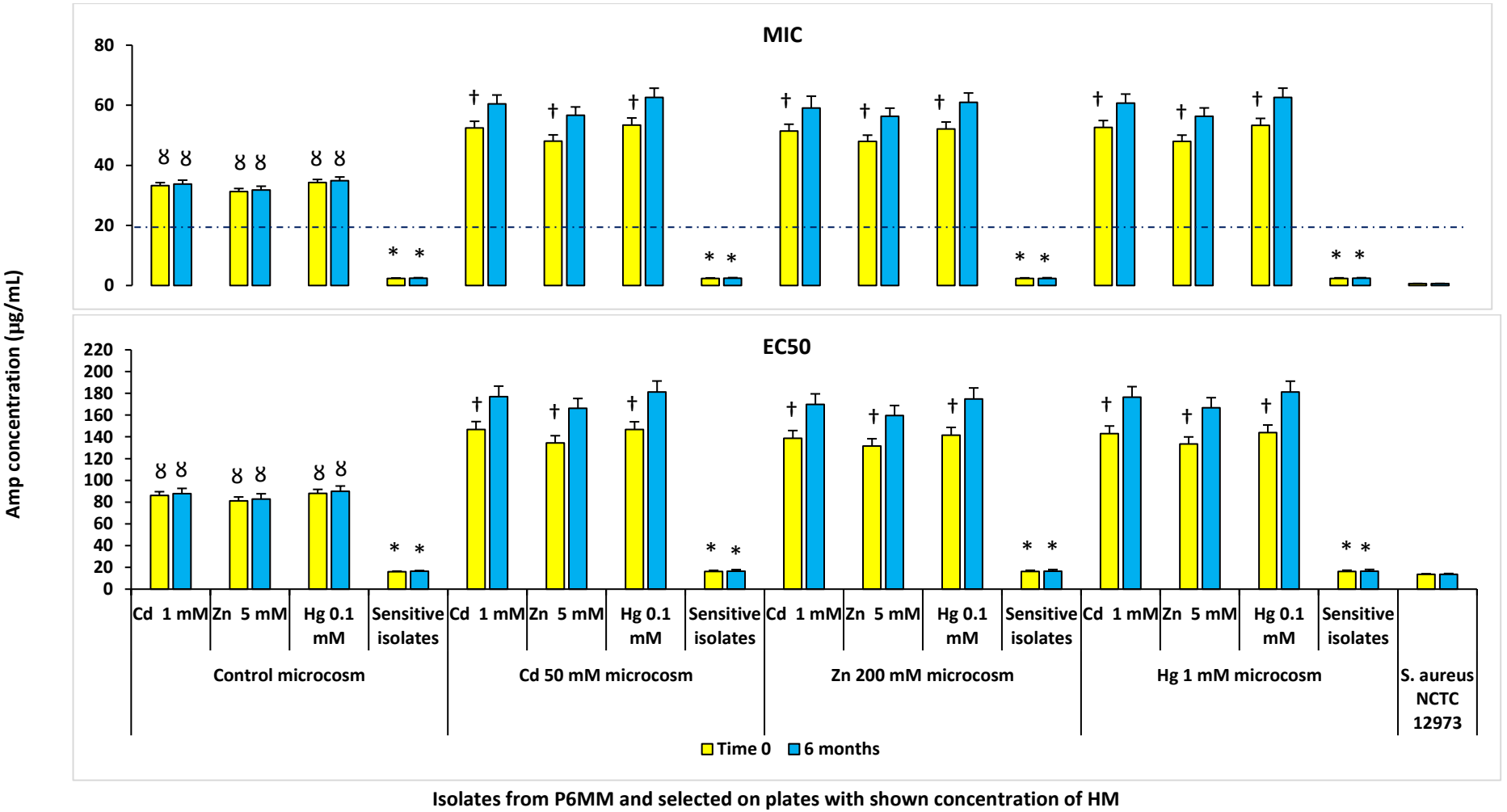

Fig. A3.76. Mean MIC and EC50 values of BM analysis with Amp for HMR isolates from P6MM. *$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm's soil; ȣ$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from HMs-spiked microcosm; †$p < 0.05$ compared to Amp MIC and EC50 value for HMR isolates from the same microcosm after 6 months of incubation. The dash line defines AbR level of soil bacteria.