Kurdistan Regional Government
Ministry of Higher Education and Scientific Research
University of Sulaimani
College of Science

# BIOREMEDIATION OF SOME HEAVY METALS BY RESISTANT BACTERIA ISOLATED FROM TANJARO RIVER WITHIN SULAIMANI CITY-KURDISTAN REGION- IRAQ

A Dissertation
Submitted to the Council of the College of Science at the University of Sulaimani in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy
In Biology
**[Environmental Molecular Microbiology]**

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بسم الله الرحمن الرحيم

وَٱللَّهُ أَخْرَجَكُم مِّنۢ بُطُونِ أُمَّهَٰتِكُمْ لَا تَعْلَمُونَ شَيْـًٔا
وَجَعَلَ لَكُمُ ٱلسَّمْعَ وَٱلْأَبْصَٰرَ وَٱلْأَفْـِٔدَةَ ۙ لَعَلَّكُمْ
تَشْكُرُونَ ۝

سورة النحل( اية ٧٨)

In the name of Allah, the Entirely Merciful, the especially Merciful

And Allah has brought you out from the wombs of your mothers while you know nothing. And he gave you hearing, sight, and hearts that you might give thanks (to Allah) ۞

Al-Nahl (Verse 78)

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We certify that the preparation of this thesis entitled" **Bioremediation of Some Heavy Metals by Resistant Bacteria, Isolated from Tanjaro River within Sulaimani City-Kirdistan region - Iraq** " accomplished by **(Laila Ibrahim Faqe Salih)**, was prepared under my supervision in the College of Science at the University of Sulaimani, in partial fulfillment of the requirements for the degree of Doctoral of Philosophy in **Biology / Environmental Molecular Microbiology**

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# DEDICATION

To my Dear Father and Mother

To my brothers and sisters

To all my friends

Laila

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# ABSTRACT

Bioremediation relies on microbes that live naturally in the environment in the presence of optimum environmental conditions to breakdown contaminants; these microbes pose no threat to people at the site or in the community.Throughout this study naturally occurring heavy metal tolerant bacteria were isolated from Tanjaro River located southwest of Sulaimani city, their potency for uptakes of (Cadmium, Lead, Copper, Chromium, Nickel, Zinc, Cobalt, and Iron) were evaluated by using inductively coupled plasma-optical emission spectrometry.

The results of physicochemical parameters of water samples obtained in this work were in the following ranges; temperature 11.9–31°C, pH 6.1–8.64 which characterized by a shift towards the alkaline side of neutrality. Electrical conductivity ranged from 525-928 µS $cm^{-1}$, total hardness 232–485 mg $l^{-1}$, alkalinity 122–324.3 mg $l^{-1}$, dissolved oxygen 3-7.75 mg $l^{-1}$, biological oxygen demand concentration 36-120 mg $l^{-1}$.

Chloride ion concentration was 13.2-77.9 mg $l^{-1}$, nitrate levels were ranged from 19.52- 48.55 mg $l^{-1}$, while sulfate concentrations were ranged from 21.16- 336.66 mg $l^{-1}$.

Among the analyzed heavy metals from Tanjaro River, Pb ions was the highest concentration, while Zn and Cd ions were the lowest concentration they were in the follows orders: Pb > Cr > Fe > Ni > Co > Cu > Zn > Cd with maximum concentrations of 0.086, 0.073, 0.071, 0.068, 0.051, 0.056, 0.031, and 0.024 ppm, respectively.

Fourty metal-tolerant bacteria were isolated that grow on heavy metal incorporated medium which included both gram-negative 23 (57.5%) and gram-positive 17 (42.5%) bacteria. Molecular identification based on 16SrRNA revealed that the isolates belong to the Bacillaceae, Moraxellaceae, Morganellaceae, Enterococcaceae, Microbacteriaceae, Enterobacteriaceae, Pseudomonadaceae, and Aeromonadaceae families.

Based on maximum tolerable concentration (MTC) values, the isolates exhibited different levels of resistance with a concentration ranging from 10-430 ppm. All the bacterial isolates showed maximum tolerance against Pb and Fe, whereas minimum tolerance was observed against Cd and Zn.

The isolates presented a diverse metal-resistant phenotype to one or more metal ions. *Leucobacter chromiiresistens* - C15T and *Bacillus safensis* - BS16L were respectively able to tolerate high Cd (90 and 80), Pb (250 and 160), Cr (210 and 100), Ni (110 and 90), and Co (160 and 170) ppm. *Raoultella ornithinolytica* - RO40LCH isolated in this study was the best

in terms of (MTC) and heavy metals uptakes, it showed high tolerance for Cd, Pb, Cr, Co, and Fe (120, 430, 230, 210, 340 ppm) respectively.

The results revealed that *R. ornithinolytica* shows the highest ability to remove the selected metals except for Cu by the percentage of (67%, 89%, 63.4%, 55.6%, 56.5%, 65%, and 61.9 %) for each of Cd, Pb, Cr, Ni, Zn, Co, and Fe respectively. These rates are influenced by different environmental conditions (temperature, pH, and incubation periods); 35°C improved the uptakes from 45 to 67%, 65 to 89%, 55 to 56.5%, and 50 to 65% for each of Cd, Pb, Zn, Fe, and Co respectively, while 25°C was optimum for Cr, Cu, and Ni uptakes.

Optimization of pH indicated that the range of 7-8 was optimum for most tested metals except for Co and Ni in which their uptakes enhanced to increase from 65 to 84% and 55.6 to73% respectively at pH 5. Change in the incubation time enhances the metal uptake from 89 to 95%, 36.4 to 45%, and 55.6 to 64% for Pb, Cu, and Ni.

Plasmid curing of *R. ornithinolytica* by each of Sodium dodecyl sulfate (SDS) and ethidium bromide (E.B) indicated that the metal tolerant ability of *R. ornithinolytica* was plasmid-encoded. Six metal resistance genes were chosen to identify the genes responsible of the metals tolerance (*czcA, pcoA, chrB, pbrT, nccA,* and *iroN*), PCR results indicated that *R. ornithinolytica* contains five genes out of the six *(pbrT, chrB, nccA, iroN,* and *czcA), pcoD* gene was absent which responsible for copper efflux, while *R. planticola* harbor only (*pcoD, pbrT, czcA* )metal resistant genes.

Field emission- scanning electron microscopy (FE-SEM) results of *R. ornithinolytica* indicates alterations in bacterial cell size and shape in comparison to the control cells. When *R. ornithinolytica* grow in medium contain eight metals collectively (multi-metal growth) their distinguishs become difficult, with the appearance of cracks on the cell wall.

Energy dispersive X-ray spectroscopy (EDS) spectral images gave visible evidence of metal ions binding on the cell wall of the bacteria which clearly showed that Cd, Pb, and Cr ions were adsorbed on the surface with different rates of binding for different metals.

Transmission electron microscopy (TEM) showed different mechanisms and localization of adsorbed metal particles within the cells, for Pb, Zn, and Co uptake, cell surface adsorption is the candidate mechanism, while Cd, Ni, and Fe were accumulated inside the cell.The results revealed that isolated bacteria particularly *R. ornithinolytica* can be used as eco-friendly biological expedients for the remediation and detoxification of metals from the contaminated environments. To the best of our knowledge, this is the first study to isolate and characterize metal resistant *R. ornithinolytica* from metal contaminated water in Iraq and neighbor countries.

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# List of abbreviations

| | |
|---|---|
| **ANOVA** | Analysis of Variance |
| **APHA** | American Public Health Association |
| **BOD** | Biochemical Oxygen Demand |
| **DO** | Dissolved Oxygen |
| **EC** | Electrical Conductivity |
| **EPA** | Environmental Protection Agency |
| **GPS** | Global Position System |
| **ICP** | Inductively coupled plasma optical emission spectrometry |
| **MCL** | Maximum Contaminant Level |
| **MEGA X** | Molecular evolutionary genetic analysis |
| **NCBI** | National Center for Biotechnology Information |
| **ppb** | part per billion |
| **ppm** | parts per million |
| **ROS** | reactive oxygen species |
| **S.E** | Standard Error |
| **SEM** | scanning electron microscope |
| **SPSS** | Statistical Package for Social Science |
| **TDS** | Total Dissolved Solids |
| **TEM** | Transmission electron microscope |
| **WHO** | World Health Organization |

# Chapter One
# Introduction

# 1. Introduction

Natural water provides a living environment for numerous plants and other organism, the presence and mutual quantitative proportions of macro- and micro-elements are determined by the chemical composition of natural waters (Rabajczyk and Namiesnik, 2014). Since water is a universal solvent, it dissolves a wide range of organic and inorganic compounds as well as contaminants in the environment, for this reason, aquatic ecosystems become vulnerable to the pollution which is one of the most pressing issues in modern human society (Ali *et al*., 2019b).

One of today's most concerning environmental problems is the contamination of the aquatic environment that causes ecological and anthropological health issues as a result of exposure to toxic levels of a variety of substances (Masindi and Muedi, 2018).

Tanjaro River is a permanent river located in Sulaimani city that is used as a source for irrigation and livestock consumption purposes (Rashid, 2010).

Oil refining, houses sewage, and animal wastes were discharged without treatment into the river and represent the main sources of Tanjaro pollution (Ahmed, 2020), other sources for Tanjaro River pollution are black water and residual materials from hospitals, industry, and agriculture run directly into the Tanjaro River through a combined sewerage system (Aziz *et al.,* 2012; Othman *et al.,* 2017; Rasheed and HamaKarim, 2017). Several studies have been conducted out on the quality of the Tanjaro river's water (Khalid and Rashid 2020; Qurbani and Hamzah 2020).Water quality index of Tanjaro River show that it is unsuitable for drinking purpose, the surface and ground water are polluted in Tanjaro basin (Al-Hasnawi, 2012)

The disposal of heavy metals to the environment is a major threat to human health; they not only produce toxic or chronic poisoning in aquatic lives but also pose threat to the environment (Ma *et al.,* 2020), they contaminated the environment as a result of rapid industrialization and urbanization, their rates of mobilization and transport in the environment have considerably accelerated recently.

The quality and quantity of wastes containing heavy metals in wastewater are determined by the sources of such wastes because heavy metals are not biodegradable and tend to accumulate in living organisms; their presence in the environment poses a serious and long-term environmental risk (Cai *et al.,* 2019). Some heavy metals are present at low concentrations but are biologically significant in the aquatic environment (Rahman and Singh, 2016), but high levels of them can be extremely harmful to living organisms due to their

effect on metabolic reaction inhibition, carcinogenic, mutagenic, and non-biodegradable nature with their ability to persist in the environment (Hussein *et al.*, 2003), they accumulate in biota or leach into groundwater since they are persistent in the environment (Ali *et al*., 2019a).

Heavy metals are major environmental contaminants, and their toxicity is a problem of increasing concern for ecological, evolutionary, nutritional, and environmental reasons (Jaishankar *et al.*, 2014).

High heavy metal concentrations can disrupt cell membranes, alter enzyme specificity, impair the function of cellular metabolic pathways, and produce reactive oxygen species that bring many changes in the repair mechanism of DNA (Zahri *et al.*, 2021), reacting as redox catalysts in the production of reactive oxygen species (ROS), destructing ion regulation, and directly affecting the formation of proteins as well as DNA (Igiri *et al.*, 2018).

An increased pollutant load in freshwater increases the nutrient level in the water and can alter the pH and other physicochemical properties of water bodies (Chaurasia and Tiwari, 2011), these surface water alterations act as a selective force on bacterial communities to develop resistance against heavy metals, enables heavy metals resistant bacteria to adapt and thrive in the area (Aktan *et al.*, 2012).

Treatment of heavy metal contaminated water is a challenging process, the removal of metal ions from aqueous solution has been intensively conducted using technology approaches which mainly consist of physical, chemical, and biological technologies that developed and optimized to utilize and remove heavy metals from contaminated environments (Wang and Chen, 2009). The common physicochemical treatment processes for metal remediation in water include: Precipitation, ion exchange and reverse osmosis. while, the chemical methods (Akpor and Muchie, 2010); however, these methods are cost-effective, inefficient when removing heavy metals from large amounts of water, and have limitations such as high energy consumption, non-selectivity, and the use of chemical products (Grenni *et al.*, 2019).

Alternatively, bioremediation has become an option to conventional remediation technologies; the use of microorganisms; bacteria, fungi, and other microorganisms to remove heavy metals has gotten a lot of interest in recent years. (Afzal *et al*., 2017); they can be used for metal remediation by removing, concentrating, and recovering metals from contaminated sites (Irawati *et al*., 2019). Microbe-related technologies provide an addition to the conventional methods for metal removal or metal recovery (Shammi and Ahmed, 2013).

Microorganisms and microbial products can be effective bioaccumulators of metals in both soluble and particulate forms (Shukla *et al.,* 2017). Indigenous microorganisms have evolved a variety of mechanisms that enable their living in the presence of toxic concentrations of metals, these mechanisms include efflux of toxic metals that enter cells via essential metal transporters, enzymatic transformations that decrease metal toxicity (Chatziefthimiou *et al.,* 2007); or biosorption to the cell walls and entrapment in extracellular capsules, precipitation, and oxidation-reduction reactions (Hussein *et al.,* 2003).

Microorganism's capacity to detoxify metal pollution can be employed for bioremediation; isolation and characterization of bacteria from the metal-contaminated environment should be carried out to find metal-resistant strain candidates for heavy metal removal and bioremediation (Rajbanshi, 2008). Various microorganisms such as bacteria (Afzal *et al.,* 2017), co-culture of fungi and bacteria (Qurbani and Hamzah 2020), microphytes (algae) by (Khalid and Rashid, 2020) have been reported to tolerate and remove heavy metals from aqueous solutions.

*Raoultella* sp. is one of the indigenous bacteria that are usually found in aquatic environments and soil (Hajjar *et al.,* 2020); in recent studies, it was isolated from a heavy metal contaminated sites in Brazil and was found to harbor silver *silA,* (cadmium, zinc, and cobalt) *czcA,* and copper *pcoD* resistant genes (Zagui *et al.,* 2020), in Germany *Raoultella* sp. is used for the remediation of cadmium from contaminated soil (Xu *et al.,* 2019).

The roles of *Raoultella* sp. were examined by many researchers in degrading some pollutants: pesticides (Xie *et al.,* 2012), uranium removal (Sklodowska *et al.,* 2018), in the precipitation of Pb (Eltarahony *et al.,* 2021).

Although *R. planticola* was isolated from metal contaminated water in Turkey (Koc *et al.,* 2013), this is the first study on isolating and characterizing metal resistant *R. ornithinolytica* from metal-contaminated water for bioremediation of heavy metals in Iraq and neighbor countries.

## The aims of the study

The main goals of this research were to:

1. Investigating some physicochemical parameters of Tanjaro River's water, and determining the concentration of some heavy metal in water samples.
2. Determining the bacterial diversity in metal-contaminated water in Sulaimani provience.
3. Isolating, analyzing, and molecularly characterize heavy metal-tolerant bacteria from the aquatic environment and assess their tolerance potential against selected heavy metals.
4. Evaluating of bioremediation potential of isolated heavy metal resistant bacteria, which could be used as a cheap and eco-friendly alternative for metal remediation methods.
5. Determining the heavy metal resistant genes in the bacteria that record higher resistance and remediation level *R. ornithinolytica*.

Chapter Two

# Literature Review

# 2. Literature Review

## 2.1 Water studies

Potable water is the basic requirement for human existence; while, polluted water may become a source of toxins that are harmful to human health (Ali *et al.*, 2019b). A constantly growing population, rapid industrialization, expanding urbanization is irresponsible use of natural resources that negatively influencing the water quality; heavy metal ions are among the most often discharged pollutants, which makes them particularly concerning (Hashem and Qi, 2021; Zamora-Ledezma *et al.*, 2021).

Water contamination by heavy metals is a serious environmental problem that has negative consequences for plants, animals, and human health. (Ali *et al.*, 2019 a), for this reason, chemical pollution monitoring of surface water aid in determining the level of environmental risk associated with the toxicity of pollutants to aquatic organisms and enables the evaluation of their accumulation in the ecosystem (Michalec *et al*., 2014). Since there are no sufficient facilities for the treatment of municipal and industrial wastes, effluents discharged into different water bodies caused water contamination, endangering biodiversity and reducing water quality (Khan and Noor, 2002).

According to recent research, long-term use of untreated wastewater of industrial sources can decrease water quality, making it unsafe for human consumption (Kapahi and Sachdeva 2019). Dumping a significant amount of industrial and household pollutants into rivers; make considerable stress on the river's physicochemical and microbiological charactristics (Haque *et al.*, 2019). Hazardous metallic elements are discharged into the water regularly from diverse natural and anthropogenic sources, not only do they cause acute or chronic poisoning in aquatic life, but they also endanger the ecosystem (Cabral *et al.*, 2019; Ma *et al.*, 2020).

Heavy metals are considered as the main group of inorganic pollutants which are continuously accumulating in the environment, their small size and the tendency for bioaccumulation in the biota may have adverse effects on animals and humans, which is a global problem that disrupts environmental balance by gaining access into ecosystems (Manasi *et al.*, 2016). When metals enter the food chain, they can cause biomagnification, which means that a low quantity can rise and become much more harmful as it passes through various trophic levels (Jaishankar *et al.*, 2014).

Many studies on water pollution by various sources and heavy metals have been conducted around the world and in Iraqi Kurdistan Region ; in a study carried out on the impact of wastewater on the Tanjaro aquatic environment, it was concluded that Tanjaro

River, Qliasan stream and groundwater were polluted with nitrate, nitrite, and heavy metals (Mustafa, 2006) Assessing the water quality parameters in the Trabzon, Turkey was done by (Bulut *et al.,* 2010) who indicated that Galyan water is classified as polluted water in terms of chromium and iron that exceed the values for safe drinking water.

Aziz *et al.,* (2012) studied Tanjero River pollution by some heavy metals generated from sewage and industrial wastewater in the Sulaimani district, they revealed that the Tanjaro River and its tributaries were polluted with heavy metals (Fe, Mn, Ni, and Cr) resulting from the impact of sewage wastewater.

Hassan and Al-Barware (2016) performed a study to assess the water quality in Duhok Valley; they classified the water as hard water and recorded zero dissolved oxygen as a result of a high load of organic material.

The average concentrations of Cr, Mn, Fe, Co, Ni, As, and Cd detected in surface water bodies in several places around the world are considerably over the maximum permitted limits for drinking water as recommended by WHO (Cabral *et al.,* 2019).

Al-Asadi *et al.,* (2020) estimated the water quality of the Shatt Al-Arab River and investigate the influences on the variations of heavy metals levels, a study was conducted by, they found that the metal concentrations were low and uniform, except for Ni.

Al-Abbawy *et al.,* (2021) conducted a study to assess the level of heavy metals in various aquatic plants of Al-Hawizeh Marsh, southern Iraq; the study showed that concentrations of cadmium, chromium, and iron in plants were above the permissible limits set by WHO (appendix 1), in contrast, zinc, copper, and lead were all below the allowable limits.

## 2.2 Heavy metals: definition and properties

Heavy metals are naturally occurring cations found throughout the earth's crust and are found in varying concentrations in all ecosystems; they have a comparatively atomic number greater than 20 and density (5 $g/cm^3$) when compared to water. Metals and semimetals (metalloids) that have been linked to contamination and potential toxicity or ecotoxicity are often referred to as heavy metals (Tchounwou *et al.,* 2012; Das *et al.,* 2018).

Heavy metals able to bind organic groups covalently; as a result, when they bind to nonmetallic components of cellular macromolecules, they generate lipophilic ions and compounds, which can have toxic effects. Due to becoming lipophilic, the metalloids distribution in the biosphere and their toxic reaction differ from the action of simple ionic forms of the same element (Briffa *et al.,* 2020).

They enter the environment through both natural and anthropogenic sources; natural weathering of the metal-bearing rocks, mining, soil erosion, industrial discharge, urban runoff, sewage effluents, pestisides and disease control chemicals applied to plants, air pollution fallout, and a variety of other sources (Morais *et al.*, 2012).

Heavy metals are classified into two categories regarding their roles in biological systems: essential and non-essential. Metals such as Co, Cu, Fe, and Zn have been reported as essential nutrients needed for various biochemical and physiological functions and may be required in the body in quite low concentrations (Elbasiouny *et al.*, 2021).

Heavy metals with no recognized biological function in living beings are known as non-essential (Ali *et al.*, 2019a), they include Cd, Ld, Cr, and Ni; although traces of these metals are required as a co-factors in enzymatic reactions, high levels of them can be extremely harmful to living organisms due to their effect on metabolic reaction inhibition (Hussein *et al.*, 2003); however, the lists of essential and nonessential heavy metals may be different for different groups of organisms such as plants, animals, and microorganisms, it means a heavy metal may be essential for a given group of organisms but nonessential for another one (Chalkiadaki *et al.*, 2014).

### 2.2.1Cadmium (Cd)

Cadmium (Cd) is considered to be one of the most harmful metals in the environment because it is an element rather it lack a known biological and physiological role in the human body, It can affect human and other organisms at relatively low concentrations and is highly mobile in the environment (Masindi and Muedi, 2018).

Cadmium is a byproduct of the zinc industry and is found with copper, and lead, found in ores; it is frequently utilized in industrial operations, as an anti-corrosive agent, a color pigment, a neutron absorber in nuclear power plants, and the manufacture of nickel-cadmium batteries. Cadmium levels in phosphate fertilizers are very high (Godt *et al.*, 2006).

Cadmium is discovered in drinking water sources as a result of galvanized plumbing degradation, as well as industrial waste pollution and surface water contamination. The Environmental Protection Agency (EPA) has established a Maximum Contaminant Level (MCL) of 0.005ppm for cadmium in drinking water.

Although trace cadmium can be chelated or sequestered like any other metal, it is more commonly present in the dissolved ionic form (Rzetata, 2016). Cadmium is highly soluble in water as compared to other heavy metals. It is a health hazard for employees who are exposed to it since it causes acute and chronic illnesses (Franko *et al.*, 2005). It is rapidly

absorbed and accumulates in tissues; its main sources in our diet are fish and cereal products. Long-term exposure to Cd can harm the kidneys, liver, testes, and prostate. Anemia, high blood pressure, circulation difficulties, bone decalcification, and muscular atrophy are all possible side effects of excessive Cd exposure (Olmedo *et al.,* 2013).

Cadmium is toxic to microorganisms, causing damage to their cell membranes and destroying DNA structure. The displacement of metals from their natural binding sites or ligand interactions causes this toxicity. Changing the nucleic acid structure, creating functional disruption, inhibiting enzyme activity, and oxidative phosphorylation all have an impact on the morphology, metabolism, and development of microorganisms (Fashola *et al* ., 2016).

### 2.2.2 Lead (Pb)

Lead is extremely soft, malleable, ductile, and has low electrical conductivity. It is corrosion-resistant but tarnishes when exposed to air (Haynes, 2015). It is utilized in a variety of industries, including cosmetics, metal products, batteries, and plumbing pipes, cable sheathing, and lead crystal glass, it is now widely used in paints and gasoline. Pb is considered a carcinogenic compound according to the environmental protection agency (Carneiro *et al.,* 2014).

Lead is the most significant toxin of heavy metals, and the inorganic forms are absorbed through ingestion by food, water, and inhalation (Jaishankar *et al.,* 2014).

A high level of lead exposure may result in toxic biochemical effects in humans which, in turn, cause problems in the synthesis of hemoglobin; effects on the kidneys, gastrointestinal tract, joints, reproductive system; and chronic damage to the nervous system. Anemia has been linked to lead poisoning in many cases because lead inhibits porphobilinogen synthase and ferrochelatase, inhibiting the creation of porphobilinogen and the integration of iron into protoporphyrin, which hinders heme synthesis (Wani *et al.,* 2015).

Paints, pesticides, vehicular emissions, mining, and coal combustion are all major contributors to lead contamination in water. As a result, it may enter the soil and run into bodies of water, where it may be absorbed by plants and hence humans. (Barbosa *et al.,* 2006).

Bacteria with the ability to modify or sequester lead may provide an option for the removal of lead from the environment. The bacteria could be used alone to detoxify the contaminant or bioremediation could be combined with current physicochemical methods to improve their efficiency (Gummersheimer and Giblin, 2003).

### 2.2.3Copper (Cu)

Copper is a trace mineral that is required for living. It is present in all bodily tissues and is involved in the production of red blood cells, as well as the maintenance of nerve cells and the immune system, it also helps the body form collagen and absorb iron, and plays a role in energy production (Hobman and Crossman, 2015). Copper is a highly common element that exists naturally in the environment and spreads throughout the environment through natural processes (Haynes, 2015).

Copper is also widely used in agriculture as wood preservatives, antifungal agents, in hospitals especially on surfaces to prevent biofilm formation and healthcare-associated infections, where copper impregnated sanitary pads are used to prevent postpartum infections, also used as animal food supplementation (Arendsen *et al.,* 2019).

Copper sulfate is used to add copper to drinking water and swimming pools. Copper can enter the environment through waste dumps, domestic wastewater, combustion of fossil fuels, wood production, and phosphate fertilizer production, since the copper in its elemental form does not degrade in the environment, the Environmental Protection Agency (EPA) has concluded that drinking water should not include more than (1.3 ppm) copper (Dorsey *et al.*, 2004).

Although copper is an essential metal for aerobic life; high cell concentrations can become toxic, drinking water with high concentrations may cause nausea, vomiting, stomach cramps, or diarrhea. Copper poisoning can result in liver and kidney damage, as well as death if consumed in excess. The high concentrations of copper, resulting from various exposure routes, can influence the high occurrence of bacteria carrying resistance genes to tolerate metals high levels (Zagui *et al.,* 2020).

### 2.2.4 Chromium (Cr)

The element chromium is the seventh most abundant element on earth, and it may be found in several oxidative states in the environment, ranging from Cr (0) (elemental chromium) to Cr (VI) (hexavalent chromium) the most abundantly occurring forms of chromium are (III), and (VI) that differ not only in their oxidation states but also in their chemical properties and toxicity.

Chromium has high environmental mobility and can originate from anthropogenic and natural sources; natural sources of chromium include burning coal, petroleum, oxidants of pigments, fertilizers, oil well drilling, and metal plating tenures. Anthropogenic sources of chromium release in the environment include fertilizers and sewage (Tunakova *et al.,* 2021).

Because chromium is highly attached to the soil and is often contained within the silt layer around or within the groundwater reservoir, water pollution is restricted to surface water and will not damage groundwater (Agarwal *et al.,* 2021).Since chromium Cr (V)is a transition metal that has the ability to interact with DNA, it causes significant DNA damage and causes hazardous illnesses (Jadoon and Malik, 2017).

By interacting with the carboxyl and thiol groups of enzymes, chromium Cr (III) can alter their structure and function. Intracellular cationic Cr (III) complexes interact electrostatically with DNA's negatively charged phosphate groups, potentially disrupting transcription and replication (Igiri *et al.,* 2018). The WHO recommended safe limits for Cr in wastewater are 0.05 ppm (Kinuthia *et al.,* 2020).

### 2.2.5 Nickel (Ni)

Nickel is the 24$^{th}$ most abundant element in the earth's crust, it is a transition metal that may exist in several oxidative states (from −1 to +4), it can be found at very low levels in the environment, including air, water, and soil. It might come from both natural and man-made sources; its release from anthropogenic sources could be in the form of oxides, sulfides, soluble compounds, and to a lesser content, as metallic nickel. Despite its abundance in the environment, the role of nickel as a trace element for animals and humans has yet to be discovered (Genchi *et al.,* 2020). It is one of the components that cannot be naturally broken down, thereby contributing to the increased risk of environmental pollution, endangering the ecological systems and living beings globally (Babar *et al.,* 2021).

Nickel and nickel compounds are most often consumed through dietary exposure and drinking water in the general population (Cameron *et al.,* 2011).

Depending on the amount, the solubility of the nickel compound, and length of exposure, accumulation of nickel and nickel compounds in the body can cause a variety of health concerns, such as contact dermatitis, cardiovascular disease, asthma, lung fibrosis, and respiratory tract cancer (Sinicropi *et al.,* 2010).

Das and Buchner (2007) have published a review on the mechanisms of nickel toxicity; nickel poisoning is mostly caused by depletion of glutathione levels and bonding to the sulfhydryl groups of proteins.

### 2.2.6 Zinc (Zn)

Zinc is a natural element found in abundance in the earth's crust, which is a transition metal commonly found in its divalent form in nature. It is a nutritionally essential metal playing a role in the biological processes of all humans, animals, and plants. It's classified as an essential mineral since it's required for the creation of hundreds of enzymes all over the body (Hurdebise *et al.*, 2015).

It's one of the body's most vital trace elements, serving as a catalytic, structural, and regulatory ion (Stefanidou *et al.*, 2006), it acts as a co-factor in enzymatic activities involving DNA expression, membrane stability, vitamin A metabolism, and the gustatory and olfactory systems (Kim *et al.*, 2010). Zinc deficiency has been recorded in a wide range of agricultural plants and animals, with serious consequences for reproduction, growth, and tissue proliferation at all stages (Sharma *et al.*, 2013).

Natural and anthropogenic sources releases zinc into the environment; however, release from anthropogenic sources is bigger than natural releases. The main anthropogenic sources of zinc in the environment include zinc mining and metallurgical activities, as well as the use of commercial goods containing zinc (Curtis *et al.*, 2003).

The fate of zinc in the environment is mostly regulated by sorption processes, whereas its bioavailability is influenced by a variety of physicochemical (temperature, hardness, pH) and biological factors. (Zhang *et al.*, 2012). It is considered that Zn is not dangerous to humans, and its possible negative impacts are rather observed on soil biota and soil functioning (De Oliveira, 2019).

### 2.2.7 Cobalt (Co)

Cobalt is a natural element found throughout the environment, it found in relatively low concentrations in the earth's crust and in natural waters, it usually occurs in the environment in association with other metals such as copper, nickel, manganese, and arsenic (Melby *et al.*, 2018).

Cobalt is an essential trace element for life and plays an important role in biochemical reactions, notably in the coenzyme cobalamin (vitamin $B_{12}$) (Pourret *et al.*, 2015), while inorganic cobalt is not required in human diets, and cobalt insufficiency has never been documented in humans (Simonsen *et al.*, 2012).

As cobalt is widely dispersed in the environment, it cannot be destroyed in the environment, rather can only change its form or become attached or separated from particles. Cobalt can enter the environment from both natural sources and human activities, it can be

released from power plants and other combustion processes is usually attached to very small particles. Humans can be exposed to cobalt through the air they breathe, drinking water, and consuming food. Skin contact with cobalt-containing soil or water can potentially increase the exposure rate; it is largely used in the manufacture of alloys, catalysts in the petroleum industry, catalytic converters, and paint pigments, thus the potential for Co releases into the environment is highly increased (Abdel-Sabour, 2003).

Cobalt released into the water may stick to particles in the water column or to the sediment at the bottom of the body of water into which it was released, or remain in the water column in ionic form. The fate of cobalt will be determined by a variety of parameters, including the chemistry of the water and sediment at a given location, as well as cobalt concentration and water velocity (Li *et al.,* 2018). In most drinking water around the world, cobalt levels are less than 1–2 ppb, Environmental Protection Agency classifies Co in the priority list of environmental risk elements (Bundy *et al.,* 2020).

### 2.2.8 Iron (Fe)

Iron is an essential redox-active transition metal that can control the geochemical cycle of other trace elements (Mills *et al.,* 2004), it is found in two oxidation states, +2 and +3, and its circulation is intertwined with that of oxygen, sulfur, and carbon (Nowack and Bucheli, 2007).

Iron is one of the most common metals found in nature, and it is classified as a macroelement for living organisms, because of the wide range of applications for this metal, as well as variables that influence its chemical transitions, various iron species can be found in an aquatic ecosystem (Rabajczyk and Namiesnik, 2014).

Iron enters the water by natural processes such as rock and soil erosion, as well as out-washing and infiltration, or through human activities such as industrial waste discharge, corrosion of containers, pipelines, and other iron parts or equipment (Mahowald *et al.,* 2009).

Iron is an essential element for the growth and survival of human beings which is an important component of enzymes and hemoglobin (Jadoon and Malik, 2017). When iron fails to bind to protein, it produces harmful free radicals; this harmful free radical destroys the digestive tract, liver, brain, and heart cells, as well as the mitochondria. Overconsumption of iron raises the risk of these free radicals causing further DNA damage (Bridges and Zalups, 2010). High iron levels have been identified as a major risk factor for myocardial infarction. According to research, the higher the iron level, the higher the synthesis of so-called bad cholesterol (Pan *et al.,* 2011).

## 2.3 Water pollution by heavy metals

Heavy metals have contaminated around 40% of the world's rivers and lakes. One of the most serious environmental concerns is the presence of hazardous heavy metals in surface water as a result of the discharge of untreated metal-containing effluents into water bodies (Irawati *et al.,* 2016; (Zamora-Ledezma *et al.,* 2021) .

Mining, smelting, energy and fuel production, fertilizer and pesticide manufacture and application, electroplating, metal surface treatment, and other industries all produce and discharge wastes containing various heavy metals into the aquatic environment. As a result, causes major environmental contamination, endangering human health and the environment (Wang and Chen, 2009).

The quality and the quantity of the wastes containing toxic heavy metals are dependent upon their sources of discharge (Rayan *et al.,* 2005). Heavy metals are transported by runoff from industries, municipalities, and urban areas and end up accumulating in the water resources, soil, and sediments of water bodies (Musilova *et al.*, 2016).

Heavy metals discharged into water sources can cause physical, chemical, and biological problems, resulting in changes in diversity, density, species population composition, and community organizations of organisms (Pratush *et al.,* 2018); because they are extremely soluble in the aquatic environment, they are easily absorbed by living organisms (Kinuthia *et al.,* 2020).

Ingestion of higher amounts of metals through the water route is of extreme significance in risk assessment studies in human health (Ali *et al.,* 2019b).

Many studies documented that several human sicknesses are directly correlated with metal intoxication that enters the food chain through the water–plant ecosystem (Hussain *et al.,* 2021). The use of industrial or municipal wastewater in agriculture is a common practice of irrigation in many parts of the world (El- Zahrani and El-Saied, 2011).

## 2.4 Major toxicity effects of heavy metals

Heavy metals are major environmental contaminants, and their toxicity is a problem of increasing concern for ecological, evolutionary, nutritional, and environmental reasons (Jaishankar *et al.,* 2014). there are a wide range of applications and play an essential part in today's industrialized society, some metals have vital physiological and biochemical roles in biological systems, and their deficiency or excess can cause metabolic problems and, as a result, a variety of diseases (Ali *et al.,* 2019b).

Toxicity of heavy metals is the ability of a metal to cause detrimental effects on organisms when consumed above the recommended limits of risk assessment that depends on the bioavailability of heavy metals, duration of exposure, the absorbed dose, the organism's age and gender; metal toxicity is of great environmental concern because of their bioaccumulation and nonbiodegradability in nature (Igiri *et al.,* 2018).

Metals in the form of free ions, metal complexes, metal particles, and poorly soluble compounds may be carcinogenic. The physicochemical characteristics of metals and their compounds determine their toxicity. The oxidation state, charge, and ionic radii of metal ions are crucial. The coordination number, shape, and type of ligands are important for toxic interactions. Regarding metals and their poorly soluble compounds, particle size and crystal structure are important (Beyersmann and Hartwig 2008).

Lead and cadmium are widely distributed in the environment, and their form can enhance their toxicity. Dimethyl mercury and tetraethyl lead are particularly harmful because they can easily enter the body and remain there due to their high lipid solubility. In humans; these elements have no beneficial effects, and no recognized homeostasis mechanism exists for them. They are the most hazardous to people and animals, the adverse human health effects associated with exposure to them, even at low concentrations, are broad (Morais *et al.,* 2012), the effect’s nature of heavy metal poisoning could be toxic (acute, chronic or sub-chronic), neurotoxic, carcinogenic, mutagenic or teratogenic (Verma and Dwivedi, 2013).

Heavy metals may enter the human body in different ways from ingestion of polluted food, inhalation of contaminated air, drinking contaminated water, and skin contact from the farm, pharmaceutical, manufacturing, residential, and industrial regions (Masindi and Muedi, 2018).

Heavy metal toxicity has proven to be a major threat and several health risks associated with it, chronic low exposures to heavy metals can have serious health effects in the long run. They may disturb the body’s metabolic systems in a variety of ways. Furthermore, they can accumulate in vital organs including the liver, heart, kidneys, and

brain, disrupting normal biological function. Once heavy metals have entered the biological systems, they block the vital activities in the body (Rehman *et al.*, 2017).

Two types of damages might occur due to metals: "direct" and "indirect" damage, causing conformational changes in the biomolecules as a result of "direct" damage. On the other hand, causes "indirect" damage as a result of the production of reactive oxygen and nitrogen species which comprise the hydroxyl and superoxide radicals, hydrogen peroxide, nitric oxide, and other endogenous oxidants, it has been noted that heavy metals activating signal pathways (Valko *et al.*, 2005).

High heavy metal concentrations can disrupt cell membranes, alter enzyme specificity, impair the function of cellular metabolic pathways, and produce reactive oxygen species that bring many changes in the repair mechanism of DNA (Zahri *et al.*, 2021), reacting as redox catalysts in the production of reactive oxygen species (ROS), destructing ion regulation, and directly affecting the formation of proteins as well as DNA (Igiri *et al.*, 2018), skin disorders, neurological diseases such as Parkinson disease, cardiovascular disorders, carcinoma, tumor, rare autoimmune disorder, degenerative disease are common examples of damage caused by heavy metals; also they may act as free radical causing damage which includes aging as a result of DNA damage (Jadoon and Malik, 2017).

In addition, they exert an inhibitory action on microbes by blocking key functional groups, displacing essential metal ions, or modifying the active conformation of biological molecules. Heavy metal uptake by biomass is often divided into three categories: cell surface binding, intracellular accumulation, and extracellular accumulation. Because cell surface binding is metabolism-independent, it can occur in both living and inactivated bacteria, whereas intracellular and extracellular metal buildups are often energy-driven processes that can only occur in living cells (Rayan *et al.*, 2005).

Most heavy metals have no known positive benefits on bacterial cells, even at low concentrations; while, some ones such as Pb, Cd, and Cr are hazardous. High pollution levels have been linked to bacteria developing resistance and detoxifying mechanisms, according to previous research. It is not difficult to identify mercury-resistant bacterial strains in high mercury settings, for example. Resistant bacteria to zinc, copper, and cobalt may be easily acquired from industrial locations with high amounts of these pollutants. These examples demonstrate how bacteria that can withstand high amounts of pollution might be effective instruments for environmental remediation (Gummersheimer and Giblin 2003).

## 2.5 Interactions of microorganisms with heavy metals

In the environment, bacteria are the most abundant microorganism. Bacteria have a high surface-to-volume ratio due to their tiny size, providing a broad contact area for interactions with the surrounding environment. In addition to their occurrence in high numbers, the negative net charge of the cell membrane makes these organisms sensitive to accumulating metals from the environment (Haferburg and Kothe, 2007).

Heavy metals, which are typically found in their ionized forms, are exposed to living organisms in nature. On microbes, these ions have a variety of toxic effects. Metal exposure both selects and maintains microbial variations that can resist their negative consequences (Cervantes *et al.,* 2006).

The bioavailability of metals in the habitat is influenced by microbial activity, and the water flow (Azubuike *et al.,* 2016). Bacteria can influence the types of heavy metals to which they are exposed to some extent; they can alter metals into more or less dangerous forms (Irawati *et al.,* 2017b).

## 2.6 Metal tolerance mechanisms

Metal-contaminated environments usually contain bacteria that exhibit a complex array of biochemical and genetically encoded mechanisms to counteract the harmful effects of heavy metals in their surroundings; So the analysis of bacterial genetic characteristics may help to a better understanding of the mechanisms involved in bacteria–metal ion interactions, as well as information on heavy metal resistance genes in metal-contaminated environments (Aka and Badalona, 2017).

The abundance and diversity of metal resistant microorganisms in diverse habitats suggests that metal resistance evolved before human activities spread metal pollutants. The existence of metal resistance genes in bacterial genomes supports microbial growth in the presence of high quantities of harmful metals, which has been going on since the evolution of life on Earth. (Sand and Gehrke, 2006); however, continuous waste disposal in aquatic environment enables heavy metals resistant bacteria to adapt and thrive in this area. Indigenous bacteria isolated from a heavy metals-contaminated site usually develop resistance mechanisms to survive under stress conditions and may potentially be used as bioremediation agents (Irawati *et al.,* 2017a).

Gram negative bacteria are more tolerant than Gram positive bacteria. These differences may be attributed to the different biochemical and morphological features of the groups. This may be reflected in the distribution of metals in cellular fractions, although

nearly most microorganisms have evolved a variety of mechanisms that enable life in the presence of toxic concentrations of metals (Figure 2.1), these include efflux of toxic metals that enter cells via essential metal transporters, enzymatic transformations that decrease metal toxicity (Chatziefthimiou *et al.,* 2007), Precipitation, complexation, and oxidation-reduction processes, biosorption to cell walls and trapping in extracellular capsules (Hussein *et al.,* 2003).

Membrane transport systems of the cell cannot differentiate between the trace elements needed for metabolic actions and toxic metals that would – once inside the cell – interfere with the phosphoryl groups of nucleic acids or the thiol groups of proteins (Haferburg and Kothe, 2007). Bacterial strains may include genetic factors that contribute to heavy metal resistance, and these determinants are frequently found on plasmids, transposons, or chromosomal DNA (Carattoli, 2003).

Adaptation to a harsh polluted environment can be natural or acquired by plasmids, and the prevalence of plasmid-bearing metal-tolerant strains is higher in polluted areas than in unpolluted areas (Manasi *et al.,* 2016).

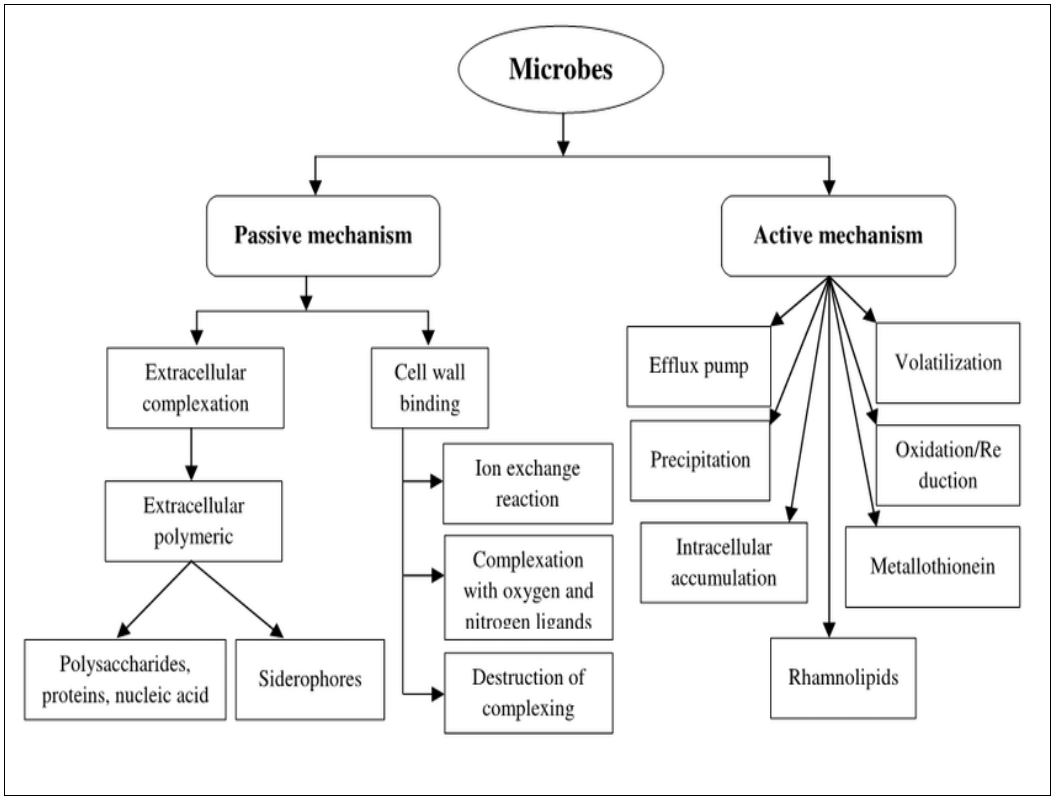



**Figure (2.1)** Mechanism of microbial metal tolerance (adopted from Rajendran *et al.,* 2003)

## 2.7 Remediation techniques for removal of heavy metals from water

Treatment of heavy metal contaminated water is a challenging process. The removal of metal ions from aqueous solution has been intensively conducted using technology approaches which mainly consist of physical, chemical, and biological technologies that developed and optimized in order to utilize and remove heavy metals from contaminated environments (Wang and Chen 2009).

Reduced bioavailability of heavy metals, and consequently their accumulation and toxicity in plants and animals, is one of the most significant aims of remediation (Elbasiouny *et al.*, 2021).

Ion exchange, chemical precipitation, reverse osmosis, evaporation, membrane filtration, and adsorption are the most common techniques for removing heavy metals from wastewaters (Kobya *et al.*, 2005).

Each of these methods has its advantages and disadvantages; however, the majority of them are costly, inefficient when removing heavy metals from large amounts of water, and have limitations such as high energy consumption, and non-selectivity (Grenni *et al.,* 2019), incomplete removal and generation of toxic sludge are generated causing secondary environmental issues (Akhter *et al.,* 2017). There is a need to develop methods that are inexpensive and result in a less secondary waste generation; microbe related technologies may provide an alternative or addition to the conventional methods of metal removal or metal recovery (Shammi and Ahmed, 2013).

### 2.8 Biological Techniques

Bioremediation is the technique of employing microbial systems to remove contaminants from polluted sites (Pratush *et al.,* 2018). Bioremediation methods such as bioaugmentation, bioaccumulation, biosorption, phytoremediation, rhizoremediation and biomethylation, or change the organic metallic complex to radionuclides (Irawati *et al.,* 2019); they are good alternatives to remove pollutants from the environment and are considered as more eco-friendly, cost-effective owing to their natural occurrence and easy availability to treat large volumes of industrial effluents and high selectivity in terms of removal and recovery of specific metals (Cai *et al.,* 2019).

Bioremediation is a natural process involving the capabilities of intrinsic bacteria to clean the environment (Ayangbenro and Babalola, 2017).

The use of microorganisms to remove heavy metals has gotten a lot of interest in recent years. Various microorganisms such as bacteria (Afzal *et al.,* 2017), microorganisms

co-culture as fungi and bacteria (Qurbani and Hamzah 2020), microphytes (algae and duckweed) by (Khalid and Rashid, 2020) have been reported to resist and remove heavy metals from aqueous solutions.

To remove heavy metals and organic compounds from wastewater, microorganisms have been utilized as biosorbent. Microbial cells, both alive and dead, are employed to transform or adsorb heavy metals and their metabolites, and they can be a very efficient bioaccumulator (Elbasiouny *et al.,* 2021).

In comparison with traditional physicochemical techniques, bioremediation have some advantages: low costs, low production of secondary wastes, and minimal risks for environments; however, bioremediation of heavy metals has some limitations; among those are the slow rates of this process in nature (Osman *et al.,* 2019).

Naturally occurring bacteria that are capable of metal accumulation have been extensively studied because that some single bacterium could be capable to remove high levels of heavy metals from polluted sites (Hussein *et al.,* 2003). The strategy of bioremediation by bacteria depends on having a bacterium with the ability to break down or transform the complex and toxic contaminant into the simpler or less toxic compound (Gummersheimer and Giblin 2003).

The heavy metal transforming microbial species can be isolated from both aerobic and anaerobic environments, in comparison to anaerobic bacteria; aerobic microorganisms are used more commonly in bioremediation methods (Azubuike *et al.,* 2016).

Heavy metal bioremediation utilizing microorganisms has received a great deal of attention, not only because of its scientific novelty but also because of its potential industrial applicability. Bisorptive (passive) absorption by nonliving, non-growing biomass or biomass products and bioaccumulation by living cells are the two types of metal accumulative bioprocesses (Doenmez and Aksu, 2001).

Once, the toxic metals are adsorbed and/or transferred within organic materials; they can be removed from wastewater (Irawati *et al.,* 2017b), heavy metal pollution can be removed by microorganisms via biosorption, covalent binding, redox interactions, extracellular precipitation, or a combination of these mechanisms (Cavalier-smith, 2005).

The direct use of microorganisms with specific catabolic potential and/or their products, such as enzymes and biosurfactants, is a novel approach to enhance and improve their remediation efficacy. Biofilm-mediated bioremediation can be applied for cleaning up heavy metal contaminated environments (Igiri *et al.,* 2018).

The biological method is limited by the difficulty in isolating microorganisms and growing plants for bioremediation, as well as the microbes' and plants' adaptation abilities, which are insufficient for practical application (Karn *et al.,* 2021).

## 2.9 *Raoultella* sp. for bioremediation

*Raoultella* sp. was initially classified in the genus *Klebsiella* as *Klebsiella ornithinolytica*, until the creation of the genus *Raoultella* in 2001, which is usually found in water and soil environments.The *Raoultella* genus is named after Didier Raoult, a French bacteriologist from the Université de la Méditerranée in Marseille, France (Hajjar *et al.*, 2020).

The incidence of human disease caused by *R. ornithinolytica* is low with no previously reported cases of clinical infections requiring treatment. The low prevalence of *R. ornithinolytica* related infections is a good point to use this bacterium as environment friend bacteria, *R. ornithinolytica* and *R. planticola* are two closely related species that are difficult to distinguish using phenotypic approaches. Data from 16S rDNA sequencing investigations revealed high DNA homology between *R. ornithinolytica* and *R. planticola*, with these bacteria clustered together. (Dang *et al.,* 2020).

Many of *Raoultella* sp. have been isolated as environmental strains, some of them have the ability to degrade different organic compounds, (Ping *et al.,* 2017) indicated that the *R. planticola* is a promising polycyclic aromatic hydrocarbons degradation strain and demonstrated its potential in the remediation of mixed PAH contamination, also in a study done by (Zhang *et al.,* 2019).

The role of *Raoultella* sp. were examined in the degrading pyrethroid pesticides for the first time, and some of them are able to remove inorganic, e.g. nitrogen and phosphorus (Xie *et al.,* 2012). Physiological analysis showed that a novel strain of *Raoultella sp* may be involved in uranium removal from contaminated waters and sediments (Sklodowska *et al.,* 2018). Recently *Raoultella* sp. is used as an ureolytic strain for the precipitation of Pb in a study performed by (Eltarahony *et al.,* 2021).

Chapter Three

# Description of the Studied Area

# 3. Description Area

## 3.1 Description of the Study Area

The present study focused on the Tanjaro river which is located in Tanjaro, Sulaimani Governorate-Iraq, Tanjaro River is a permanent river situated 7 km southwest of Sulaimani city with the geographical coordinates of 35°16'35" N 45° 5 '9" E, as shown in (Figure 3.1 and 3.2)The river is formed by linking two major streams Kani-Ban and Qiliasan with other small tributaries, it starts in the Sulaimani Governorate between the Azmar and Baranan mountains and runs near the NW to SE border of Sulaimani city crossing many urban and agricultural regions (Mustafa, 2006) and passes through Tanjero valley until it reached Darbandikhan Dam (Rasheed and HamaKarim 2017).

In this study, six sampling sites were selected that designated as S1 to S6 (Table 3.1) that located at Qaragol, which is representing Tanjaro downstream. Along the area agricultural fields is present, different small factories and sewage inlet points, that discharg waste directly into the river.

Sampling was carried out and samples were analyzed for the determination of physicochemical and bacteriological parameters.

## 3.2 Climate

Iraqi Kurdistan is characterized by cold and rainy winter, long warm and dry summer. Autumn and spring are very short. Mediterranean cyclones move east to north-east over the region throughout the winter, invading the region, while Arabian Sea cyclones moving northward are passing over the gulf and carry a great amount of moisture which causes a large number of precipitations (Mustafa, 2006).

**Table (3.١ ) List of sampling sites and their geographical specification**

| Sites | Coordinates (North (N) , East(E | Site description |
|---|---|---|
| S1 | 35°35'44.44"N 45°60'19.55"E | Beginning of Qaragol region |
| S2 | 35°35'37.62" N 45°60'90.93"E | Near greenhouses |
| S3 | 35°35'25.64" N 45°61'43.19"E | Near small factories and sewage inlet points |
| S4 | 35°35'23.44" N 45°62'04.24"E | Close agricultural area |
| S5 | 35°35'65.74" N 45°62'26.11"E | Before Qaragol bridge |
| S6 | 35°35'64.31"N 45°62'75.25" E | After Qaragol bridge |

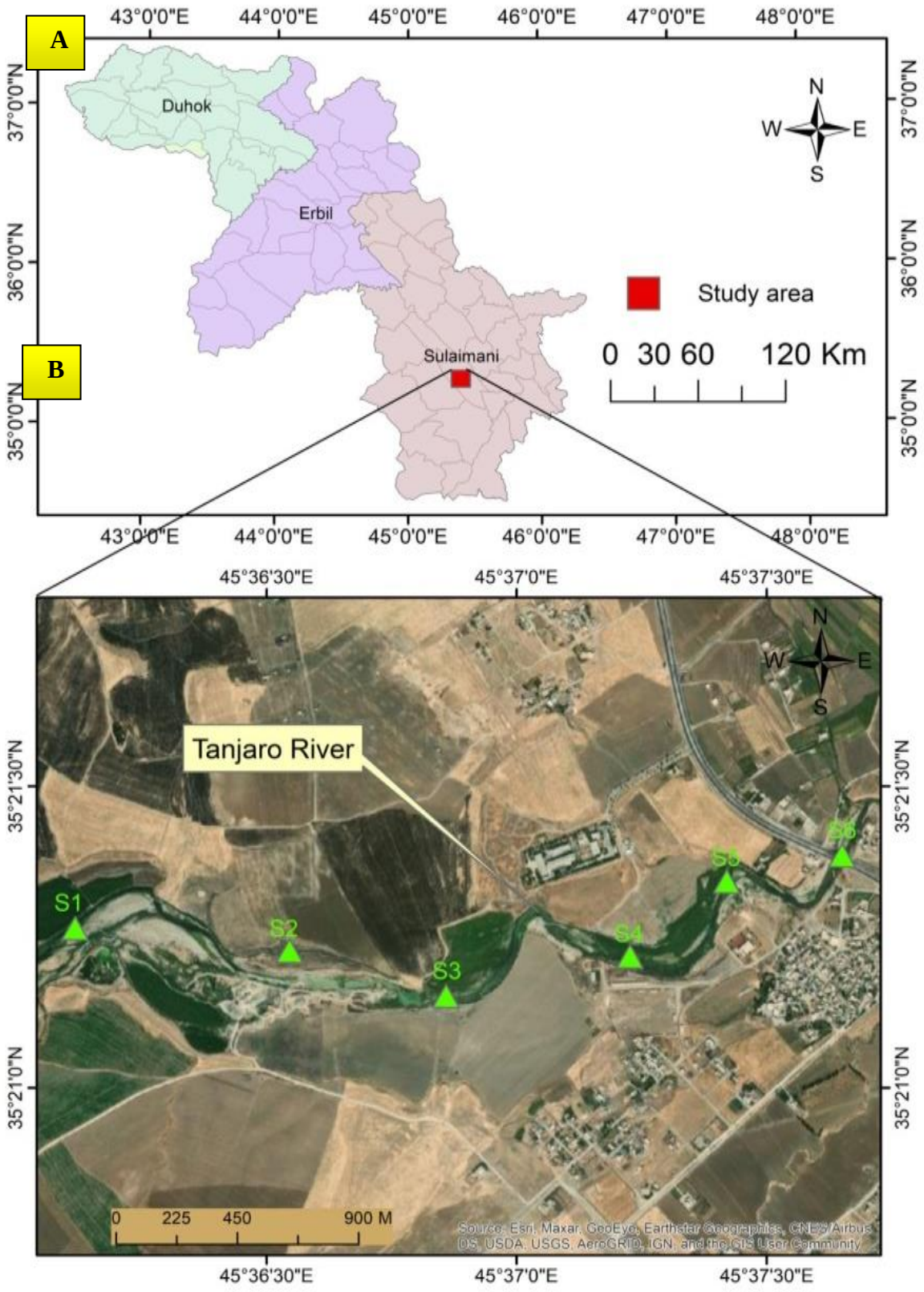



**Figure (3.1)** Map shows: **A-** Iraqi Kurdistan Region and the location of studied area, **B-**studied sites along the Tanjaro River (Google map 2019).


**Figure (3.2)** Shows water sampling sites S1-S6.

Chapter Four

# Materials and Methods

# 4. Materials and Methods

## 4.1 Materials

### 4.1.1 Apparatus and Equipment

The following Apparatus and Equipment were used in the present study:

**Table (4. 1) List of Apparatus and Equipment**

| No. | Apparatus and Equipment | Company | Origin |
|---|---|---|---|
| 1. | Autoclave | Memmert | Germany |
| 2. | Centrifuge | Sigma S-16P | UK |
| 3. | Dissolve oxygen meter | HANNA | USA |
| 4. | Genetic Analyzer 3500xl | Applied Biosystems | USA |
| 5. | hotplate | Harry Gestigkeit GmbH | Germany |
| 6. | Hotplate stirrer | Keison | UK |
| 7. | Incubator | EVOTEK | USA |
| 8. | Inductively coupled plasma optical emission spectrometer (ICP-OES) | Perkin Elmer-Optima 7300 | USA |
| 9. | Microcentrifuge | EVOTEK | USA |
| 10. | Multi meter (Temperature, pH, EC, TDS) meter | HANNA | USA |
| 11. | Oven | Shell lab | USA |
| 12. | spectrophotometer | Thermo Fisher Scientific | USA |
| 13. | Scanning Electron Microscope | Carl Zeiss SIGMA VP | Germany |
| 14. | Transmission Electron Microscope | Carl Zeiss-EM10C-100Kv | Germany |
| 15. | MultiDoc-it Digital Imaging system | BIO-RAD Gel Doc™ | USA |
| 16. | Sensitive balance | Sartorios | Germany |
| 17. | Shaker incubator | Bibby Scientific | UK |
| 18. | Shaker water bath | Labocon lswb-103 | UK |
| 19. | Vortex | Dragon Lab | Israel |

### 4.1.2 Chemical and Reagents

**Table (4. 2) List of Chemicals materials**

| No. | Chemical materials | Company | Origin |
|---|---|---|---|
| 1. | Nitric acid | Carl ROTH | Germany |
| 2. | Hydrochloric acid | Carl ROTH | Germany |
| 3. | Sulfuric acid | Carl ROTH | Germany |
| 4. | Phosphoric acid | Carl ROTH | Germany |
| 5. | Ammonia | Carl ROTH | Germany |
| 6. | Ethanol 96% | Carl ROTH | Germany |
| 7. | Ethanol absolute 100 | Carl ROTH | Germany |
| 8. | $Na_2SO_3$ | BDH chemicals Ltd | England |
| 9. | NaCl | Carl ROTH | Germany |
| 10. | Methyl orang | Polska Przychodnia | Poland |
| 11. | Phenophthaline | Merck | Germany |
| 12. | methanol | Carl ROTH | Germany |
| 13. | NaOH | Carl ROTH | Germany |
| 14. | $AgNO_3$ | Merck | Germany |
| 15. | Luria-Bertani Agar | Carl ROTH | Germany |
| 16. | Luria-Bertani broth | Carl ROTH | Germany |
| 17. | Nutrient agar | Carl ROTH | Germany |
| 18. | Nutrient broth | Carl ROTH | Germany |
| 19. | Cetrimide agar | Carl ROTH | Germany |
| 20. | Eosin methylene blue agar | BIOCHEM | France |
| 21. | MacConkey agar | Carl ROTH | Germany |
| 22. | Glycerol | IVDCE | Turkey |
| 23. | Copper sulfate anhydrate $CuSO_4$ | Carl ROTH | Germany |
| 24. | $CdSO_4 \cdot 4H_2O$ | Carl ROTH | Germany |
| 25. | Pb $(CH_3COO)_2 \cdot 3H_2O$ | IVDCE | Turkey |
| 26. | $K_2Cr_2O7$ | Carl ROTH | Germany |
| 27. | $Ni(NO_3)_2 \cdot 4H_2O$ | Carl ROTH | Germany |
| 28. | $Zn(CH_3CO_2)_2$ | Carl ROTH | Germany |
| 29. | $COCl_3 \cdot 6H_2O$ | Carl ROTH | Germany |

| | | | |
|---|---|---|---|
| 30. | Iron chloride | Carl ROTH | Germany |
| 31. | Primers | Macrogen | Korea |
| 32. | 10x Tris-Borate-EDTA Buffer (TBE buffer) | GeNet Bio | Korea |
| 33. | Agarose standard | Carl ROTH | Germany |
| 34. | Gram stain | ATOM SCIENTIFIC | UK |
| 35. | Sodium Dodecyl Sulfate (SDS) | Carl ROTH | Germany |
| 36. | Ethidium bromide | Carl ROTH | Germany |

**Table (4. 3) List of Kits and Enzymes**

| No. | Items | Company | Origin |
|---|---|---|---|
| **1.** | Presto mini gDNA extraction kit | Geneaid Biotech Ltd | Taiwan |
| **2.** | Proteinase K | TransGen Biotech | China |
| **3.** | 1x Gel loading Buffer | Carl ROTH | Germany |
| **4.** | 100bp DNA Ladder | GeNet Bio | Korea |
| **5.** | 1Kb DNA Ladder | GeNet Bio | Korea |
| **6.** | 2X PCR Mastermix | GeNet Bio Korea | Korea |

**Table (4 .4) List of primers, all primers were synthesized by Macrogen, Korea**

| No. | Target gene | Primer (Forward and Reverse) | No. of samples analyzed | Amplified region (bp) | References |
|---|---|---|---|---|---|
| **1-** | 16SrRNA | F- AGAGTTTGATYMTGGCTCAG<br>R- ACGGYTACCTTGTTACGACTT | 40 | 1401 | Satokari *et al.,* 2001 |
| **2-** | *czcA* | F- GTTCACCTTGCTCTTCGCCATGTT<br>R- ACAGGTTGCGGATGAAGGAGATCA | 2 | 320 | Chen *et al.,* 2019 |
| **3-** | *pcoD* | F- CTGGCCACACTTGCCTGGGG<br>R- CACGCTACGGCGCCCAGAAT | 2 | 500 | Mourao *et al.,* 2015 |
| **4-** | *pbrT* | F- AGCGCGCCCAGGAGCGCAGCGTCTT<br>R- GGCTCGAAGCCGTCGAGRTA | 2 | 448 | Chen *et al.,* 2019 |
| **5-** | *chrB* | F- GTCGTTAGCTTGCCAACATC<br>R- CGGAAAGCAAGATGTCGATCG | 2 | 450 | Chen *et al.,* 2019 |
| **6-** | *nccA* | F- ACGCCGGACATCACGAACAAG<br>R- CCAGCGCACCGAGACTCATCA | 2 | 1141 | Abou-Shanab *et al.,* 2007 |
| **7-** | *iroN* | F- AAGTCAAAGCAGGGGTTGCCG<br>R- GACGCCGACATTAAGACGCAG | 2 | 667 | Messaili *et al.,* 2019 |

## 4.2 Methods

### 4.2.1 Preparation of Culture Media and reagents

#### 4.2.1.1 Nutrient, Luria Bertani agar and broth

According to the instructions of the manufactures of (Carl ROTH/ Germany), nutrient agar and broth (N.A, N.B), Luria Bertani Agar and broth (LBA, LB) culture media were prepared and autoclaved at 121°C (15 lb / inch2) for 15 minutes for subculturing, purification, checking macroscopic morphology of the isolates on the plate and for preservation purposes.

#### 4.2.1.2 MacConkey Agar

A differential medium used to prevent the growth of gram-positive bacteria; 51.5gm of the medium were dissolved in 1000ml distilled water and autoclaved for 15 minutes at 121°C, as directed by the manufacturer (NEOGEN/ USA). 20ml of the medium was poured into a Petridish after cooling to 45-50°C and allowed to harden for 20 to 30 minutes before being stored in the refrigerator (4°C).

#### 4.2.1.3 Eosin-Methylene Blue Agar (EMB)

Eosin-Methylene Blue Agar was used to isolate and identify the lactose fermenter *Escherichia coli*, colonies with a brilliant green metallic sheen. It was made by dissolving 36gm of the medium in one liter of distilled water and autoclaving for 15 minutes at 121°C, as mentioned by instruction manufacture Company (BIOCHEM/France). The sterilized medium was then cooled to 45-55°C, shaken to oxidize the methylene blue, and dispensed into sterilized Petri plates to solidify.

#### 4.2.1.4 Cetrimide agar

Cetrimide agar is used as selective and differential medium for the isolation and identification of *Pseudomonas sp.* It was made by dissolving 45.3gm of the medium in one liter of distilled water; adding 10ml of glycerol and boil to dissolve completely and autoclaving for 15 minutes at 121$^{o}$C as mentioned by instruction manufacture Company (Carl ROTH/ Germany), then cooling the medium to approximately 50°C and pour into sterile Petri dishes.

### 4.2.2 Gram stain set

Gram stain set kit is composed of Crystal violet solution, Gram iodine solution; Gram decolorized alcohol and safranin (ATOM SCIENTIFIC/ UK).

### 4.2.3 Preparation of 20% Sodium dodecyl sulfate (SDS) (w/v)

Sodium dodecyl sulfate was prepared by adding 20gm of SDS to 90ml of distilled water(D.W), then heated to 68°C and stirred with magnetic stirrer to assist dissolution, the volume was adjusted to 100ml with D.W and stored at room temperature (Shahriar *et al.*, 2012).

### 4.2.4 Preparation of Ethidium bromide

A stock solution of 1mg $ml^{-1}$ was prepared by dissolving 1gm of Ethidium bromide in D.W stirred with magnetic stirrer several hours to ensure that the dye has dissolved then the solution preserved in a dark bottle and store at room temperature as described by (Thabit *et al.*, 2020).

### 4.2.5 Preparation of metal solutions

The salts of $CdSO_4 \cdot 4H_2O$, $Pb(CH_3COO)_2 \cdot 3H_2O$, $CuSO_4$, $K_2Cr_2O_7$, $Ni(NO_3)_2 \cdot 4H_2O$, $Zn(CH_3CO_2)_2$, $CoCl_3 \cdot 6H_2O$, and $FeCl_3$ were used as a sources for (Cadmium-Cd, Lead-Pb, Copper-Cu, Chromium-Cr, Nickel-Ni, Zinc-Zn, Cobalt-Co, and Iron-Fe) respectively. Stock solutions of (1000 ppm) were prepared by dissolving certain amount of metal salts in distilled water. The metallic salts were of analytical grade, the stock solutions were filter-sterilized with 0.22 μm pore size Millipore membranes and added to 45°C sterilized medium (Silva *et al.*, 2012).

## 4.3 Sample collection and preparation for ecological study

Water samples were regularly collected during 10 months from January to October 2019; samples were analyzed monthly for physicochemical parameters and once per season for heavy metals and bacterial examination. All sample containers and laboratory glasses used in analytical processes were cleansed with hot water and soaked with 10% HCl solution followed by rinsing with distilled water, rinsed twice with the water sample, and then transferred to the laboratories of Charmo Center for Research and Training for the analysis. The samples were acidified with 1:1 $HNO_3$:D.W to a pH value of 2 for heavy metals detection to minimize the precipitation and adsorption to the container wall, and then were stored in refrigerators for later determination (APHA, 2017).

## 4.4 Field Analysis

The parameters of the site elevation, water temperature, hydrogen ion concentration (pH), Electrical conductivity (EC), and Total dissolved solids (TDS) were measured in the field.

### 4.4.1 Sites coordination

The Coordinates of the sites longitude, latitude and elevation were measured in the field using a Global Positioning System (GPS), Garmin model eTrex legend HCx.

### 4.4.2 Water temperature, Hydrogen Ion Concentration (pH), Electrical Conductivity (EC), Total Dissolved Solid (TDS) and Dissolved oxygen (DO)

These parameters were analyzed in situ with a portable water quality tester (HANNA) after calibration by appropriate solutions, results were expressed as μS/cm for EC and (mg $l^{-1}$) for TDS and DO measurement according to (APHA, 2017).

## 4.5 Laboratory analysis

### 4.5.1 Total Hardness (mg $CaCO_3$ $l^{-1}$)

The total hardness was determined using the EDTA–titrimetric method, as reported by (APHA, 2017). The titration was performed with a buffer solution of pH 10 and the Erichrom Black –T indicator against a 0.01M EDTA (di-sodium salt) solution. The following equation was used to calculate the results in mg $CaCO_3$ $l^{-1}$:

Total hardness (mg $CaCO_3$/l) = A× N×50 ×1000/ ml of sample

Where: A=volume of EDTA titrant

N=Normality of. EDTA

### 4.5.2 Total Alkalinity mg $l^{-1}$

After adding (5) drops of methyl orange to 50 ml of water samples and mixing with $H_2SO_4$ (0.01N), total alkalinity was evaluated using the titration method as specified by (APHA, 2017). Results were reported as mg $l^{-1}$ using the following equation:

Alkalinity as mg $CaCO_3$ $l^{-1}$= A×B×50000/ ml of sample

Where: A=ml of $H_2SO_4$ titrant

B=Normality of $H_2SO_4$

### 4.5.3 Biological Oxygen Demand Concentration ($BOD_5$)

The basic principle underlying the $BOD_5$ determination is the measurement of dissolved oxygen content before and after five days incubation at 20-21°C as recommended by (APHA, 2017), the was wastewater diluted and results were reported as mg $l^{-1}$ (Aniyikaiye *et al.,* 2019) using the following equation:

$BOD_5$ (mg $l^{-1}$) = ($DO_0$ - $DO_5$) * Volume of BOD bottle/ Volume of sample

### 4.5.4 Chloride ($Cl^-$) in mg $l^{-1}$

Argentometric method was used to determine $Cl^-$ anion by using silver nitrate ($AgNO_3$) as a titrant with the potassium chromate ($K_2Cr_2O_7$) as indicator (APHA, 2017).

### 4.5.5 Nitrate ion ($NO_3^-$) in mg $l^{-1}$

The nitrate nitrogen concentration was determined by chromotrophic acid method, in which the reaction between nitrate and the reagent causes a yellow tint in the sample, and the results examined using HI 83214 multiparameter bench photometer.

### 4.5.6 Sulfate ion ($SO_4^{-2}$) in mg $l^{-1}$

The turbidimetric method as described by (APHA, 2017) was used for sulfate determination when barium chloride was used, and results were recorded at the wavelength 420 nm within 30 seconds intervals and the $SO_4^{-2}$ concentration is determined by comparison of the readings with a standard curve of sulfate concentration in the range 0.0 to 40 mg $l^{-1}$.

### 4.5.7 Heavy metal measurement in the water samples (ppm)

The acidified samples were digested by adding 2 ml of 1:1 $HNO_3$ and 10 ml of 1:1 HCl, heating on a hot plate until the volume was decreased to 25 ml, cooling overnight, then adjusting the content to 100 ml by adding distilled water, whattman filter paper No.42 was used for sample filtration as described by (APHA, 2017). The analysis was conducted using an Optima 7300V inductively coupled plasma-optical emission spectrometer (ICP-OES) according to manufacture instructions. Argon gas with purity of 99.996 % was applied for analysis of all samples. The flow rate of the argon gas for axillary ICP torch was 0.2 L.$min^{-1}$. The nitrogen gas with purity of 99.999 % was applied for removing of water and air from the optic system of the ICP instrument. The axial view of the plasma was used for obtaining the results. All blanks, standards and samples were introduced to the ICP instrument using a peristaltic pump and nebulizing system. Before introducing each sample the nitric acid 2% W/W was introduced to remove the memory effect of the previous samples. Before

introducing the samples the instrument was calibrated using 24 element standards (ICP multi-element standard solution from Merck Company). The linear ranges for all elements were in the range of 0.01 to 500 $mg.l^{-1}$. The R2 values for all analysis were higher than 0.99.

## 4.6 Sample collection and preparation for microbiological study

During the study period, samples for bacterial analysis were collected once per season in sterilized pyrex glass containers with stopper and kept airtight to avoid any contamination and transferred in a cool box when the air temperature was more than 25°C.

### 4.6.1 Primary screening of heavy metal-resistant bacteria

Aseptically collected water samples were used to inoculate Erlenmeyer flasks containing L.B medium separately supplemented with 10 ppm of various heavy metal salts (Cd, Pb, Cu, Cr, Ni, Zn, Co, and Fe). After adjusting the pH to 7.0, the flasks were incubated in a shaker incubator at 37°C/120 rpm for 24–48 hrs. The growth culture was diluted 5 fold and spread on LB agar plates, incubated for 48 hrs at 30°C. Preliminary identification of bacteria was done based on standard microbiological techniques including microscopic examination, colony characteristics of the bacteria, Gram's stain, colonies were selected for further isolation to obtain single colonies (Aktan *et al.*, 2012; Aka and Babalola, 2017).

#### 4.6.1.1 Gram Stain

The overnight incubated pure colonies were identified using gram staining. Thin smears were prepared, air-dried, heat-fixed, stained for one minute with crystal violet, and softly rinsed with distilled water. It was then flooded for one minute with iodine solution and decolorized for one minute with 95% ethanol; the slide was washed with distilled water, air-dried, and observed under a light microscope at $100_X$ magnification using oil immersion (Prescott, 2002).

#### 4.6.1.2 Oxidase Test

The filter paper strip was saturated with oxidase reagent (1% of dimethyl-p-phenylenediamine-dihydrochloride) and placed in a petridish, an overnight colony from the tested organism was transferred to the filter paper and rubbed onto the reagent with an applicator stick. A purple color should develop in 10 sec. which is the positive reaction that indicate the presence of oxidase enzyme in bacterial isolates (Faraj, 2011).

#### 4.6.1.3 Catalase Test

A loopful of pure growth was deposited onto the surface of a clean, dry glass slide, and then a drop of freshly prepared 3% $H_2O_2$ was instantly applied onto the apportion of the colony on the slide, the development of gas bubbles indicated a positive result (Alexander *et al.,* 2001).

### 4.6.2 Determination of maximum tolerable concentration of heavy metals

The maximum tolerable concentration to eight selected metal salts was carried out separately using the 96-well microtiter plate method. Bacterial isolates were precultured for 24 hr in liquid L.B medium at 37℃/120 rpm till reach an optical density of 0.6 at 600 nm. Next, 50 µl of the preculture was added to 150 µl of L.B broth containing 20 ppm of a separate heavy metal compound as a starter. The mixture was transferred into a 96-well microplate and incubated at 37℃/120 rpm for 48 hr, at which point the maximum tolerable concentration (MTC) was determined using a microplate reader. The MTC was defined as the maximum heavy metal concentration that permitted for development after two days. Heavy metal removal efficacy was tested on strains with the highest tolerance to each heavy metals as described by (Sultan *et al.,* 2020; Cai *et al.*, 2019) with some modifications.

### 4.6.3 Multiple metal resistance capacity

Metal resistance isolates were grown separately on autoclaved and cooled L.B agar medium integrated with filter-sterilized solutions of the eight heavy metals collectively (Cd, Pb, Cu, Cr, Ni, Zn, Co, and Fe) in equal ratio of (1:1:1:1:1:1:1:1ppm) with the pH adjustment to 7.0 and incubated at 37±2℃/120 rpm for 24 hr; whereas the resistance potential of multiple heavy metals was assessed after incubation, adapted from (Afzal *et al.,* 2017) with slight modifications.

### 4.6.4 Determining of heavy metal removal efficacy

The heavy metals removal potential of the tolerant bacteria was evaluated in a batch experiment process. A 500 ml bottle containing 200 ml of L.B broth and eight metal ions (Cd, Pb, Cu, Cr, Ni, Zn, Co, and Fe) were separately prepared according to MIC value and inoculated with 2 ml of 18-24 hr old bacterial culture with OD600 of 0.6. The cell culture was incubated at 37°C and 120 rpm for 24 hr. The culture was then centrifuged (Sigma S-16P) at 5000 rpm for 20 min. The supernatant was digested with $HNO_3$ at 100°C. ICP-OES (Optima 7300 V) was used to identify heavy metal concentrations in the medium before bacterium inoculation and after 24 hours of culture. The same treatment without the inoculation of bacterial strains

was used as a control for each heavy metal as described by (Afzal *et al.*, 2017; Marzan *et al.*, 2017).

For the multi-metal removal assays, 20 ppm of each metal (Cd, Pb, Cu, Cr, Ni, Zn, Co, and Fe) was used. The assay was carried the same way as the mono-metal system, except that the dialyzed cultures were transferred to aqueous solutions containing a combination of all eight metals. (Cd, Pb, Cu, Cr, Ni, Zn, Co, and Fe) as mentioned by (Bowman *et al.*, 2018).

The results were compared with the control to calculate the heavy metal remediation capacity (%) as follows: % of heavy metal utilized =

The heavy metal utilized / Heavy metal added to the L.B broth ppm ×100

The heavy metal utilized = Heavy metal added to the LB broth – Heavy metal remaining at the end of culture.

## 4.7 Molecular Bacterial identification

### 4.7.1 Extraction of Genomic DNA from Bacterial Isolates

Genomic DNA from all the 40 bacterial isolates were extracted and purified by using Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., New TaipeiCity, Taiwan) according manufacturer's protocol. A single colony of heavy metal resistance bacterial isolates was grown in 5ml of L.B broth for 24hr at 35±2°C. The over night culture that has 0.6 optical density at 600nm was transferred to 1.5 ml microcentrifuge tube. Then genomic DNA was extracted as follows: cells were collected in the microcentrifuge tube by centrifugation at 14000 rpm for 1 minute; the supernatant was discarded by pipetting.

After adding GT buffer for gram negative bacteria; the pellet was re-suspended by vortexing or pipetting. While, for gram positives; 200 µl of Gram+ Buffer was added and incubated at 37ºC for 30 minutes. During incubation, the tube was inverted every 10 minutes.

The mixtures were vortexed after adding (20 µl) of Proteinase K and incubated for at least 10 minutes at 60°C. GB buffer was added to the samples and mix by vortexing in order to lysis the bacterial cells. For DNA binding, 200 µl of absolute ethanol was used and mixed then transferred to the GD Column and centrifuged at 14000 rpm for 2 minutes. The 2 ml collection tube containing the flow-through was discarded and the GD column was put in a new 2 ml collection tube. Washing buffer used several times to remove any debris found.

The column was dried by centrifugation; 100µl of pre-heated elution buffer was added into the center of the column matrix and incubated for at least e minutes at room temperature and centrifuged for 30 seconds at 14000 rpm to be eluted. The binding column was discarded and the genomic DNA was stored at 4°C.

### 4.7.2 DNA amplification

Conventional PCR (polymer chain reaction technique) analysis was performed for fourt bacterial isolates using universal bacterial 16S rRNA primers, forward 7F (5′AGAGTTTGATYMTGGCTCAG-3′) and 1015R (5′ACGGYTACCTTGTTACGACTT-3′) designed by (Satokari *et al.*, 2001). Ready-to-use PCR mixtures were prepared to conform to manufacturer protocol and the reaction constituent concentration were as presented in (Table 4.5)

The PCR reactions were performed in a thermocycler (Mega Cycler PCR) according to (Zagui *et al.*, 2020), it was run under an optimized condition of amplification using the following cycling instructions: 95°C for 5 min (initial denaturation), and 30 cycles of 95°C, 30 sec (denaturation), 60°C, 30 sec (annealing), 72°C, 30 sec (extension) and a final extension of 72°C for 5 min, finally a 4°C hold. The PCR product was run on gel electrophoresis.

**Table (4. 5) PCR master reaction for the identification of bacterial isolates**

| No. | Reaction Components | Volume |
|---|---|---|
| 1 | Template DNA 50 ng. | 3 µl |
| 2 | Forward primer 10 pmol/ µL | 1 µl |
| 3 | Reverse primer 10 pmol/ µL | 1 µl |
| 4 | EasyTaq® PCR SuperMix(2×) | 10 µl |
| 5 | dH2O ( DNase , RNase free) | 5 µl |
| 6 | Total Volume | 20 µl |

### 4.7.3 Gel Electrophoresis

The gel electrophoresis was performed by dissolving 1.5gm of pure agarose powder (Carl ROTH/Germany) in 100 mL of 1X Tris Borate EDTA (TBE) buffer to make a 1.5% agarose gel. In a microwave oven, the mixture was boiled until the agarose was dissolved and fully combined by gentle swirling. After cooling, safe dye was added to the gel and mixed thoroughly. The melted agarose solution was carefully poured into the casting chamber and left at room temperature to solidify. 5µl of PCR products were mixed with 1µl of 6X loading buffer and loaded into the wells.

DNA ladder was run alongside the samples to serve as an indicator for the sizes of the bands. The DNA was electrophoresed using 90 Volts for 1.5 hours. Finally, for DNA visualization, the gel was examined and documented, the fluorescent safe dye-intercalated DNA bands and the gel image was captured via BIO-RAD Gel Doc™ XR+ Imaging System (USA).

### 4.7.4 DNA sequencing

The purified amplicons of the 40 bacterial samples were sequenced using the Sanger method using a 3500xl Genetic Analyzer (Applied Biosystems), including the same forward and reverse PCR primers strands by the Macrogen Inc. (Daejeon, Republic of Korea). Multiple sequence alignment of all the sequences obtained in the present study was carried out using the Bio-Edit version 7.2.5 software program.

The consensus sequences were submitted to GenBank (National Center for Biotechnology Information, Bethesda, MD, USA) to assign accession numbers and then Blasted against each other as well as the contents of the GenBank database (http://www.ncbi.nlm.nih.gov/Blast.cgi).

### 4.7.5 Phylogenetic analysis

The phylogenetic trees of all the sequence data collected from metal resistant bacterial strains were created based on the sequences of 16S rDNA genes using MEGA X version 10.7.1 software program (Kumar *et al.,* 2018). The trees of all isolated species were constructed based on the neighbor-joining method with 1000 bootstrap replicates (Tamura *et al.,* 2013).

## 4.8 Optimization of heavy metal removal factors of *Raoultella ornithinolytica:*

Temperature, incubation time, and pH are the factors which affects the metal removal process according to (Das and Kumari, 2016).

### 4.8.1 Effect of different incubation temperature

The bacterial isolate that record the highest rate of metal removal *(R. ornithinolytica)*. 0.5 ml od over night cultur that have optical density of 0.6 at 600 nm, was inoculated into a flask containing 100 ml of L.B medium supplemented separately with the eight metal ions according to MTC concentration. After the addition of metal solutions, media was adjusted at pH=7 by using 0.1 N NaOH. The cultures were incubated at different temperatures (15, 25, 35, 45 °C) at 120 rpm for 24 hr. The incubated cultures were centrifuged at 5000 rpm for 20 min. The supernatants were used for the determination of the residual metal ion contents by using ICP-OES (Optima 7300 V). Control cultures without the inoculation of bacteria were prepared to detect the initial metal concentration. Heavy metal concentrations in the medium before and after bacterial inoculation were determined as previously.

### 4.8.2 Effect of contact times

The percentage removal of metals was determined for a different time intervals (18, 24, 48, and 72hr) by incubating the selected isolate at 35°C, The initial and the residual concentrations were measured as mentioned before.

### 4.8.3 Effect of different pH values

To find out the optimum pH for maximum metal uptake, various pH were used (4, 7, and 9) by adjusting the medium supplemented with different types of metal ions. All the cultures were incubated at 35°C for 24 hr. In the batch culture, the culture conditions were maintained for optimal microbial growth. All the tests were performed in triplicates. Heavy metal concentrations in the medium before and after bacterial inoculation were determined as previously.

## 4.9 Effect of different heavy metals on *R. ornithinolytica* growth

A growth curve experiment was conducted in L.B broth for the isolate that record the highest rate of metal removal *R. ornithinolytica*. For this purpose 250 ml. flasks containing 100 ml L.B medium supplemented with different heavy metals (Cd, Pb, Cr, Cu, Ni, Zn, Co, and Fe) separately according to MTC value. The control flask was not supplemented with any metals. Flasks inoculated with 0.5 ml of overnight culture, incubated in shaking incubator at 37°C/120 rpm. After 0, 4, 8, 12, 16, 20, 24 and 28hr. Growth was monitored as a function of biomass by measuring the absorbance at 600 nm using the spectrum SP-2000UV spectrophotometer, Growth curve was plotted by the readings obtained from the experiment and compared (Afzal *et al.,* 2017).

## 4.10 Field emission scanning electron microscopy (FE-SEM) and energy dispersive X-ray spectroscopy (EDS) analysis

Field emission scanning electron microscopy and dispersive X-ray spectroscopy were conducted for characterization of *R. ornithinolytica* before and after treatment with heavy metals to detect any change in the morphology of the cells as a result of metal treatment. The bacterial cultures with and without heavy metals were centrifuged for 5min at 8000 rpm/min. Collected bacteria and sediments were rinsed three times in Phosphate Buffer Saline PBS for 5 min. each time, and then pre-fixed on a grid with an aldehyde (2.5 % (v/v) glutaraldehyde) in PBS for 3hrs at 4 °C. The fixative was rinsed and washed three times in PBS for 5min. each time. After that, 1hour at room temperature was spent post-fixing with 0.5 % (v/v) osmium tetroxide in de-ionized water. The fixative was then removed and washed 3 times in

de-ionized water for 5 min. each time. Samples were dehydrated in a series of ethanol and hexamethyldisilazane (HMDS) solution (Sigma, Australia) as follows: 50%, 70%, 80%, 90%, 95%, 100% ethanol, the samples were dried in 2:1 analytical grade 1:1 ethanol/HMDS, followed by drying twice in pure HMDS (100%) for 10 min for each treatment, then samples were left in a fume hood overnight. Subsequently, the dried samples were sputter-coated with gold for 120 sec at 22 Kv (JunYe *et al.,* 2015), samples were scanned with FESEM in a low-vacuum mode using (ZEISS MODEL SIGMA VP-Germany) Field Emission Scanning Electron Microscope (FE-SEM) and Energy-Dispersive X-ray Spectroscopy (EDS) detector (Oxford instrument), with the accelerating voltage applied at 15 kV for FE-SEM and 20 Kv for EDS images (Jiang *et al.,* 2019).

## 4.11 Transmission Electron Microscope (TEM)

Transmission electron microscopy (TEM) is used to identify the location of heavy metal particles within the cells. The bacterial cell of *(Raoultella ornithinolytica)* was inoculated in LB broth and grown at 37°C/120 rpm until the optical density (OD) reached 0.6. (600nm). Heavy metals were subsequently added to the growth medium according to MIC's value and cultured for additional 24 hours at 37°C, the cells without any treatment served as control. The 48-hour-old bacterial culture was harvested by centrifugation and washed with PBS several times. The cells were fixed with an equal volume of 3 % glutaraldehyde and left at room temperature for 2 hr and incubated overnight at 4°C, followed by post-fixed with 1% osmium tetroxide ($OsO_4$)for 2 hr and rinsed with PBS. After washing, the specimen was dehydrated using a series of ethanol treatments (30, 50, 70, 80, 90, 95, and 100 %). The dehydrated specimen was embedded in spurs resins and incubated for 4 hours at 25 °C. Polymerization was achieved by incubating the specimen at 65°C for 24 hr. The solidified specimen was sectioned and stained for 5 and 10 minutes with uranyl acetate and alkaline lead, respectively, and examined by (TEM Carl Zeiss-EM10C-100Kv-Germany) Modified procedure of (Upadhyay *et al.,* 2017).

## 4.12 Extraction of plasmid DNA

The *Raoultella* sp. isolates was analyzed for its plasmid content according to the protocol provided by the manufacturer High-Speed Plasmid Mini Kit (Geneaid Biotech Ltd., New Taipei City, Taiwan).the extraction process consist of harvesting, suspension, lysis, neutralization, DNA binding, wash, and DNA elusion steps.

## 4.13 Plasmid curing

To determine if the resistance genes were encoded by plasmids, 0.5 ml of overnight cultures were used to inoculate 4.5 ml L.B containing different concentrations of curing agents, Sodium Dodecyl Sulfate SDS (8, 10, 12 % w/v) and Ethidium bromide (1.0 to 10 µg/ml) as described by (Raja and Selvam, 2009). An orbital shaker with 120 rpm was used to incubate the culture for 48 hours. After incubation, 0.5 ml of the culture was spread on L.B agar without heavy metals and another L.B agar contains 10 ppm of different heavy metals. After a 24-hour incubation at 37°C, the cured plasmid cells were detected comparing the development of bacterial colonies on heavy metal-containing plate with that of the normal (without heavy metals) plate. The samples that showed colonies on normal LB agar but failed to grow on LB agar supplemented with different heavy metals were the possible cured isolates (Zaman *et al.*, 2010).

## 4.14 PCR Amplification of heavy metal resistance genes

Primers that targeting the (cadmium, zinc, and cobalt efflux pump) genes *czcA*; copper resistance genes *pcoA* (copper efflux pump); chromate resistance genes *chrB*, lead resistance gene *pbrT*, Nickel resistant gene *nccA*, and iron resistant gene *iroN* were used to amplify metal-resistance encoding genes as described in (Table 4.4). The primers can amplify 320, 500, 450,448, 1141, and 667 base pair respectively (Chen *et al.*, 2019). Ready-to-use PCR mixtures were prepared to conform to the manufacturer protocol and the reaction constituent concentrations were as presented in (Table 4.6).

**Table (4. 6) PCR master reaction for identification of bacterial resistant genes**

| No. | Reaction Components | Volume | |
|---|---|---|---|
| 1 | Template DNA | 3 µl | |
| 2 | Forward primer 10 pmol/ µL | 1 µl | For each genes |
| 3 | Reverse primer 10 pmol/ µL | 1 µl | |
| 4 | EasyTaq® PCR SuperMix(2×) | 10 µl | |
| 5 | dH2O ( DNase , RNase free) | 5 µl | |
| | Total Volume | 20 µl | |

The reaction tubes were placed in Thermal cycler (Mega Cycler PCR) and it was run under an optimized condition of amplification as summarized in (Table 4.7)

**Table (4.7) Thermocycler PCR condition for detecting metal resistant genes**

| Reaction | Cycling conditions | | | | |
|---|---|---|---|---|---|
| Gene (*chrB* ) | Initial denaturation | denaturation | Annealing | Extension | Final extension |
| | 94°C 5min | 94°C 30 sec | 58°C 30 sec | 72°C 30 sec | 72°C 5 min |
| Gene (*nccA, iroN* ) | 94°C 5min | 94°C 30 sec | 60°C 30 sec | 72°C 30 sec | 72°C 5 min |
| Gene (*pcoA, czcA, pbrT* ) | 94°C 5min | 94°C 30 sec | 62°C 30 sec | 72°C 30 sec | 72°C 5 min |
| Number of cycles 30 | | | | | |

## 4.15 Preservation of bacterial isolates

Following the complete identification, a pure culture of each isolate was retained and conserved for further research using glycerol freezing, as stated by (Prakash *et al.*, 2012), bacterial preservation is achieved by mixing 500µl of an overnight growth culture with 500 µl of 30% glycerol solution (sterilized by autoclaving), ensure that the glycerol is evenly distributed, the mixture was vortexed and stored at -20°C for long term storage, which keeps them viable under all freezing temperatures.

## 4.16 Statistical analysis

The Statistical Package for the Social Sciences (SPSS) program version 23 was used to statistically analyze the results. The analyzed parameters were processed using the variance method (ANOVA) followed by Tukey's-b tests, then data were expressed as mean ± standard error (Ravanbakhsh *et al.,* 2009). Two-way analysis of variance (ANOVA) multiple comparison test at the 95% confidence level ($P<0.05$) was used to evaluate significant differences between the various treatment options. while, Spearman's test was used to compare between physicochemical parameters and heavy metal levels in the water samples.

Chapter Five

# Results and Discussion

# 5. Results and Discussion

## 5.1 Physical and Chemical Characteristics

### 5.1.1 Water Temperature (ºC)

Water temperature is one of the important factors that affects the rate of numerous biological and chemical processes in the water system, as well as the amount of oxygen gas that can dissolve in the water (Al-Enazi, 2016), also directly or indirectly influences the biological species that can survive in a given aquatic environment (Iram *et al.*, 2013).

Water temperature in the current study ranged between 11.9 - 31°C in all studied sites during the study period. The lowest water temperature was 11.9°C recorded in January 2019 in S1, while the highest was 31°C in August in S1 and S6 (Table 5.1). No abnormal water temperatures were recorded for the water samples. Statistical analysis indicated that there were only significant differences between months in the studied area (P≤0.05). It appears that the coldest temperature was recorded in January, while the warmmer one was during August; similar temperature ranges have been previously documented by (Mustafa, 2006) at Tanjaro River.

**Table (5.1) Water temperature (ºC) represented as (mean ±S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean ±SE** |
| **Jan.** | 11.9 | 12.1 | 12.1 | 12.3 | 12.5 | 14.3 | 12.54±0.24 a |
| **Feb.** | 15.4 | 15.4 | 16.1 | 16.6 | 15.8 | 15 | 15.75±0.16 b |
| **Apr.** | 19 | 19.9 | 19.9 | 20.3 | 20.3 | 20.6 | 20.02±0.15 c |
| **May** | 21.1 | 21.1 | 21.5 | 21.4 | 22.2 | 21.4 | 21.54±0.1 d |
| **Jun.** | 26.6 | 27.3 | 27.9 | 28 | 27.8 | 25.7 | 27.24±0.24 f |
| **Jul.** | 30 | 30 | 30.1 | 29 | 29 | 30 | 29.69±0.14 g |
| **Aug.** | 31 | 30 | 30 | 30 | 30.6 | 31 | 30.44±0.13 h |
| **Sep.** | 27.9 | 27 | 27.8 | 27.5 | 28.2 | 28 | 27.73±0.11 f |
| **Oct.** | 24 | 24 | 29 | 24 | 25 | 24 | 24±0.000 e |
| **Mean ±SE** | 22.99±1.5 a | 23.03±1.4 a | 23.28±1.4 a | 23.24±1. a | 23.39±1.4 a | 23.35±1.3 a | 23.21±1.38 |

### 5.1.2 Hydrogen Ion Concentration (pH)

Hydrogen ion (pH) indicates the level of acidity, it is a measure of the concentration of hydrogen ($H^+$) ions in a given aquatic ecosystem, in the aquatic system any increase or decrease in the pH rate leads to disturbance the chemical balance of water (Hantoush, 2006). It is an important factor in assessing water quality because it affects on the other chemical properties such as mineral solubility and metal toxicity (AL- Taei *et al*., 2020). The results of pH values of wastewater are shown in (Table 5.2) the minimum value (6.1) was recorded in S6 during October, while the maximum (8.64) was recorded in S2 during August.

Statistical analysis of the results indicated that there were significant differences among studied months and sites at ($P \leq 0.05$). The recommended pH range of surface water according to WHO (2017) is 6.5–8.5 where keeps most trace elements immobilized, while the ideal pH value for bacterial growth is usually between 6.5 and 7.5. Most of the collected samples had pH values within the WHO range except samples from S2 and S6.

In the present study pH of wastewater is characterized by a shift towards the alkaline side of neutrality, due to the geological formation of the area which is composed mainly of $CaCO_3$ and this may be related to the soil and watershed characters (Abdullah *et al.,* 2017), similar results obtained by (Ahmed, 2020). The pH was highest in the samples of S2 (8.64) during August, which are slightly higher than the WHO recommended range for wastewater, (Besharati *et al.*,2018) suggested that it is likely because of the reduced rainfall and river volume during that time, while Aziz *et al.*, (2012) indicated that this elevation in pH level may be resulted from an increase in both photosynthetic activity and sewage disposal with high detergent concentrations, then a sharp decrease in the pH level was observed in September after a rainfull, which may be due to the fertilizers washing out from the agricultural lands along the area, (Hassan and Al-Barware, 2016) also concluded that the water pH is affected by the nature of pollutants that reach the water sits, such as fertilizers.

**Table (5.2) pH values represented as (mean ±S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean ±SE** |
| **Jan.** | 7.32 | 7.3 | 7.34 | 7.33 | 7.18 | 7.16 | 7.27±0.22 b |
| **Feb.** | 7.28 | 7.28 | 7.3 | 7.2 | 7.35 | 7.35 | 7.29±0.01 b |
| **Apr.** | 6.88 | 6.94 | 7.01 | 7.14 | 7.23 | 7.22 | 7.07±0.04 b |
| **May** | 7.2 | 7.2 | 7.27 | 7.36 | 7.31 | 7.27 | 7.26±0.01 b |
| **Jun.** | 7.4 | 7.2 | 7.19 | 7.1 | 6.9 | 7 | 7.13±0.04 b |
| **Jul.** | 7.1 | 7.04 | 6.8 | 6.7 | 6.7 | 6.5 | 6.81±0.06 a |
| **Aug.** | 8.39 | 8.64 | 8.54 | 8.35 | 8.26 | 8.39 | 8.42±0.03 c |
| **Sep.** | 6.8 | 6.9 | 6.7 | 6.3 | 6.8 | 6.6 | 6.68±0.05 a |
| **Oct.** | 7.1 | 7.5 | 6.5 | 6.3 | 6.5 | 6.1 | 6.69±0.14 a |
| **Mean ±SE** | 7.28±0.1 bc | 7.33±0.12 c | 7.18±0.13 abc | 7.08±0.14 a | 7.14±0.11 ab | 7.07±0.14 a | 7.18±0.05 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.3 Electrical Conductivity (EC) µS.cm$^{-1}$and Total Dissolved Solid (TDS) mg l$^{-1}$:

The ability of an aqueous solution to convey an electric current is expressed numerically as conductivity (Solanki *et al.,* 2011); this ability is affected by total dissolved solids and also depends upon the number of ions in the water.

According to the results obtained during the studied period (Table 5.3), electrical conductivity values were ranged from 525 µS.cm$^{-1}$ to 928 µS.cm$^{-1}$, the lowest value was observed in February 2019 in S6; while, highest value of 928 µS.cm$^{-1}$ recorded during September and October 2019 in S1and S6 with the mean of 689.1µS.cm$^{-1}$. The differences in EC values could be related to the dilution and the highest flow of wastewater during studied period, similar results were observed by (Ahmed, 2020), while the maximum mean of 837.6 µS.cm$^{-1}$ recorded during October.

Values of TDS in the water samples aspresented in (Table 5.4) ranged between 268 mg $l^{-1}$and 464 mg $l^{-1}$, the highest value was recorded in S1 during September 2019, and the lowest value was recorded in S6 in February.

From the statistical analysis view, it appeared that the maximum value of TDS for the studied sites was 361 mg $l^{-1}$ recorded in S5, while the minimum mean value 276.83 mg $l^{-1}$was recorded in February. The statistical analysis for EC and total dissolved solids, showed significant differences ($P \leq 0.05$) between months only during the studied period.

The conductivity is highly depending on the amount of total dissolved solids (such as salt), particulate mobility, and temperature (APHA, 2017) this was confirmed by observing a maximum value of EC and TDS in September. The electrical conductivity started from 525 µS $cm^{-1}$ in February which was relatively lower than that recorded by (Rashid, 2010) but higher than the results of (Aziz *et al.,* 2012; Ahmed, 2020), then it was increased as the study period progressed, reached 928 µS $cm^{-1}$ in September. The high EC ranges in water could be due to the nature of municipal pollutants, industrial wastes, and land use activities in the area, interactions between compounds created by oxidation and biological breakdown, decrease in water level and high evaporation balance, low water flows during warmer months, and high temperature due to climate change., as found by (Lateef *et al*., 2020).

The presence of a high concentration of dissolved solid elements could affect water density, osmoregulation, reduces the solubility of gases, and limits the use of water for drinking and irrigation (Azeez, 2021). As the water from the Tanjaro River is used for irrigation, much of the water will be taken up by the crop and transpired; a proportion of the salts will be left behind in the soil and lead to the build-up of salts in the root zone of the crop (Rashid, 2010).

**Table (5.3) Electrical conductivity (µS cm-1) at (25 ºC) represented as (mean ±S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
| Jan. | 583 | 580 | 576 | 570 | 570 | 589 | 578±2.067 a |
| Feb. | 560 | 562 | 556 | 556 | 555 | 525 | 552.3±3.7 a |
| Apr. | 555 | 674 | 740 | 804 | 779 | 601 | 692.1±27.4 b |
| May | 563 | 563 | 567 | 660 | 568 | 559 | 580.5±10.7 a |
| Jun. | 687 | 678 | 657 | 673 | 686 | 693 | 677.6±3.4 b |
| Jul. | 699 | 706 | 702 | 700 | 704 | 706 | 702.8±0.8 bc |
| Aug. | 745 | 571 | 748 | 750 | 739 | 745 | 746.3±1.2 c |
| Sep. | 928 | 922 | 796 | 794 | 794 | 775 | 834.8±19.3 d |
| Oct. | 796 | 794 | 794 | 794 | 922 | 928 | 837.6±18.6 d |
| **Mean ±SE** | 679.5±29.4 a | 692.6±27.1 a | 681.7±22.0 a | 699.8±21. a | 701±28.2 a | 680.1±29.0 a | 689.1±10.5 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

**Table (5.3) Total dissolved solids (mg l-1) represented as (mean ± S.E) of the water studied sites during the period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean ±SE** |
| **Jan.** | 291 | 290 | 289 | 278 | 286 | 295 | 288.17±1.5 ab |
| **Feb.** | 280 | 280 | 278 | 278 | 277 | 268 | 276.83±1.2 a |
| **Apr.** | 272 | 330 | 364 | 397 | 390 | 300 | 342.17±13.82 c |
| **May** | 281 | 281 | 283 | 330 | 384 | 279 | 306.67±11.7 b |
| **Jun.** | 344 | 339 | 328 | 336 | 339 | 348 | 339±1.8 c |
| **Jul.** | 350 | 350 | 346 | 350 | 351 | 350 | 349.5±0.48 cd |
| **Aug.** | 372 | 375 | 374 | 370 | 370 | 372 | 372.17±0.5 d |
| **Sep.** | 464 | 461 | 398 | 397 | 397 | 400 | 419.5±9.1 e |
| **Oct.** | 398 | 397 | 397 | 396 | 461 | 464 | 418.83±9.3 e |
| **Mean ±SE** | 339.1±14.9 a | 345±13.6 a | 339.6±10.9 a | 348±10.8 a | 361±13 a | 341.7±14.6 a | 345.87±5.2 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.4 Total hardness (TH) mg $CaCO_3$ $l^{-1}$:

Water hardness is caused by multivalent cations, but calcium and magnesium are the most abundant cations in natural waters. Water hardness can be of two types: temporary hardness, which is caused by the presence of calcium and magnesium carbonates and bicarbonates, and permanent hardness, which is caused by the presence of calcium and magnesium sulfates, chlorides, and nitrates (Bartram and Balance, 1996).

Total hardness of the water samples has been taken for all sites as shown in (Table 5.5), the lowest value was 232 mg $l^{-1}$, recorded at site 5 in April, while the highest was 485 mg $l^{-1}$recorded in June at S2 and April in site S5.

Dissolved calcium and, to a lesser degree, magnesium, which is expressed as an equal amount of calcium carbonate, causes water hardness (WHO, 2017).

| **Sites** | **Studied sites** |
|---|---|

Tanjaro River has hard water according to WHO guidelines, our recorded data exceeded 200 mg CaCO3.$l^{-1}$ WHO maximum recommendation. Results determined in this study agreed with (Ahmed, 2020) who record total hardness ranged between (210-585) mg $l^{-1}$ in Tanjaro River.

Ebrahimpour *et al.*, (2010) stated that water hardness affects the solubility and toxicity of heavy metals. Metals are more toxic in soft water than in hard water because their solubility increases with the decreasing of water hardness as in the present study, and it is known that the dissolved forms of heavy metals are the active toxic agents. Heavy metal concentration obtained by (Al-Asadi *et al.*, 2020) in shatt Al-Arab was lower than those obtained in our study with a higher level of calcium and magnesium hardness, In our study, the heavy metal concentrations decreased in spring, during which higher levels of hardness were recorded and this confirms the finding of (Aziz *et al.*, 2012) that shows a decrease in metal toxicity with the increasing of water hardness.

| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
|---|---|---|---|---|---|---|---|
| **Jan.** | 269 | 282 | 304 | 305 | 285 | 332 | 296.17±6.2 a |
| **Feb.** | 300 | 258 | 278 | 264 | 248 | 257.5 | 267.58±6.7 a |
| **Apr.** | 294 | 300 | 264 | 306 | 232 | 266 | 277.00±8.6 a |
| **May** | 415 | 355 | 430 | 410 | 485 | 380 | 412.50±12.4 c |
| **Jun.** | 430 | 485 | 310 | 410 | 391 | 390 | 402.67±15.9 c |
| **Jul.** | 322 | 342 | 310 | 371 | 380 | 308 | 338.83±6.8 b |
| **Aug.** | 364 | 314 | 280 | 326 | 354 | 360 | 333.00±9 b |
| **Sep.** | 354 | 368 | 358 | 338 | 322 | 352 | 348.67±4.5 b |
| **Oct.** | 356 | 340 | 324 | 345 | 364 | 320 | 341.50±4.7 b |
| **Mean ±SE** | 344.8±54.5 a | 338.2±63.8 a | 317.8±49.7 a | 341.6±48.0 a | 340.1±76.3 a | 329.5±45.4 a | 335.32±5.4 |

**Table (5.4) Total hardness (mg CaCO3 l-1) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.5 Alkalinity mg $l^{-1}$

Alkalinity is a measure of water's ability to neutralize acids; it is required to maintain the neutral pH (buffer) during biological, chemical, and physical treatment procedures (Wang *et al.*, 2005).

As shown in (Table 5.6) values of Tanjaro water alkalinity during the studying period was between 122 and 324.3 mg $l^{-1}$, the minimum value obtained at site 2 in January, while the maximum value was in October at site 6.

Statistical analysis revealed that the minimum mean value of the studied sites was 204.1mg $l^{-1}$ which was recorded in site 3. Regarding the monthly mean, the minimum value was 136.5 mg $l^{-1}$ recorded in January, while the maximum mean was 283.6mg $l^{-1}$ during September, with significant differences (P≤0.05).

During January and February, the water's alkalinity was lower than the permissible level, after February, the alkalinity increased to exceed the permissible level for freshwater used for drinking which is 200 mg $l^{-1}$ (WHO, 2017). The high alkalinity level in some of the studied samples

may be due to the action of carbonate on the basic material, also alkalinity is strongly related to the amount of carbon dioxide present in water and the geological formation of the area which is composed mainly of CaCO3 (Amro, 2004).

**Table (5.5) Alkalinity (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean ±SE** |
| **Jan.** | 126 | 122 | 124 | 140 | 126 | 181 | 136.5±6.4 a |
| **Feb.** | 127 | 158 | 156 | 129 | 156 | 138 | 144±4.05 a |
| **Apr.** | 229 | 260 | 225 | 245 | 231 | 218 | 234.66±4.3 c |
| **May** | 207 | 208 | 197 | 209 | 184 | 216.2 | 203.54±3.1 b |
| **Jun.** | 235.2 | 228.9 | 203.5 | 227.3 | 209.8 | 241.6 | 224.4±4.2 c |
| **Jul.** | 205.1 | 198.7 | 201.9 | 208.2 | 186 | 203.5 | 200.60±2.1 b |
| **Aug.** | 241.6 | 197.1 | 219.4 | 240.09 | 251.2 | 238.5 | 231.3±5.4 c |
| **Sep.** | 303.6 | 255.9 | 248 | 249.6 | 287.2 | 320 | 283.6±10.02 d |
| **Oct.** | 254.4 | 244.8 | 254.4 | 262.5 | 298.9 | 324.3 | 273.2±8.6 d |
| **Mean ±SE** | 216.6±60.1 ab | 213.4±52.6 a | 204.1±41.6 a | 212.1±46.2 a | 210.2±51.9 a | 231.2±58.4 b | 214.6±4.9 |

### 5.1.6 Dissolved oxygen (DO) mg $l^{-1}$

Dissolved oxygen is an important factor used to regulate water quality, the impact of the waste release on a surface water supply is primarily determined by the system's oxygen balance and its presence is crucial to sustaining biological life within the water body (Mustapha and Halimoon,

2015), it is used as an indicator of water quality, high concentrations of oxygen usually indicate good water quality, which generally depends on water temperature, air pressure, consumption rate in the process of organic matter degradation, salinity, photosynthesis, organism respiration, and oxygen gas exchange between air and water (Nasir, 2007).

Dissolved oxygen concentration in Tanjaro River shown in (Table 5.7) ranged from 3mg $l^{-1}$ to 7.75mg $l^{-1}$, the overall mean of dissolved oxygen concentration recorded for the study period during the entire sampling time was 5.34mg $l^{-1}$.

Statistical analysis revealed that 4.98mg $l^{-1}$ is the minimum value for the studied sites recorded at S6 and showed a significant different ($P \leq 0.05$) as compared with the other sites, while for the months understudy, the minimum value was observed in August.

The equilibrium concentration of dissolved oxygen in the water in contact with air is a function of temperature (Bartram and Balance, 1996), depletion of dissolved oxygen during August confirm the negative relation between temperature and the amount of dissolved oxygen, or probably due to the large number of organic materials resulting from effluent discharge into the water, leading to an increase in the number and activity of microorganisms, increasing decomposition and oxidation processes for organic matters by bacteria, and thus a reduction in dissolved oxygen in the water occur (Aniyikaiye *et al* .,2019) nearly similar results were obtained by (Aziz *et at.*, 2012; Mustafa, 2006) in surface water samples of Tanjaro River and its tributaries with the values ranging from ( 2.4- 4.8 mg $l^{-1}$) and (4.4 to 5.15mg $l^{-1}$) respectively.

The maximum means obtained during April and showed significant differences with other months excep January and October. Increases in dissolved oxygen content during April 2019 could be due to self-purification activities in the water, heavy rainfall, wind action and photosynthetic processes, similar results were obtained by Ahmed (2020) that ranged between (3.1 and 7.1mg $l^{-1}$) and (Hann and ASheka, 2017) that record 7.67 mg $l^{-1}$ in the rivers within Erbil city the higher result was obtained by Hassan and Ali, 2016) that record 9.01mg $l^{-1}$ of DO at Zea river, in contrast (Hassan and Al-Barware, 2016) recorded zero dissolve oxygen at some investigation sites in Duhok Valley.

**Table (5.6) Dissolved oxygen concentration (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
| Jan. | 6.65 | 5.77 | 7.75 | 5.64 | 5.37 | 6.5 | 6.13±0.24 cd |
| Feb. | 6.5 | 6.2 | 6.1 | 5.64 | 6.6 | 5.8 | 6.14±0.1 cd |
| Apr. | 6.24 | 6.83 | 6.56 | 6.7 | 6.88 | 5.11 | 6.38±0.18 d |
| May | 5.12 | 6.06 | 5.76 | 6.1 | 6.31 | 4.65 | 5.66±0.17 bc |
| Jun. | 4.49 | 5.93 | 5.7 | 5.96 | 5.89 | 5.75 | 5.62±0.15 bc |
| Jul. | 4.35 | 5.5 | 5.1 | 5.6 | 5.3 | 5 | 5.14±0.12 b |
| Aug. | 3.15 | 4.1 | 3.8 | 3.57 | 3.33 | 3 | 3.51±0.1 a |
| Sep. | 4.8 | 3.3 | 3.8 | 4 | 3.5 | 3.15 | 3.7±0.17 a |
| Oct. | 5.6 | 6.35 | 4.6 | 5.1 | 6.1 | 6.7 | 5.7±0.22 bcd |
| Mean ±SE | 5.21±0.26 ab | 5.56±0.26 b | 5.46±0.29 ab | 5.36±0.22 ab | 5.47±0.29 ab | 4.98±0.28 a | 5.34±0.11 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.7 Biological oxygen demand ($BOD_5$) mg $l^{-1}$

Biological oxygen demand is one of the most important indicators of pollution level of waters used to measure the quality of water in terms of organic matter present in both suspended and dissolved form (Ahipathy and Puttaiah, 2006), it is the quantity of oxygen required by microorganisms to decompose the organic substances in the water system, therefore, the more organic matter, the higher biological oxygen demand.

Biological oxygen demand is a standard 5-day value that is often used to describe the strength of municipal wastewaters, to estimate the amount of organic pollution in water, and to evaluate the efficacy of treatment by measuring oxygen demand remaining in the effluent (Mara, 2013).

According to the results shown in (Table 5.8) water's $BOD_5$ values were between 36 and 120 mg $l^{-1}$ for S5 and S2 as a minimum and maximum during January and August respectively for the current study.

From statistical analysis of the studied months, it appeared that the minimum value of 43.1mg $l^{-1}$ was recorded in January, while the maximum value of 103.1 mg $l^{-1}$was obtained in August, with a significant difference with all other studied months.

$BOD_5$ in clean water is less than 1ppm, 3 ppm is an acceptable range when 5ppm is critical limits, but when it became more than 10ppm is an indicator for water pollution (Al-Asadi, 2020). DO is greatly influenced by the $BOD_5$ level in the water. The higher $BOD_5$ concentration means the greater the extent of oxygen depletion in the water bodies (Bhateria and Jain, 2016), this confirmed by the recorded data of our study in which data during August contain the higher $BOD_5$ level with the lower DO level and this results in agreement with (Al-Enazi, 2016).

Higher $BOD_5$ recorded during the hot months, which may be due to the increase of the activity of microorganisms that consumes DO in oxidation processes, similar output was found by Rasheed and Hama Karim (2008), or due to the effluent discharge enriched with untreated domestic waste, and industrial wastewater from Sulaimani sewage and wastewater. These results are proportional to the data revealed and reported by (Rashid, 2010), while disagreeing with (Ahmed, 2020) data that ranged between 52 to 360 mg $l^{-1,}$ with the highest value being during October.

**Table (5.7) $BOD_5$ concentration (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
| Jan. | 43 | 45 | 42 | 39 | 36 | 54 | 43.1±2.05 a |
| Feb. | 53 | 64.2 | 88.8 | 89.8 | 111.4 | 100.8 | 84.6±6.08 de |
| Apr. | 54 | 56.4 | 68.4 | 65.2 | 60.8 | 57.6 | 60.4±1.5 bc |
| May | 64 | 78 | 80 | 90 | 80 | 82 | 79±2.3 de |
| Jun. | 86 | 100 | 90 | 96 | 100 | 80 | 91.9±2.2 ef |
| Jul. | 66 | 80 | 76 | 70 | 80 | 56 | 71±2.5 cd |
| Aug. | 89 | 120 | 104 | 116 | 110 | 80 | 103.1±4.3 f |
| Sep. | 62 | 50 | 60 | 50 | 42 | 38 | 50.3±2.6 ab |
| Oct. | 83 | 63.2 | 110.6 | 37.8 | 69.8 | 79.8 | 74.03±6.6 cd |
| Mean ±SE | 66.6±3.6 a | 72.9±5.5 ab | 79.9±4.9 b | 72.6±6.23 ab | 76.6±6.22 ab | 69.8±4.4 ab | 73.12±2.1 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.8 Chloride ($Cl^-$) mg $l^{-1}$

The chloride ion is one of the most common inorganic anions found in water as a result of leaching from various rocks, but it can also be produced from a number of agricultural, industrial, and domestic sources; combined sewerage systems is the main seasonal source of chloride (Huang *et al.*,2020).

Tanjaro water data for chloride reoresented in (Table 5.9) it appeared that the ranges were between 13.2 and 77.9 mg $l^{-1}$, the minimum value detected at S4 during April and the maximum at S1 during October.

Statistical analysis of the data revealed that the minimum value for the studying sites was 45.9 mg $l^{-1}$ in S5 which show no significant difference (P≤0.05) with other sites except for site 6, while the studying months show that the maximum means of 74.98 mg $l^{-1}$ recorded during September with significant difference at (P≤0.05) from all investigated months.

Throughout the study period, the chloride concentrations were less than the maximum WHO recommended value of clean water which is 250 mg $l^{-1}$, the excessive use of chloride as a disinfectant in different water purification systems, as well as industrial pollutants dumped into the river, may be contributing to the rise in chloride levels in the water (Rashid 2010).

**Table (5.8) Chloride concentration (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
| Jan. | 29.9 | 31 | 31.6 | 34 | 35.5 | 42.5 | 33.59±1.2 c |
| Feb. | 21.6 | 17.7 | 16.6 | 17.3 | 17 | 21.9 | 18.7±0.7 a |
| Apr. | 27.5 | 36.5 | 29.4 | 13.2 | 21.5 | 25.7 | 25.54±2.1 b |
| May | 24.4 | 26.1 | 29 | 47.2 | 26.1 | 43.9 | 32.81±2.7 c |
| Jun. | 57.4 | 58.1 | 53.88 | 48.2 | 51.04 | 59.5 | 54.70±1.4 d |
| Jul. | 57.4 | 58.1 | 53.8 | 48.2 | 51 | 56.7 | 54.23±1.3 d |
| Aug. | 75.8 | 64.5 | 62.3 | 67.4 | 65.9 | 75.1 | 68.66±1.5 e |
| Sep. | 73.7 | 76.5 | 78.6 | 76.5 | 72.3 | 72 | 74.98±0.8 f |
| Oct. | 77.9 | 76.5 | 73.7 | 74 | 75.1 | 72.3 | 74.95±0.5 f |
| **Mean ±SE** | 49.5±23.04 ab | 49.5±21.3 ab | 47.7±21.4 ab | 47.2±22.4 a | 45.9±21.7 a | 52.2±19.3 b | 48.69±2.03 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.9 Nitrate ($NO_3^-$) mg $l^{-1}$

Nitrate is the most oxidized form of nitrogen compounds. The determination of nitrate aids in the evaluation of the kind and degree of oxidation in biological processes. It is commonly present in surface and ground waters because it is the end product of the aerobic decomposition of nitrogenous organic matter (Walakira and Okot- Okumu, 2011).

The MCLG (Maximum contaminant level goals) for nitrate in drinking water is 10mg $l^{-1}$, although nitrate concentration greater than 5mg $l^{-1}$ reflects unsanitary condition according to (WHO, 2017).

Nitrate concentrations of Tanjaro River were displayed in (Table 5.10). The observed data was ranged between 19.52 and 48.55mg $l^{-1}$, the minimum value was obtained in S4 during July and the maximum in S1 during February. The statistical analysis for the investigated sites revealed that a maximum value of 36.11 mg $l^{-1}$ was recorded in S1 and showed a significant difference ($P \leq 0.05$) with other studied sites.

For the investigated months the maximum mean of 37.8 mg $l^{-1}$ was recorded during January, and the minimum mean value of 29.2 mg $l^{-1}$ was recorded during April, the lower values of $NO_3$ during April are mostly due to the dilution of the wastewater by heavy rainfall during this month which closed to the results obtained by (Ahmed, 2020) at Tanjaro river, but higher than those obtained by (Mustafa, 2006) which observed $NO_3$ values ranged between (21.5-24.9) mg $l^{-1}$. High nitrate concentrations may result from agricultural, sewage disposal from households, cleaning products, detergents, and the presence of a landfill site near the Tanjaro River (Rashid, 2010).

**Table (5.9) Nitrate concentration (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the studied period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | Mean ±SE |
| Jan. | 36.06 | 34.35 | 34.18 | 38.11 | 38.8 | 45.46 | 37.8±1.1 b |
| Feb. | 48.55 | 38.46 | 27.34 | 31.96 | 31.45 | 35.04 | 35.4±2.04 ab |
| Apr. | 24.44 | 31.28 | 27.17 | 30.93 | 31.45 | 30.26 | 29.2±0.7 a |
| May | 29.23 | 27 | 35.21 | 31.76 | 28.4 | 31.44 | 30.5±0.8 a |
| Jun. | 32.13 | 35.04 | 44.78 | 23.58 | 27.86 | 25.64 | 31.5±2.1 ab |
| Jul. | 37.5 | 43.94 | 25.46 | 19.52 | 33.84 | 22.05 | 30.38±2.6 a |
| Aug. | 42.73 | 42.73 | 33.24 | 27.52 | 28.2 | 20.05 | 32.41±2.4 ab |
| Sep. | 39 | 38.5 | 35 | 30 | 32 | 25 | 33.2±1.4 ab |
| Oct. | 35.4 | 32.7 | 33 | 27 | 29.3 | 20 | 29.5±1.5 a |
| Mean ±SE | 36.11±1.6 b | 35.9±1.2 b | 32.8±1.3 ab | 28.9±1.2 a | 31.2±0.7 ab | 28.3±1.8 a | 32.2±2.0.6 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.10 Sulfate ($SO_4^{2-}$) mg $l^{-1}$

Sulfate is a common ion in the earth's crust, and its concentration in water can range from a few milligrams per liter to several thousand milligrams per liter, it is discharged into the water through industrial wastes and atmospheric deposition (Bartram and Balance, 1996).

Sulfates are readily broken down under anaerobic conditions to hydrogen sulfide gas resulting in increased toxicity, odor, and corrosion. Typical Sulfate levels in domestic wastewater are 20-50 mg $l^{-1}$. No guideline for health risk due to sulfate ions in water is proposed by WHO, however drinking water containing high concentration of sulfate ions can cause a gastrointestinal effect (WHO, 2017), it is recommended that the sulfate concentration must be lower than 500 mg $l^{-1}$, while according to EPA, (2011) the allowable concentration must be lower than 250 mg $l^{-1}$.

The mean value of $SO_4^{2-}$ concentration in the Tanjaro River was 167.07 mg $l^{-1}$ as illustrated in (Table 5.11) with the minimum value of 21.16 mg $l^{-1}$ that was recorded at site 5

during June and increased until reach the maximum value of 336.66 mg $l^{-1}$ at site 6.

When the data was statistically analyzed for the studied sites, the minimum value was 103.3 mg $l^{-1}$ recorded at S5 but with no significant difference ($P \leq 0.05$) from other sites except for sites 4 and 6, while the maximum value of 267.4 mg $l^{-1}$ was recorded in site 6. In the studying months, 214.2 mg $l^{-1}$was the maximum value recorded during August. The results of this study were higher than those obtained by (Mohammed, 2020; Rasheed and Hama Karim, 2017) with the mean value of 94.57 mg.$l^{-1}$ and 141.5 mg.$l^{-1}$, but agreed with those reported by (Faqi Salih, 2013) at Bazian area. Higher level of $SO_4$ was recorded by (Hanna and Ali, 2017) in Zar Cali stream, Bekhal and Khalan Rivers with in Erbil city that ranged from 840.4 to 869.8 mg $l^{-1}$. Tanjaro River contamination with $SO_4$ may results from sewage wastewater, fertilizers, insecticide, and industrial waste disposal to Tanjaro River and it is tributaries (Mustafa, 2006).

**Table (5.10) Sulfate concentration (mg $l^{-1}$) represented as (mean ± S.E) of the studied sites during the period from January to October 2019.**

| Sites / Months | Studied sites | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **Mean ±SE** |
| **Jan.** | 111.7 | 119.1 | 123.33 | 320 | 130 | 170 | 162.35±21.9 a |
| **Feb.** | 206.6 | 51.66 | 51.66 | 276.66 | 61.66 | 286.66 | 155.8±31.3 a |
| **Apr.** | 50.1 | 40 | 43 | 386.66 | 26.66 | 336.66 | 147.1±45.9 a |
| **May** | 70.33 | 78.2 | 124.33 | 280 | 63.33 | 283.33 | 149.9±86.7 a |
| **Jun.** | 63.33 | 48.33 | 170 | 313.33 | 21.16 | 323.33 | 156.5±37.1 a |
| **Jul.** | 110 | 85 | 90 | 193.33 | 95 | 280 | 142.2±21.6 a |
| **Aug.** | 205.3 | 208.1 | 203.33 | 204.7 | 203.4 | 260.5 | 214.2±6.2 a |
| **Sep.** | 208.8 | 119.1 | 180 | 200 | 170 | 265 | 189.8±13.3 a |
| **Oct.** | 200.5 | 182.5 | 194 | 172.5 | 162.5 | 201.5 | 185.5±4.8 a |
| **Mean ±SE** | 136.29±15.6 a | 103.54±13.6 a | 131.07±13.7 a | 260.7±16.5 b | 103.3±14.9 a | 267.4±12.1 b | 167.07±8.8 |

Note: Means followed by the same latter are not significantly different at (P<0.05) according to Tukey'-b multiple range test.

### 5.1.11 Metal content of the water samples

Tanjaro River is contaminated by municipal sewage outlets of the areas and industrial effluent of factories in the area, Albisaka, Qalawa, Wluba, Shekh-Abbas, and Bakrajo boxes are discharged directly to Tanjaro River without any pretreatment that leads to heavy metal accumulation (Majid *et al.*, 2018).

The ICP-OES results of heavy metal are shown in (Figure 5.1), several variations in heavy metal concentrations were observed between sampling sites but with no significant differences; that may be due to the nonpoint sources of waste discharge along with the sampling sites within Tanjaro River.

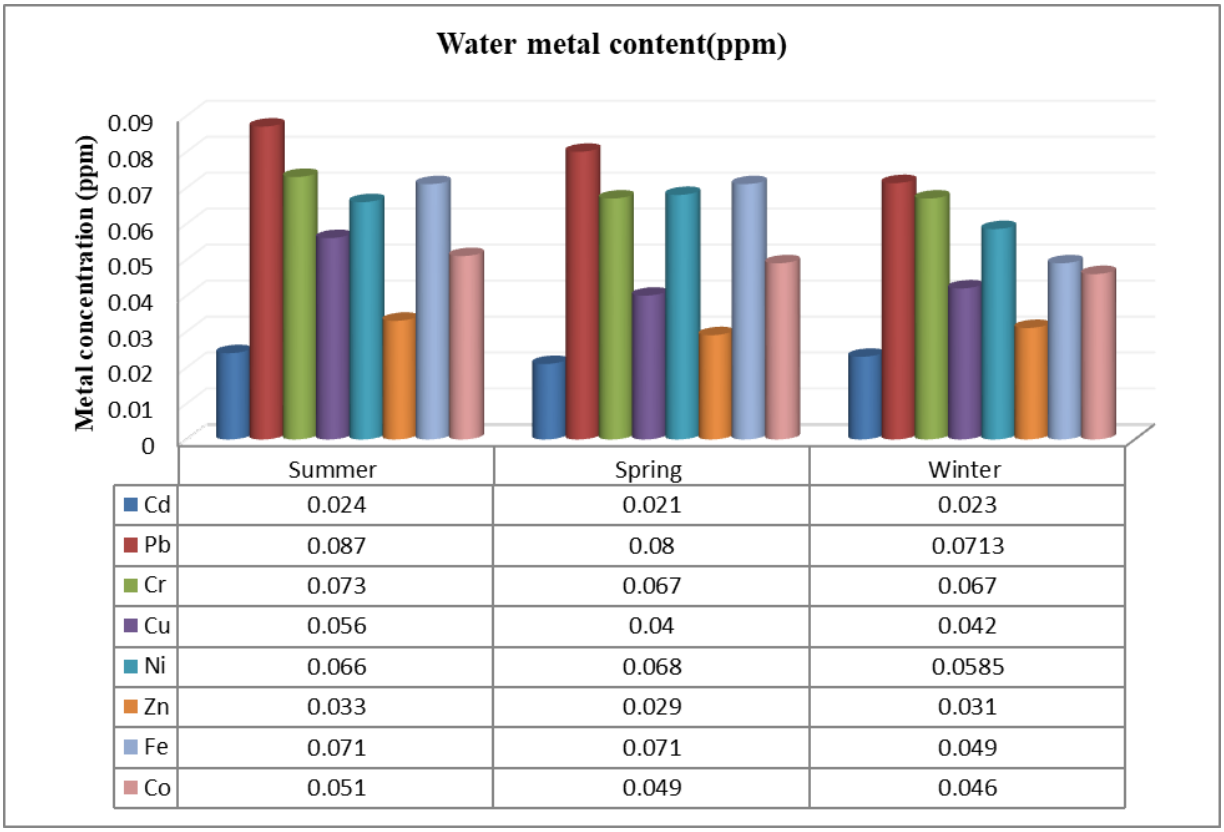
Stormwater run-off from the surrounds of the river catchment has a big impact on metal levels in rivers during the rainy season, and this typically leads to an increase in heavy metal

concentration. Another factor that might affect positively the concentration of metals in a river during the wet season is enhanced dilution of heavy metals owing to increased water volume and velocity, in this study the higher concentration of most metals was recorded during the dry season in (summer months) which may be attributed to that heavy metals concentrated as a result of reduced water volume and movement, as well as increased evaporation from water bodies, similar results were obtained by (Aziz *et al*., 2012; Edokpayi *et al.,* 2017).

Among the analyzed heavy metals, Pb ions had the highest concentration, while Zn and Cd ions had the lowest concentrations as in the follows order: Pb > Cr > Fe > Ni > Co > Cu > Zn > Cd with maximum concentrations of 0.086, 0.073, 0.071, 0.068, 0.051, 0.056, 0.031, and 0.024 ppm, respectively, but in a study performed by (Jahanshahi and Zare, 2015) for assessing heavy metal pollution in Iran it was found that the mean concentration was in the order of Fe >Zn >Pb > Cu > Ni.

The hydrological formation of the sampling site had a notable impact on water quality; also changes in the metal concentrations were primarily influenced by the time of year (Saran *et al.,* 2018), which may be the reason behind that metal concentration during Cd, Pb, Cr, and Ni were present in higher concentrations than that stated by WHO in the water samples, while Co, Cu, Fe, and Zn were found within the normal range of (EPA 2011; WHO 2017) for freshwater.

The results were lower than those observed by (Rashid, 2010; Mustafa, 2006) but higher than those obtained by (Rasheed and Hama Karim, 2017) at the same river, and those obtained by (Hamdan, 2020; Al-Abbawy *et al.,* 2021) at both Shatt- Alarab and Al-Hawizeh Marsh, southern of Iraq, the low observation may be attributed to the fact that most factories stopped operating during their study period. In a study performed on the Gaylan stream in Turkey by (Bulut *et al.,* 2009) it was observed that each of Cu and Pb concentrations did not exceed the values proposed by WHO guidelines for drinking water, but total Cr and total Fe concentrations exceed the values for safe drinking water. The continuous use of contaminated water for irrigation may cause the accumulation of metals to concentrations that are toxic for plants and animals (Iram *et al.,* 2013).




| | Summer | Spring | Winter |
|---|---|---|---|
| Cd | 0.024 | 0.021 | 0.023 |
| Pb | 0.087 | 0.08 | 0.0713 |
| Cr | 0.073 | 0.067 | 0.067 |
| Cu | 0.056 | 0.04 | 0.042 |
| Ni | 0.066 | 0.068 | 0.0585 |
| Zn | 0.033 | 0.029 | 0.031 |
| Fe | 0.071 | 0.071 | 0.049 |
| Co | 0.051 | 0.049 | 0.046 |

**Figure (5.1)** Mean concentrations of heavy metals (ppm) in water samples during different seasons.

### 5.1.12 heavy metals correlation with physicochemical parameters

Spearman's test was used to compare among physicochemical parameters and heavy metal levels in Tanjaro river water. The results are presented in (Table 5.12). According to the obtained data, there were significant positive relationships ($P \leq 0.05, 0.01$) among temperature, pH, EC, TDS, and $BOD_5$.

Temperature had negative relationships with DO, $NO_3$ and approximately with all metals except Cd and Cr. When the pH values increased, each of $BOD_5$, $NO_3$, CO, Pb and Ni increase and show significant positive correlation, On the other hand negative significant correlation was observed between pH , EC, TDS, alkalinity and chloride, also negative relation were observed between pH and the metal dissolution. As stated by (Li *et al.*, 2013 ), with pH decreasing in the environment, the competition between $H^+$ and the dissolved metals for ligands ($OH^-$, $Cl^-$, $S_2^-$, and phosphates) becomes more and more significant.

The bioavailabilities and adsorption abilities of the metals subsequently decrease and then increase the mobility of heavy metal. Dissolved oxygen levels had no relationship with all studied metals except Co and Pb. There were positive relationships between T.H with the level of alkalinity and chloride; but it have significant negative impact on the dissolving of four metals (Cu, Ni, Pb, and Zn).

Ebrahimpour *et al.,(*2010) showed that toxicity of Cu and Zn decreased with increasing water hardness. There were positive correlation between nitrate concentration and all the studied metals with the exception of Ni. In the view of cobalt metal, it is observed that when its concentration increases each of (Cr, Pb, Ni, and Zn) will significantly increase.

**Table (5.11) Spearman correlation matrix showing the relationships of metal in water and some physicochemical parameters in water.**

| | Tem | pH | Ec | TDS | DO | BOD | TH | Alkalinity | Chloride | NO3 | SO4 | Cd | Co | Cr | Cu | Fe | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| water tem | **1** | **-.147** | **.565**** | **.572**** | **-.674**** | **.372**** | **.448**** | **.467**** | **.681**** | **-.198*** | **.178** | **.234** | **-.414*** | **.131** | **-.542**** | **-.426**** | **-.330*** | **-.387*** | **-.572**** |
| pH | | **1** | **-.389**** | **-.378**** | **.055** | **.374**** | **-.132** | **-.421**** | **-.270**** | **.299**** | **.088** | **-.251** | **.369*** | **-.206** | **.250** | **.060** | **.342*** | **.379*** | **.150** |
| EC | | | **1** | **.960**** | **-.369**** | **-.065** | **.201*** | **.775**** | **.777**** | **-.079** | **.188** | **.456**** | **-.097** | **.117** | **.339*** | **-.111** | **-.398*** | **-.262** | **.156** |
| TDS | | | | **1** | **-.357**** | **-.085** | **.288**** | **.765**** | **.756**** | **-.115** | **.148** | **.317** | **-.161** | **.010** | **.067** | **-.263** | **-.291** | **-.219** | **-.080** |
| DO | | | | | **1** | **-.085** | **-.292**** | **-.321**** | **-.615**** | **-.062** | **-.344**** | **-.261** | **.452**** | **-.274** | **.260** | **.038** | **.276** | **.536**** | **.251** |
| BOD | | | | | | **1** | **.217*** | **.217*** | **.036** | **-.052** | **.074** | **-.134** | **.201** | **-.194** | **-.624**** | **-.423*** | **.312** | **.111** | **-.618**** |
| TH | | | | | | | **1** | **.363**** | **.435**** | **-.140** | **.048** | **-.112** | **-.190** | **.028** | **-.803**** | **-.569**** | **.160** | **.012** | **-.763**** |
| Alkalinity | | | | | | | | **1** | **.686**** | **-.326**** | **.147** | **.280** | **-.300** | **.233** | **-.474**** | **-.326** | **-.367*** | **-.438**** | **-.470**** |
| chloride | | | | | | | | | **1** | **.002** | **.223*** | **.321** | **-.057** | **.059** | **.159** | **.023** | **-.362*** | **-.414*** | **-.019** |
| NO3 | | | | | | | | | | **1** | **-.032** | **.277** | **.331*** | **.422*** | **.584**** | **.384*** | **-.032** | **.111** | **.576**** |
| SO4 | | | | | | | | | | | **1** | **.269** | **.167** | **.102** | **.169** | **.350*** | **-.361*** | **-.041** | **.100** |
| Cd | | | | | | | | | | | | **1** | **.015** | **.317** | **.120** | **.105** | **-.161** | **.117** | **.323** |
| Co | | | | | | | | | | | | | **1** | **-.123** | **.337*** | **.264** | **.373*** | **.356*** | **.382*** |
| Cr | | | | | | | | | | | | | | **1** | **-.103** | **.131** | **-.310** | **-.119** | **.283** |
| Cu | | | | | | | | | | | | | | | **1** | **.511**** | **-.040** | **.084** | **.750**** |
| Fe | | | | | | | | | | | | | | | | **1** | **-.200** | **.236** | **.685**** |
| Ni | | | | | | | | | | | | | | | | | **1** | **.010** | **-.179** |
| Pb | | | | | | | | | | | | | | | | | | **1** | **.292** |
| Zn | | | | | | | | | | | | | | | | | | | **1** |

Note: -** strong negative correlation; +** strong positive correlation; * weak correlation; Yellow color: positive correlation; Blue color : negative correla

## 5.2 Identification of Bacterial Isolates

### 5.2.1 Isolation of heavy metal-resistant bacteria

The initial screening process of Tanjaro's water samples during the studied period (winter, spring, and summer) resulted in the isolation and purification of 40 metal-resistant bacteria that could tolerate and grow on heavy metal-containing Luria Bertani (L.B) agar. The isolates were (originated from 200 metal-resistant colonies). For further purification, the morphologically distinct colonies were chosen for identification; based on diagnostic keys and molecular tools.

These heavy metal-resistant isolates included both gram-negative and gram-positive bacteria. Gram staining identified 17 (42.5%) isolates as gram-positive, while the other 23 (57.5%) were gram-negative. The isolates and their cultural, microscopic, and gram stain properties are presented in (Table 5.13).

Many studies showed that heavy metal resistances indigenous bacteria could be isolated from heavy metal-contaminated sites (Anusha *et al.,* 2021) used indigenous bacteria for cleaning contaminated soil (Kabir *et al.,* 2018) isolated and characterized chromium reducing bacteria from industrial effluents, (Irawati *et al.,* 2019) isolated eight heavy metal tolerant bacteria from Kemisan River, (Mustapha and Halimoon, 2015) screened different indigenous bacteria that have the ability to resist metals in Malaysia.

The toxic effect of metal ions exerts selection pressure on microorganisms whereby those bacteria are resistant to these metals survive (Zhang *et al.*, 2019). Overall, 40 bacterial isolates were able to grow on heavy metal-spiked L.B agar. The isolated strains in this study were widely reported to possess heavy metal resistance and have been isolated from different heavy metal contaminated environments except for *(Raoultella* sp.*)* which is agreed with (Cai *et al.*, 2019) findings.

Gram staining revealed the presence of both gram-positive and gram-negative bacteria. This indicates that both types of bacteria can tolerate the presence of metals in their environment; however, a predominance of gram-negative bacteria strains was found among the heavy metal-tolerant strains isolated from the Tanjaro River for all studied metals, which is in accordance with previous findings of (Bennisse *et al.,* 2004) which found that the majority of isolates subjected to selection pressures in the presence of toxic compounds were gram-negative. However, (Silva *et al.*, 2012) was disagreed with the results obtained in this study. It has been proposed that the cell wall of gram-negative bacteria is an effective barrier against toxic metals and that the cell wall's surface structures interact with metal ions, resulting in their detoxification. By contrast, the peptidoglycan cell wall of gram-positive

bacteria absorbs contaminants, overloading the bacterial cell and destroying it (Alegbeleye *et al.*, 2017). The majority of the isolated bacteria belonged to the Bacillaceae and Enterobacteriaceae families, which is similar to the results obtained by (Besharati *et al.*, 2018; Cai *et al.*, 2019). However, a predominance of Proteobacteria was reported by (Karelove *et al.*, 2011).

### 5.2.2 Molecular characterization (PCR amplification and 16S rRNA sequencing)

Amplification of the 16S rRNA genes was performed for the 40 bacterial isolates using universal primers that demonstrated ~ 1401 bp band size (Figure 5.2). The 16S rRNA gene sequence of each isolated strain was searched in the National Center for Biotechnology Information (NCBI) database. The nearest identities of all bacterial isolates, their codes, and accession number are presented in (Table 5.13). On molecular basis, the bacteria isoltaes were belong to divers groups of bacteria.The isolates were matched with the bacteria in the mentioned table with the curry cover range of 95-100%.

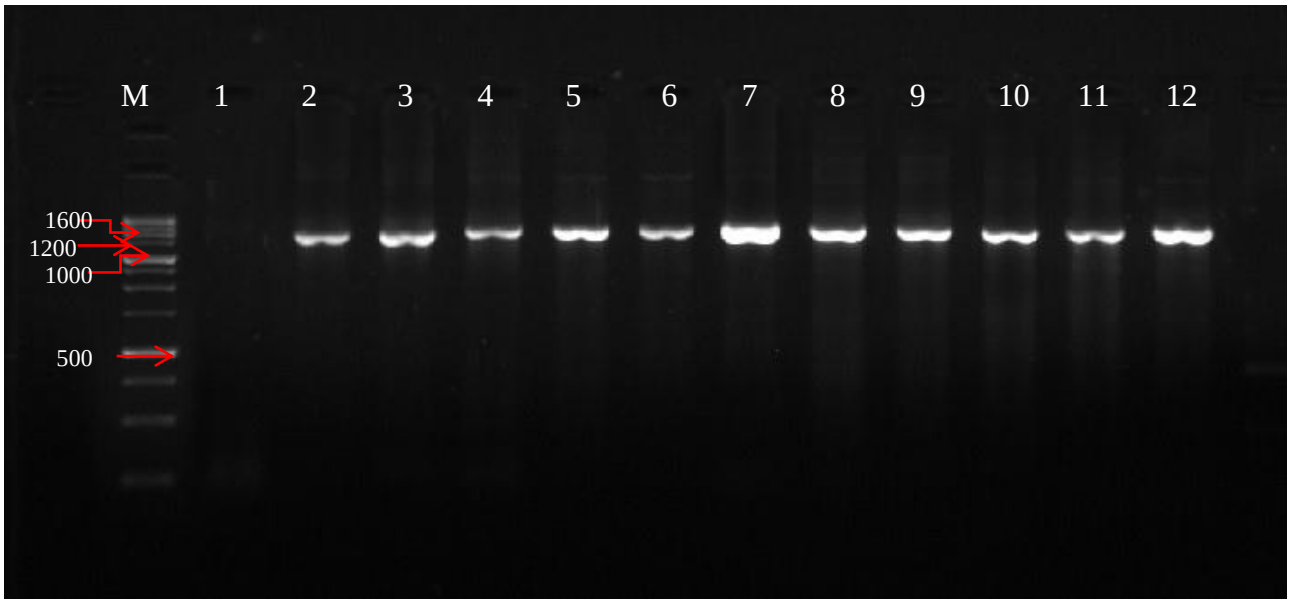



**Figure (5.2)** Agarose gel showing amplified DNA sequence of ~ 1401pb. Lane M (100bp) molecular weight marker . Lane 1: (DNA- free) negative control; Lane 2-12 bacterial isolates.

**Table (5.12) Cultural, Microscopic and Biological characteristics of bacterial isolates.**

| No | Bacterial isolates | Shape | Gram stain | Oxidase test | Catalase | Isolate's code | Query cover % | Accession no. |
|---|---|---|---|---|---|---|---|---|
| **1.** | *Acinetobacter junii* | Rod | - | - | + | AJ10T | 98 | MZ447090 |
| **2.** | *Acinetobacter junii* | Rod | - | - | + | AJ24T | 100 | MZ447104 |
| **3.** | *Aeromonas caviae* | Rod | - | + | + | AC31T | 99 | MZ447111 |
| **4.** | *Aeromonas caviae* | Rod | - | + | + | AC36T | 100 | MZ447116 |
| **5.** | *Bacillus cereus* | Rod | + | - | + | BC04I | 100 | MZ447084 |
| **6.** | *Bacillus cereus* | Rod | + | - | + | BC14L | 99 | MZ447094 |
| **7.** | *Bacillus pumilus* | Rod | + | - | + | BP01L | 90 | MZ447081 |
| **8.** | *Bacillus safensis* | Rod | + | - | + | BS16L | 99 | MZ447096 |
| **9.** | *Bacillus safensis* | Rod | + | - | + | BS23L | 99 | MZ447103 |
| **10.** | *Bacillus safensis* | Rod | + | - | + | BS39L | 99 | MZ447119 |
| **11.** | *Bacillus tropicus* | Rod | + | - | + | BT20L | 99 | MZ447100 |
| **12.** | *Bacillus zhangzhouensis* | Rod | + | - | + | BZH21L | 99 | MZ447101 |
| **13.** | *Bacillus zhangzhouensis* | Rod | + | - | + | BZH22L | 98 | MZ447102 |
| **14.** | *Bacillus zhangzhouensis* | Rod | + | - | + | BZH38L | 99 | MZ447118 |
| **15.** | *Enterobacter tabaci* | Rod | - | - | + | ET29T | 100 | MZ447109 |
| **16.** | *Enterobacter tabaci* | Rod | - | - | + | ET30T | 100 | MZ447110 |
| **17.** | *Enterobacter tabaci* | Rod | - | - | + | ET35 | 99 | MZ447115 |
| **18.** | *Enterococcus faecalis* | cocci | + | - | - | EF02I | 99.22 | MZ447082 |
| **19.** | *Enterococcus faecalis* | cocci | + | - | - | EF28I | 99 | MZ447108 |
| **20.** | *Enterococcus gallinarum* | cocci | + | - | + | EG05I | 98 | MZ447085 |
| **21.** | *Escherichia fergusonii* | Rod | - | - | + | EF08T | 99 | MZ447088 |
| **22.** | *Klebsiella quasipneumoniae* | Rod | - | - | + | KQ09T | 100 | MZ447089 |
| **23.** | *Leucobacter chromiiresistens* | Rod | + | - | + | LC15T | 99 | MZ447095 |
| **24.** | *Lysinibacillus fusiformis* | Rod | + | + | + | LF19T | 95 | MZ447099 |
| **25.** | *Microbacterium maritypicum* | Rod | + | - | + | MM03F | 98 | MZ447083 |
| **26.** | *Microbacterium oxydanse* | Rod | + | - | + | MO32I | 100 | MZ447112 |
| **27.** | *Morganella morganii* | Rod | - | - | + | MM11T | 97 | MZ447091 |
| **28.** | *Morganella morganii* | Rod | - | - | + | MM25T | 97 | MZ447105 |
| **29.** | *Proteus mirabilis* | Rod | - | - | + | PM17T | 96 | MZ447098 |
| **30.** | *Proteus mirabilis* | Rod | - | - | + | PM34T | 90 | MZ447114 |
| **31.** | *Proteus vulgaris* | Rod | - | - | + | PV06T | 99 | MZ447086 |
| **32.** | *Proteus vulgaris* | Rod | - | - | + | PV37T | 100 | MZ447117 |
| **33.** | *Providencia vermicola* | Rod | - | - | + | PV07T | 99 | MZ447087 |
| **34.** | *Pseudomonas aeruginosa* | Rod | - | + | + | PA12T | 99 | MZ447092 |
| **35.** | *Pseudomonas aeruginosa* | Rod | - | + | + | PA13T | 99 | MZ447093 |
| **36.** | *Pseudomonas aeruginosa* | Rod | - | + | + | PA33T | 99 | MZ447113 |
| **37.** | *Pseudomonas plecoglossicida* | Rod | - | + | + | PP27T | 100 | MZ447107 |
| **38.** | *Pseudomonas taiwanensis* | Rod | - | + | + | PT26T | 99 | MZ447106 |
| **39.** | *Raoultella ornithinolytica* | Rod | - | - | + | RO40LCH | 96 | MZ447120 |
| **40.** | *Raoultella planticola* | Rod | - | - | + | RP17T | 100 | MZ447097 |

### 5.2.3 Phylogenetic analysis

A phylogenetic analysis and the evolutionary history of the isolates were built based on the alignment and comparing 16S rRNA gene sequences of different bacterial isolates with others in the GenBank databases using the NCBI BLAST (www.ncbi.nlm.nih.gov), the sequences closely related to those with the bacterial species isolated in the current study were attained from the NCBI and aligned using Clustal W. The bootstrap consensus reliability was inferred from 1000 replicates using the neighbor-joining distance method by MEGA X (Kumar *et al.,* 2018) and applying Tamura-Nei model (Tamura and Nei, 1993).

Phylogenetic analyses that conducted in Blast queries revealed that the strains belonged to the Bacillaceae, Moraxellaceae, Morganellaceae, Enterococcaceae, Microbacteriaceae, Enterobacteriaceae, Pseudomonadaceae, and Aeromonadaceae families.

The query cover percentage of isolated bacteria to their closest match and their accession numbers were described in Table (5.13).The tree was rooted with *Staphylococcus aureua* and *Salmonella bongori* for both gram positive and gram negative bacteial phylogenetic tree respectively (Figure 5.3 and 5.4), the species that belong to the same family or genus were grouped into the same cluster and their phylogenetic relationships were highly compatible, similar results were obtained by (Takahashi *et al.,* 2009).

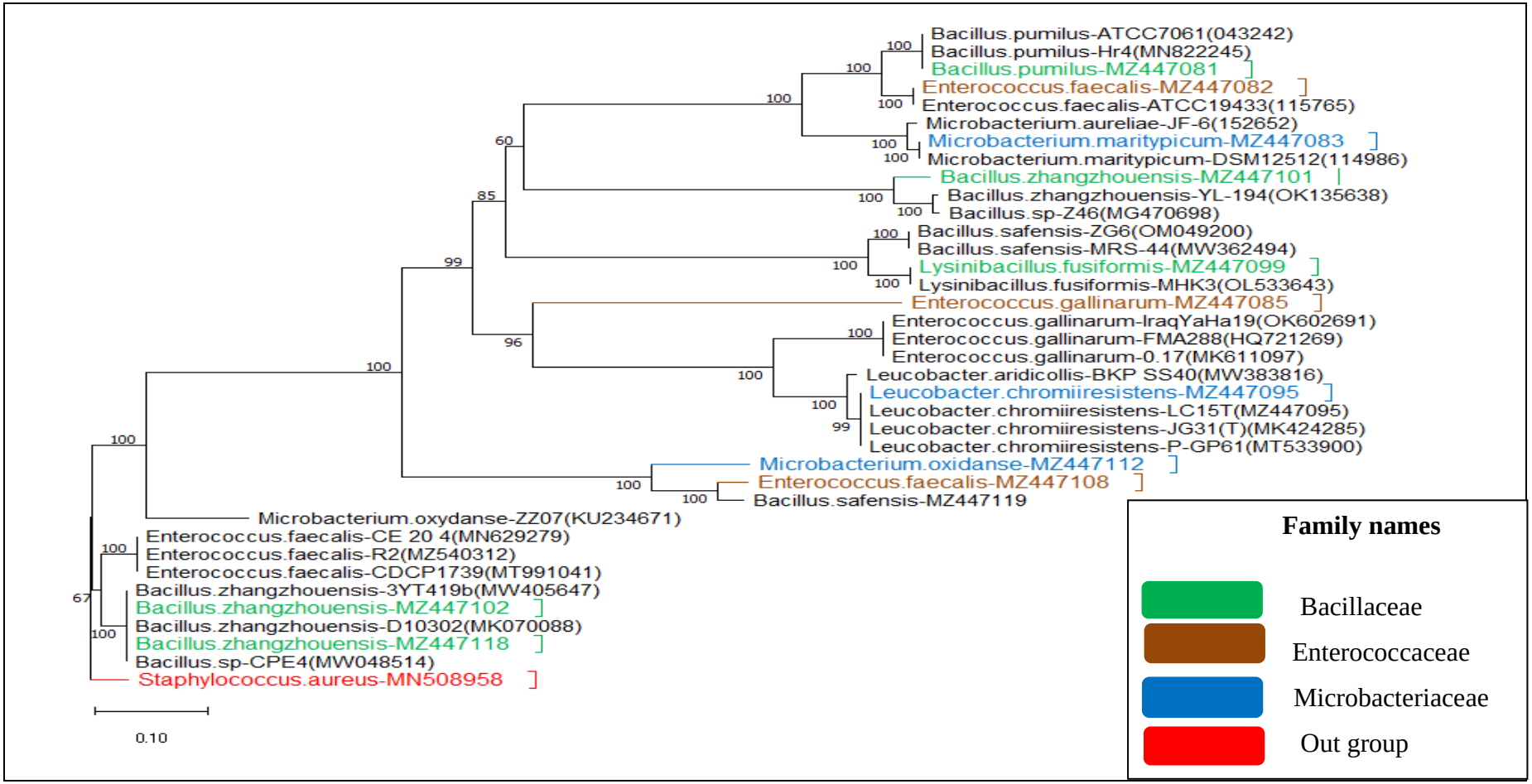



**Figure (5.3)** 16S rRNA gene sequence- based phylogenetic tree of the gram positive metal tolerant bacterial isolates. The tree was generated by the neighbor- joining methods. Genus names and the GenBank accession numberare on the right side of each tree. Scale bar represents the number of inferred nucleotide substitution per site.

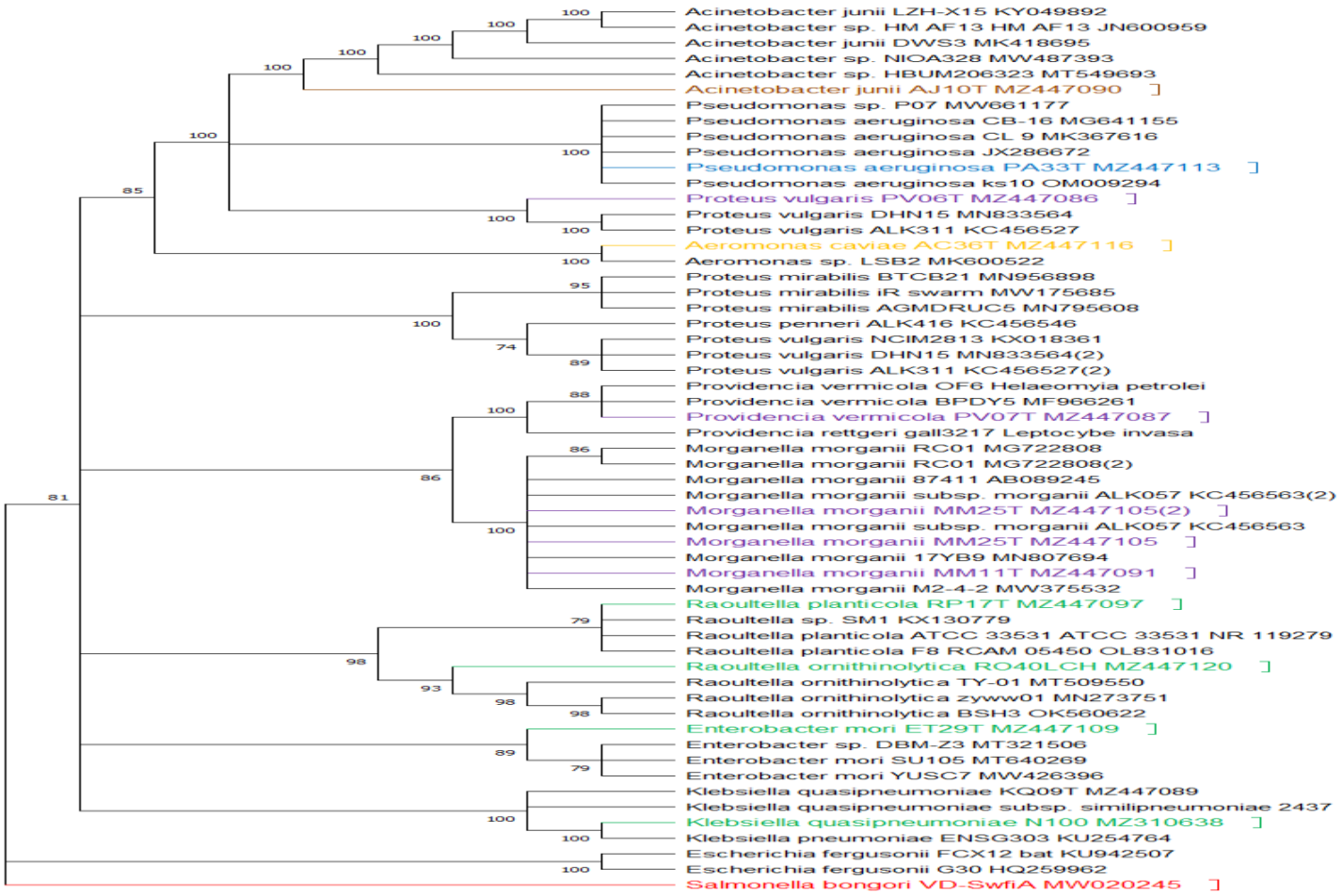



**Figure (5.4)** 16S rRNA gene sequence- based phylogenetic tree of the gram positive metal tolerant bacterial isolates. The tree was generated by the neighbor- joining methods. Genus names and the GenBank accession numberare on the right side of each tree. Scale bar represents the number of inferred nucleotide substitution per site.

## 5.3 Assessment of heavy metal tolerance:

The maximum tolerable concentration (MTC) is the highest concentration of metal which does not effect on the growth of the resistant bacteria. Because it is directly related to the survival and proliferation of bacteria in metal-contaminated water, high bacterial metal tolerance is an important factor to consider for heavy metal remediation (Aka and Babalola, 2017). The ability of the bacterial isolates to resist different concentrations of heavy metals was evaluated by determining maximum tolerable concentrations (MTCs).

The (MTCs) of the bacterial isolates against the tested metal salts are summarized in (Table 5.14). The isolated metal tolerance bacterial strains have the ability to resistant the selected metals, but they exhibited different levels of resistance with a concentration ranging from 10–430 ppm. *R. ornithinolytica* - RO40LCH isolated in this study showed higher tolerance for Cd, Pb, Cr, Co, and Fe (120, 430, 230, 210, 340 ppm) respectively in comparison to other metal tolerance bacterial isolates as reported by (Shammi and Ahmed, 2013; Kabir *et al.,* 2018), which make this strain more potential in bioremediation of heavy metal contamination.

Among the heavy metals, cadmium and copper were highly toxic, while, nearly all bacterial isolates could tolerate high concentrations of lead and iron. Other isolates presented a diverse metal-resistant phenotype to one or more metal ions. *L. chromiiresistens* - C15T *and B. safensis* - BS16L were respectively able to tolerate high Cd (90, 80), Pb (250, 160), Cr (210, 100), Ni (110, 90), and Co (160, 170) concentrations (all values in ppm). In addition, *P. mirabilis*-PM18T could tolerate 90 ppm Cd.

High tolerance variations have been observed between different strains although they belong to the same genera, same results have been obtained by (Cai *et al.,* 2019) which isolate metal-resistant bacteria from an electroplating wastewater treatment plant.

Exposure to toxic heavy metals makes the microorganism’s cell develop resistance mechanisms and metalion homeostasis so that microbial populations in metal polluted environments adapt to toxic concentrations of heavy metals and become metal resistant (Chatziefthimiou *et al.,* 2007). Factors such as the culture media used, pH value, temperature change, and incubation length, as well as the diverse forms and concentration of metals, may influence the metals' *in vitro* toxicity. Due to these facts, there are no universally accepted metal concentrations to define bacterial tolerance or resistance (Silva *et al.,* 2012), also the variation in metal tolerance might be due to the presence of different tolerance mechanisms (Irawati *et al.,* 2017b).

*R. ornithinolytica, B. safensis, L. chromiiresistens* showed the highest heavy metal tolerance and were resistant to heavy metals in the order of Pb > Fe >Cr> Co > Ni, approximate results were found by Selvi *et al.,* (2012) that isolated and characterized HMT bacteria from tannery effluents and discovered that all isolates (*Escherichia coli, Bacillus* spp., *Pseudomonas* spp., *Flavobacterium* spp., and *Alcaligenes spp*.) were resistant to heavy metals in the following order: Pb > Cu > Zn > Cr > Hg. The bacterial isolates of this study were also resistant to higher concentrations than those recorded by (Mandal *et al.*, 2020).

Among the investigated heavy metals, Pb and Fe were the most tolerable, whereas Cd, Cu, and Zn were highly toxic to all strains. Similar results were found by (Afzal *et al.,* 2017). The isolates identified in the current study were resistant to high levels of Pb (approximately 430 ppm), this may be attributed to the site where the water samples were taken being polluted with high levels of lead. Othman (2017) stated that lead is one of the heavy metals of special concern in Iraqi Kurdistan because of many emission sources, including low-quality petrol, widespread use of leaded paints in industry, unsafe disposal of car batteries and other batteries with lead products into water sources, while (Mustafa, 2006) revealed that besides the pollution from sewages, Sulaimani oil refinery wastes are the second most significant source of (Pb) pollution in Tanjaro river. This high level of Pb potentially allows a diverse range of bacteria to adapt to the environment, either through convergent evolution of resistance mechanisms or through the plasmid-based transmission of resistance genes. A similar finding was obtained by (Gummersheimer and Giblin, 2003) which concluded that a higher concentration of metals produces a greater metal resistant population of bacteria in that environment.

Resistance mechanisms can be encoded in plasmid genes, facilitating the transfer of toxic metal resistance factors from one cell to another. Because heavy metals cannot be degraded or destroyed, their introduction into the environment in various forms can cause significant changes in microbial communities and their activities, compromising their ability to survive (Samanta *et al.*, 2012).

High bacterial metal tolerance is an important factor to be considered for the remediation of heavy metals because it is directly related to the survival and growth of bacteria in metal-contaminated environments (Kang *et al.,* 2016). Generally, the ability of microbes to grow in environments with high metal concentrations is linked to several complex resistance mechanisms and environmental factors, such as microbial surface sorption, enzymatic transformation, precipitation by oxidation/reduction reactions, and biosynthesis of metal-binding proteins or extracellular polymers (Srinath *et al.*, 2002).

**Table (5.13) Heavy metals maximum tolerable concentration (MTCs) of the bacterial isolates.**

| | Bacterial Isolates | Metal concentration in ppm | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | **Cd** | **Pb** | **Cu** | **Cr** | **Ni** | **Zn** | **Co** | **Fe** |
| 1. | *Acinetobacter junii*-AJ10T | 30 | 140 | 50 | 50 | 70 | 60 | 110 | 160 |
| 2. | *Acinetobacter junii*-AJ24T | 40 | 130 | 40 | 30 | 70 | 10 | 10 | 150 |
| 3. | *Aeromonas caviae*-AC31T | 50 | 120 | 30 | 100 | 60 | 20 | 50 | 140 |
| 4. | *Aeromonas caviae*-AC36T | 40 | 150 | 60 | 60 | 80 | 50 | 70 | 150 |
| 5. | *Bacillus cereus*-BC04I | 30 | 130 | 40 | 60 | 70 | 40 | 60 | 150 |
| 6. | *Bacillus cereus*-BC14L | 20 | 130 | 40 | 60 | 70 | 30 | 20 | 140 |
| 7. | *Bacillus pumilus* strain BP01L | 30 | 120 | 40 | 60 | 70 | 50 | 60 | 170 |
| 8. | *Bacillus safensis*-BS16L | 80 | 250 | 80 | 210 | 110 | 60 | 160 | 250 |
| 9. | *Bacillus safensis*-BS23L | 40 | 120 | 20 | 30 | 50 | 30 | 40 | 140 |
| 10. | *Bacillus safensis*-BS39L | 20 | 150 | 60 | 70 | 90 | 50 | 70 | 170 |
| 11. | *Bacillus tropicus*-BT20L | 30 | 130 | 40 | 30 | 70 | 40 | 30 | 140 |
| 12. | *Bacillus zhangzhouensis*-BZH21L | 20 | 120 | 30 | 30 | 70 | 30 | 20 | 150 |
| 13. | *Bacillus zhangzhouensis*-BZH22L | 30 | 120 | 20 | 30 | 60 | 20 | 10 | 150 |
| 14. | *Bacillus zhangzhouensis*-BZH38L | 20 | 120 | 20 | 50 | 80 | 50 | 30 | 150 |
| 15. | *Enterobacter tabaci*-ET29T | 50 | 130 | 70 | 160 | 90 | 60 | 90 | 260 |
| 16. | *Enterobacter tabaci*-ET30T | 40 | 130 | 60 | 140 | 60 | 60 | 70 | 170 |
| 17. | *Enterobacter tabaci*-ET35 | 40 | 140 | 50 | 60 | 80 | 60 | 30 | 170 |
| 18. | *Enterococcus faecalis*-EF02I | 30 | 140 | 30 | 60 | 70 | 30 | 60 | 170 |
| 19. | *Enterococcus faecalis*-EF28I | 50 | 120 | 20 | 30 | 40 | 10 | 80 | 140 |
| 20. | *Enterococcus gallinarum*-EG05I | 40 | 130 | 40 | 40 | 70 | 40 | 60 | 160 |
| 21. | *Escherichia fergusonii*-EF08T | 30 | 140 | 60 | 60 | 80 | 60 | 60 | 170 |
| 22. | *Klebsiella quasipneumoniae*-KQ09T | 30 | 130 | 40 | 40 | 70 | 50 | 130 | 160 |
| 23. | *Leucobacter chromiiresistens*-LC15T | 90 | 160 | 50 | 100 | 90 | 50 | 170 | 150 |
| 24. | *Lysinibacillus fusiformis*-LF19T | 30 | 130 | 40 | 40 | 70 | 30 | 60 | 150 |
| 25. | *Microbacterium maritypicum*-MM03F | 20 | 130 | 40 | 60 | 70 | 40 | 60 | 150 |
| 26. | *Microbacterium oxydanse*-MO32I | 30 | 120 | 40 | 30 | 70 | 30 | 30 | 140 |
| 27. | *Morganella morganii*-MM11T | 30 | 140 | 30 | 50 | 60 | 40 | 60 | 160 |
| 28. | *Morganella morganii*-MM25T | 40 | 120 | 40 | 30 | 80 | 10 | 80 | 140 |
| 29. | *Proteus mirabilis*-PM18T | 90 | 130 | 40 | 40 | 70 | 50 | 60 | 150 |
| 30. | *Proteus mirabilis*-PM34T | 40 | 150 | 70 | 80 | 90 | 50 | 30 | 160 |
| 31. | *Proteus vulgaris*-PV06T | 50 | 100 | 10 | 80 | 40 | 20 | 30 | 150 |
| 32. | *Proteus vulgaris*-PV37T | 40 | 120 | 30 | 30 | 60 | 30 | 40 | 150 |
| 33. | *Providencia vermicola*-PV07T | 30 | 140 | 40 | 60 | 80 | 50 | 110 | 180 |
| 34. | *Pseudomonas aeruginosa*-PA12T | 40 | 130 | 40 | 60 | 70 | 40 | 60 | 140 |
| 35. | *Pseudomonas aeruginosa*-PA33T | 20 | 130 | 40 | 40 | 70 | 50 | 70 | 160 |
| 36. | *Pseudomonas plecoglossicida*-PP27T | 30 | 130 | 40 | 30 | 70 | 50 | 50 | 150 |
| 37. | *Pseudomonas taiwanensis*-PT26T | 40 | 120 | 30 | 100 | 90 | 10 | 80 | 115 |
| 38. | *Pseudomonas.aeruginosa*-PA13T | 50 | 130 | 40 | 30 | 70 | 60 | 60 | 160 |
| 39. | *Raoultella ornithinolytica*-RO40LCH | 120 | 430 | 90 | 230 | 100 | 90 | 210 | 340 |
| 40. | *Raoultella planticola*-RP17T | 50 | 130 | 40 | 40 | 70 | 50 | 50 | 150 |

## 5.4 Multi metal resistance

The long term effect of pollutants has led to the emergence of multi-metal resistant bacteria, all the 40 mono-resistant bacterial isolates had multiple metal-resistant to various heavy metal ions specifically *R.ornithinolytica*- RO40LCH, *B. safensis*-BS16L , *P.mirabilis*-PM18T, *L. chromiiresistens*-LC15T and were exhibit high tolerance to eight heavy metals collectively with concentration of ( 100,85,85 , 80 ppm) respectively, which is similar to the finding of (Abu shanab *et al.,* 2007, Thacker *et al.,* 2007) who reported a large variety of bacteria with multiple metal tolerance to Ni, Pb, and Zn metal ions. These reports support that the metal resistances of the bacteria were interrelated to each other. The bacteria detected in this work were isolated from river's water with relatively high levels of heavy metals which may explain their high level of tolerance to various metal ions. Moreover, bacteria exhibit several physiological and genetic mechanisms to counteract the toxic effects of metal ions (Figure 5.5).

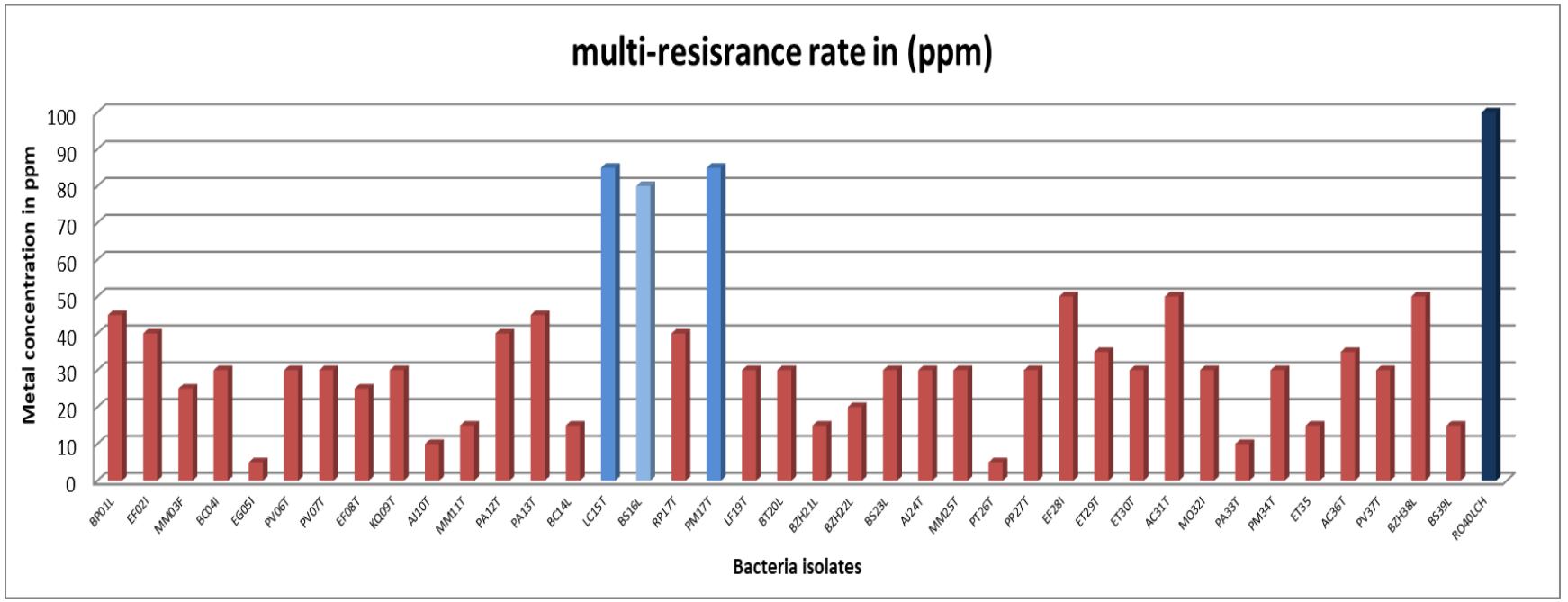



**Figure (5.5) Multi-resistance rate of bacterial species against eight heavy metal ions collectively.**

*** Complete bacterial names and their codes are mentioned in table 5.13.**

## 5.5 Heavy metal removal efficacy

The ability of the bacterial isolates to remove heavy metals from the medium was measured by an inductively coupled plasma optical emission spectrometer (ICP-OES). The results showed that *R. ornithinolytica* shows the highest ability to remove the selected metal in the present study except for Cu by the percentage of (67%, 89%, 63.4%, 55.6%, 56.5%, 65%, 61.9 %) for each of Cd, Pb, Cr, Ni, Zn, Co, and Fe respectively (figure 5.3, 5.4 and 5.5), implicating that this isolate could be a promising candidate for practical bioremediation of heavy metal polluted environments.

The maximum rate of Cu reduction was detected by *E. tabaci*-ET29T with a ratio of (55.8%). Besides *R. ornithinolytica*, each of *P. plecoglossicida*-PP27T and *E. gallinarum*-EG05I removed the highest amount of cadmium (41.9% and 41.1%, respectively), while *B. safensis* -BS16L removed 55.4% of pb as shown in (Figure 5.6).

Also, *B. safensis* -BS16L removed the high level of Cr, Ni, Fe, and Co (53.1%, 53.7%, 47.7%, and 61.4%, respectively) (Figures 5.7 and 5.8). Among the metals, zinc had the lowest amount of removal, which did not exceed 29.3% except the reduction rate by *R. ornithinolytica* as shown in (Figure 5.7). The ability of isolates to uptake heavy metals was higher than the previous studies, (*K. variicola*) isolated from industrial effluents could remove 50% of Ni and 68.6% of Co (Afzal *et al.,* 2017), while the removal effectiveness of (Pb 45% and Cu 62%); ( Cd 56%, Ni 34%, and Co 53%) was detected by *E. coli* and *P. aeruginosa* respectively in a study done by Gawali *et al.,* (2014).The results are in agreement with the work conducted by Das and Kumari (2016) who found that *Enterobacter* sp. and *Klebsiella* sp. isolated from industrial effluents have the ability to uptake Pb when studied *in vitro*.

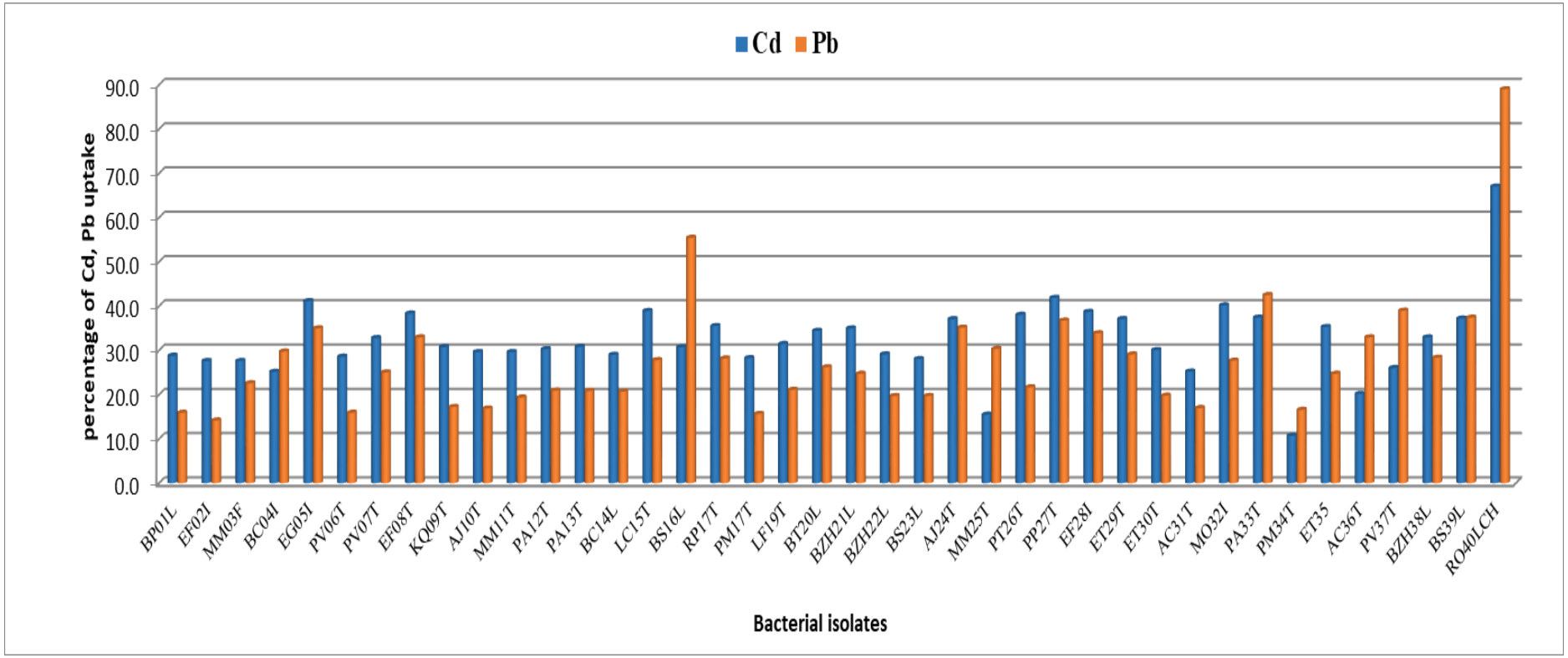



**Figure (5.6)** Percentage of Cadmium and Lead uptaked by isolated bacteria.

*** Complete bacterial names and their codes are mentioned in table 5.13**

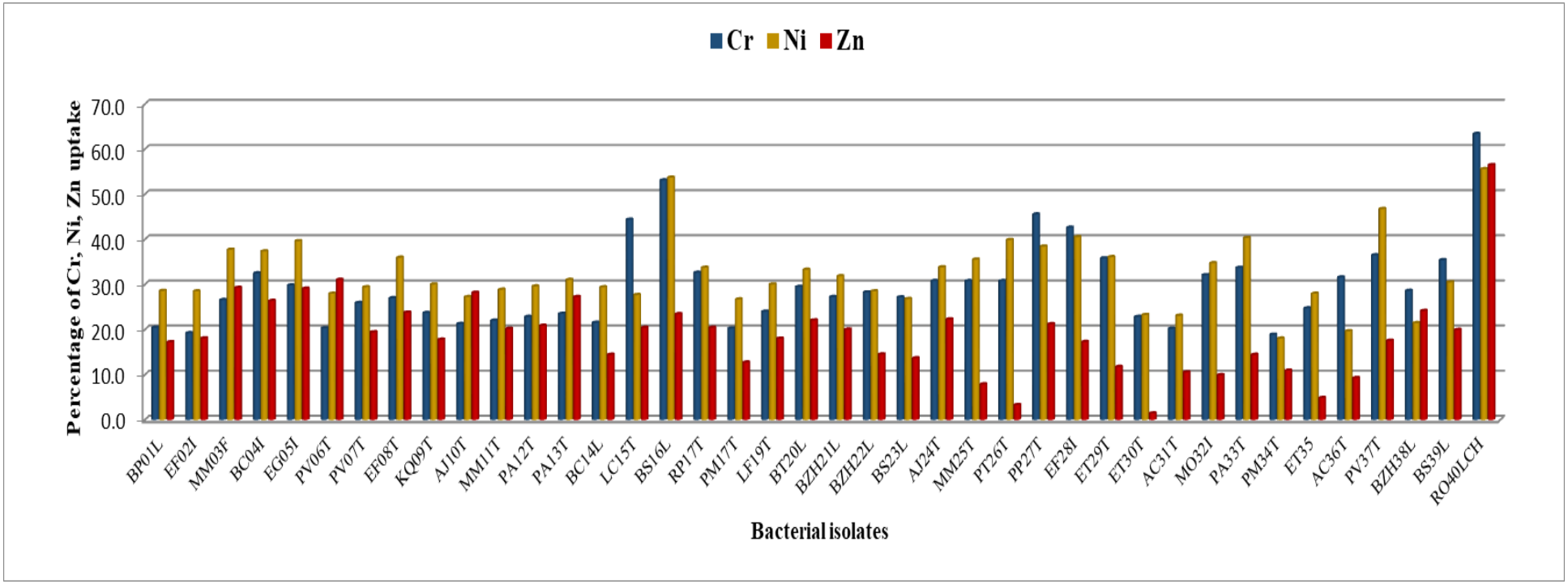



**Figure (5.7)** Percentage of Chromium, nickel, and zinc uptaked by isolated bacteria.

*** Complete bacterial names and their codes are mentioned in table 5.13**

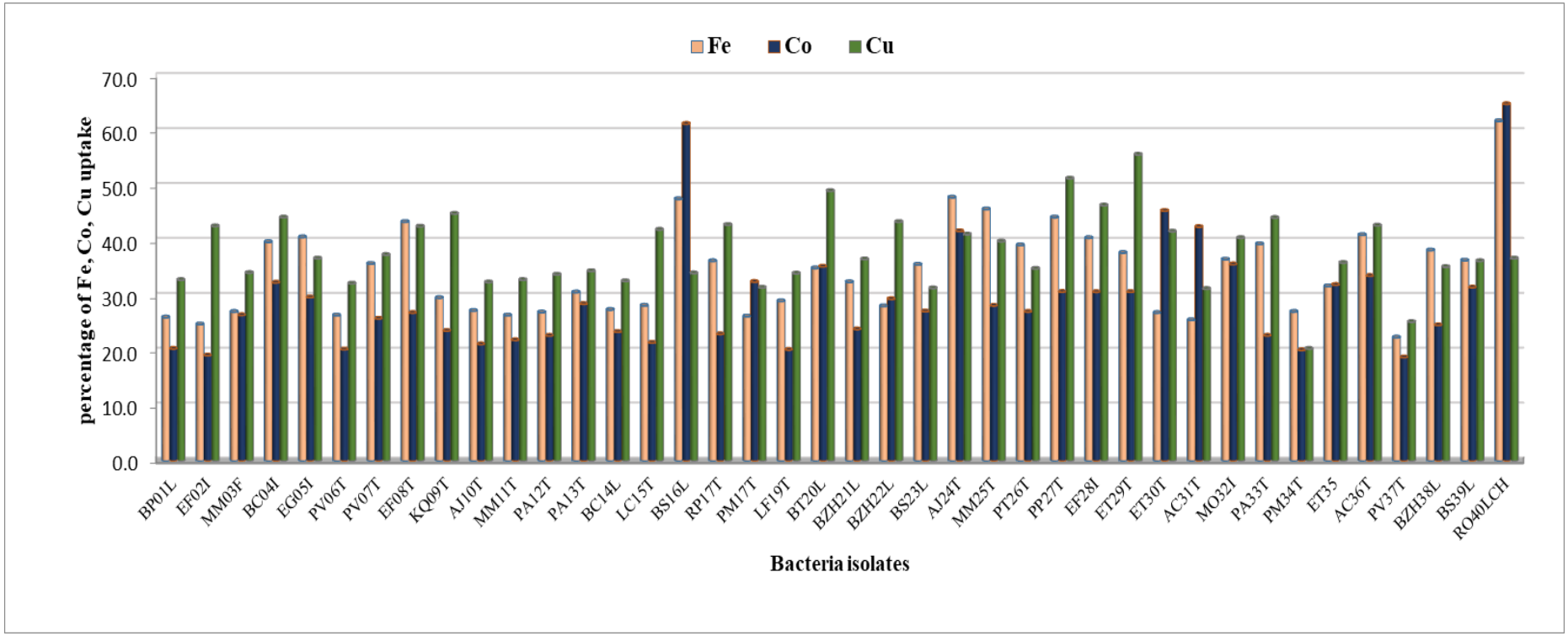



**Figure (5.8)** Percentage of iron, cobalt, and copper uptaked by isolated bacteria.

*** Complete bacterial names and their codes are mentioned in table 5.13**

## 5.6 Optimum condition for heavy metal removal

### 5.6.1 Effect of Temperatures

The capacity of living cells to remove metal ions from aqueous solutions is influenced by the type and concentration of heavy metals and environmental growth conditions, as temperature, pH, and contact time of the microorganisms with toxic metal (Aka and Babalola, 2017).

In this study, the ability of metal uptake by the highly heavy metal resistant isolate *(R. ornithinolytica)* was affected by different environmental conditions (Temperature, pH, and incubation periods). The effect of different incubation temperatures on the uptake of the eight selected metals in (Figure 5.9) revealed that 35°C was the optimum temperature for Cd, Pb, Zn, F, and Co uptake.

Metal removing ratios were changed according to the temperature variation from 45 to 67%, 65 to 89%, 55 to 56.5%, and 50 to 65% for each of Cd, Pb, Zn, F, and Co respectively.

While 25°C was optimum for Cr, Cu, Ni uptake, in which the maximum rate of these metal reductions was 75, 50, 65% for each of Cr, Cu, and Ni respectively, and this in agreement with the study of (El-Shanshoury *et al.,* 2013) who mentioned that maximum biosorption rates for Cd, Co, and Pb by *Enterobacter* sp. could be obtained at 35°C, the best temperature for Zn and Cu uptake was found at 25°C. Furthermore, *Arcanobacterium bernardiae* and *B. amylolikuefaciens* achieve their maximum capacity for Pb up taking at 35°C (Jackson *et al.,* 2011).

Metal solutions at high temperatures can inhibit or denature enzymes, as well as harm structural components of the plasma membrane, limiting bacterial growth and their activity to uptake the metals from the medium (Whiteley and Lee, 2006); this can be attributed to a decrease in metabolic activity caused by the increase in temperature above optimum.

On the other hand, when temperature decrease under the optimum level the bacterial activity is also reduced because most enzymes are inactivated at low temperatures (Aka and Babalola, 2017).

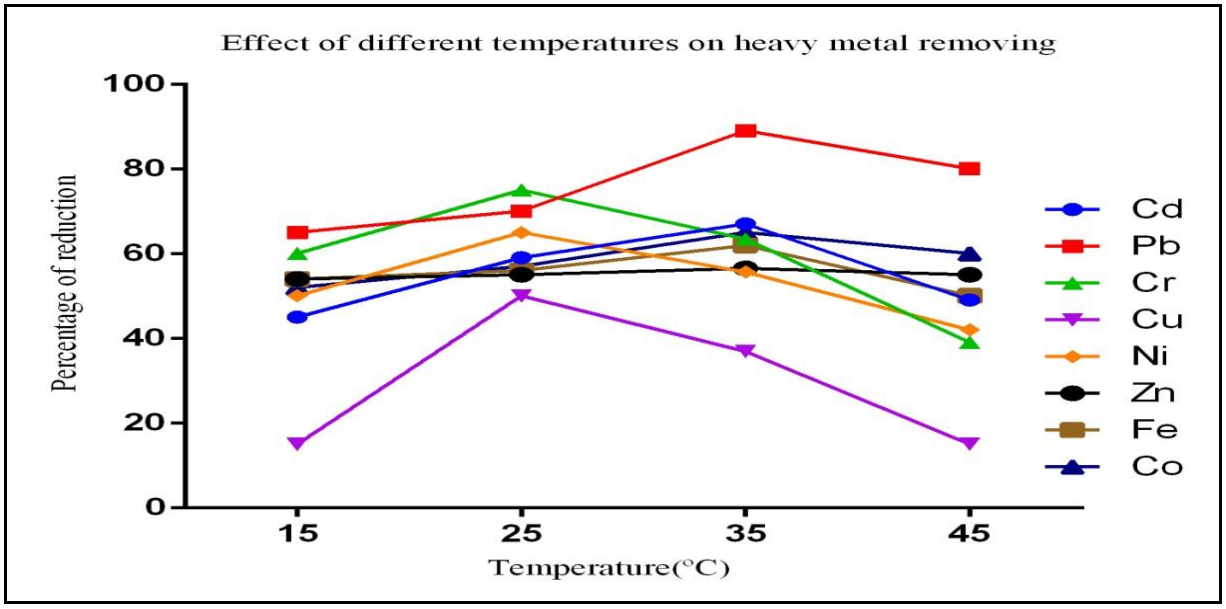



**Figure (5.9)** Effect of different temperature on heavy metal uptake by *R. ornithinolytica.*

### 5.6.2 Effect of different pH value

The bacterial growth, activity, metal bioaccumulation, and biosorption capabilities are influenced by pH, which is an important environmental factor not only affects bacterial activity but also the chemical behavior of metal ions in solution (Dharanguttikarit, 2018), it affects the uptake efficiency of heavy metals and their binding to microorganisms, in which the changes in pH deeply affect the nature of binding sites and solubility of the metals as it influences the solution chemistry of metals (Hussein *et al.,* 2003).

The results of pH variation in this study indicate that pH in the range 7-8 is optimum for most selected metals (Cd, Pb, Cr, and Fe) uptake (Figure 5.10), which agrees with that of (Ozdemir *et al.,* 2003; El-Shanshoury *et al.,* 2013) in which the optimum adsorption of Cd and Cu by *Enterobacter* sp. and *Ochrobactrum anthropi* was at pH 7-8.

similar results were obtained by (Bhattacharyya and Gupta, 2008) who suggested that the adsorption of Cd increased with increasing the pH due to increased negative surface charges, the adsorption of Cd was influenced by the pH of the aqueous medium, and the adsorbed amount gradually increased with decreasing acidity.

At low pH, Cd and Cu accumulations decreased and caused increased competition between hydrogen and Cd, Pb ions for binding sites on the cell surface or by an increase in metal efflux pump activity due to an increase in the proton gradient that drives the efflux pump (El-Shanshoury *et al.,* 2013).

The highest removal of cobalt (Co) and nickel (Ni) obtained at a pH 5, this is agreed with the results obtained by Silva *et al.*, (2009) who revealed that maximum metal removal obtained at pH 6.25, however, higher pH values led to decrease in removal efficiency, because metal hydroxide would precipitate out of solution at alkaline pH.

Amin and Selmy (2017) indicated that at pH higher than 8, the formation of hydroxide ions causes precipitation of Zinc, the hydrolyzed species including $Zn(OH)^{+}$, zinc bicarbonate ($ZnHCO_3^{+}$), $Zn(OH)_3^{-}$ of zinc will be present in sufficient amounts relative to $Zn^{+}$ to be available for the organism to transport or adsorb.

For Cu the variations of pH almost do not effect on the rate of it is removing from the medium, which is disagreed with the results obtained by (El-Shanshoury *et al.*, 2013), that pH 5 was optimum for Cu uptake.

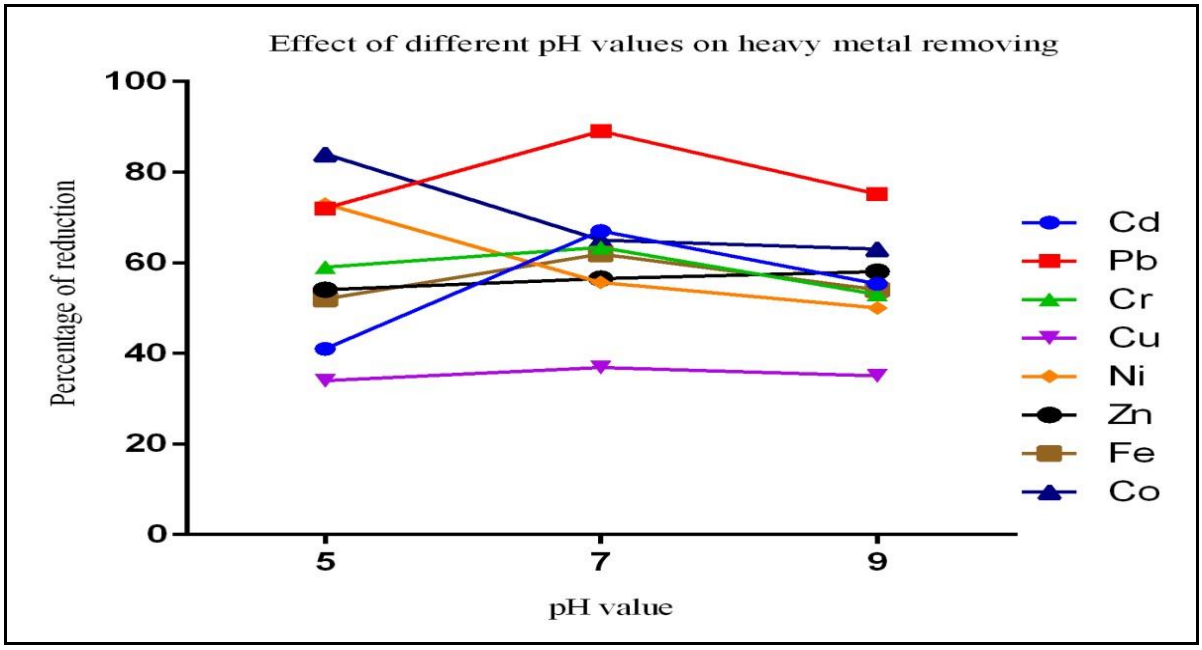



**Figure (5.10)** Effect of Different pH value on heavy metal uptake by *R. ornithinolytica*

### 5.6.3 Effect of contact time

The contact time between the bacterial cells and the metal solutions is an important factor affecting the metal uptake. (Figure 5.11) shows the uptake for heavy metals by *R. ornithinolytica* in the range from 0-72 hr. the maximum removal of Pb, Cu and Ni were reached after 18hr incubation in which the percentage of their uptake was 95, 45, and 64% respectively and this agrees with the results obtained by (Yetis and Ceribass, 2001) who reported that the biosorption of Pb by *Phanerochaete chrysporium* was rapid in the first incubation hours until equilibrium was attained.

On the other hand, *R. ornithinolytica* has the ability to remove the highest percentage of each of Cr, Zn, and Fe after 24hr incubation. Only Cu showed 46% uptake after 72hr

incubation. In a study done by Akhter *et al.*, (2017) it was concluded that the percent removal capacity of Ni and Cd reached the maximum at 24 hr and 48 hr; similar findings were reported concerning Cd biosorption by (Vijayadeep and Sastry, 2014).

The effect of contact time on metal uptake revealed that each heavy metal had an optimum period, and once this time had passed, uptake remained steady or slightly decreased, this agrees with metal uptake models, where the process can be considered as an equilibrium that involves adsorption and desorption due to saturation, as a result, exposing tested organisms to metal ions for longer than the optimum time may not improve metal uptake (Odokuma and Akponah, 2010).

When *B. altitudinis* was used to remove Ni from contaminated industrial effluents, its concentration begin to decrease in the medium after 8-9 hr in which the bacteria started to uptake it (Babar *et al.*, 2021), a similar result was obtained by (Kabir *et al.*, 2018) who observed chromium uptake by chromium resistant bacteria after 72 hr was higher when compared with those after 24 and 48 hr.

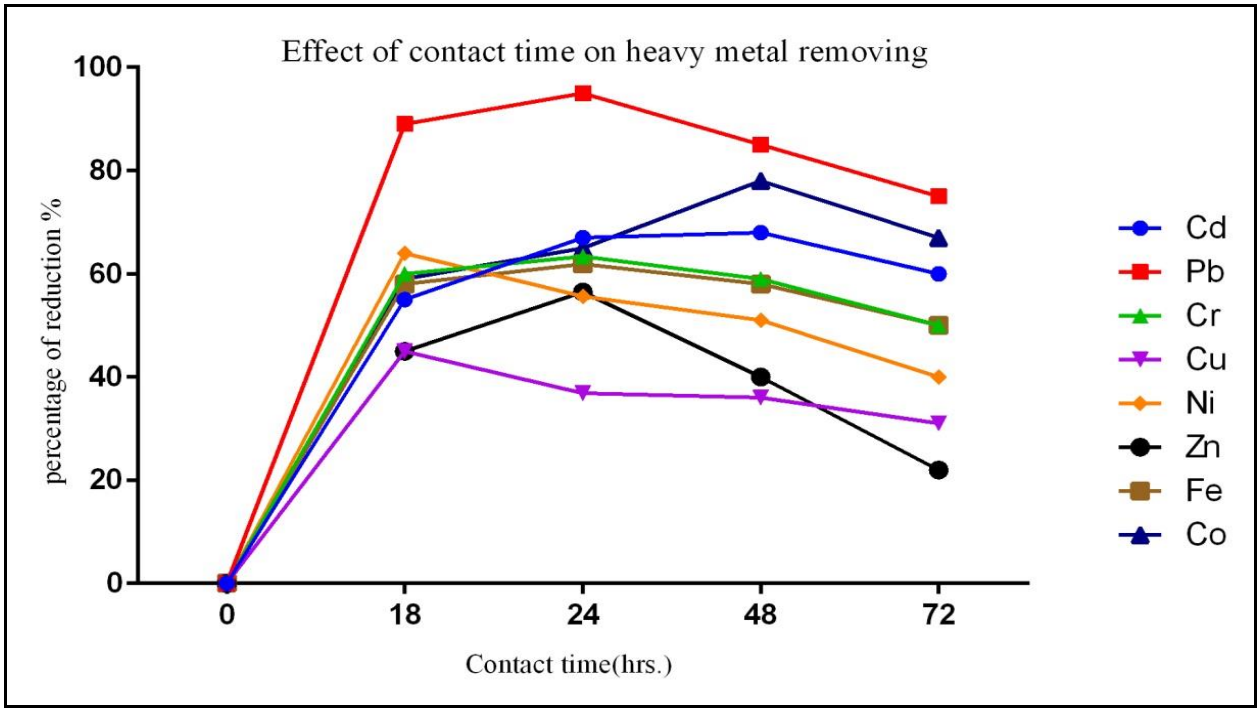



**Figure (5.11)** Effect of contact time on heavy metal uptake by *R. ornithinolytica.*

## 5.7 Effect of heavy metal on the *R. ornithinolytica's* growth

The presence of heavy metals acts as a stress for the bacterial growth, as observed from the overall reduction of the growth (Figures 5.12). The growth curves of *R.*

*ornithinolytica* against selected eight metals separately compared to its respective growth patterns in the absence of heavy metal addition.

Generally, the growth of the isolate in medium containing heavy metals was slower than that in medium without metal addition which reduced the rate of growth of bacteria as compared to the control group. This may attribute to the toxic effect of heavy metals that inhibit the growth and reproduction of some bacteria and reduce their biomass if it reaches concentration above the tolerance level of the bacteria (Wang *et al.*, 2020). *R. ornithinolytica* grew well in medium containing lead which might happen due to the well-development of lead tolerance mechanism as Tanjaro river's water contains a high concentration of Pb resulted in the lead-tolerant bacteria, while the lower concentration of Cd in the water samples resulted in a lower tolerance rate for this metal ion and the growth was slower than that in medium with the addition of other heavy metal, a similar finding was observed by (Irawati *et al.*, 2017a).

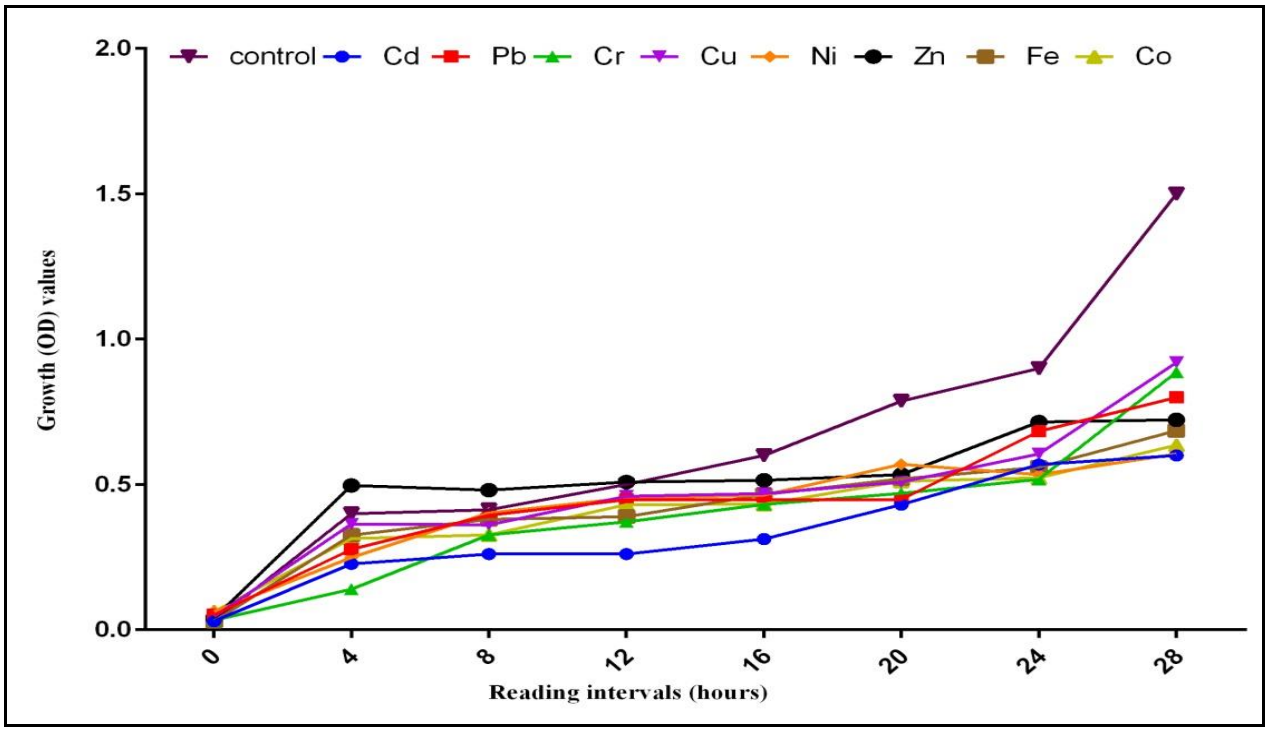



**Figure (5.12)** Effect of heavy metal on the *R ornithinolytica's* growth.

## 5.8 Plasmid curing

The presence of chemicals with antimicrobial potential (e.g. antibiotics and heavy metals) in wastewater creates a high selectivity environment for resistant microorganisms; bacteria that survive in this selective matrix can exchange genetic elements and disperse to the environment if they are not removed in wastewater treatment plants (Manaia *et al.,* 2018).

Heavy metal resistance could be mediated by genes on chromosomes, plasmids, or transposons, the plasmids carried genes responsible for resistance to high levels of toxic heavy metals (yang *et al* .,2020)

In the present study, each of SDS and Ethidium bromide were used as curing agent, after a 24-hour incubation at 37°C the capacity of living cells to remove metal ions from aqueous solutions were detected comparing the development of bacterial colonies on heavy metal-containing plates with that of the normal (without heavy metals) plates as shown in (Figure 5.13), the ability of *R. ornithinolytica* to grow in the presence of different heavy metals was plasmid-encoded and this ability is lost after treating the bacteria with 12% SDS or 10µg/ml of ethidium bromide.

Zolgharnein *et al.,* (2007) reported that the frequency of the occurrence of plasmids in heavy metal resistant bacteria was more than that in common bacteria. Similar results were concluded by (El-Shanshoury *et al.,* 2013) who worked on *Enterobacter sp.* ability for metal uptake from polluted industrial wastewater in Egypt.

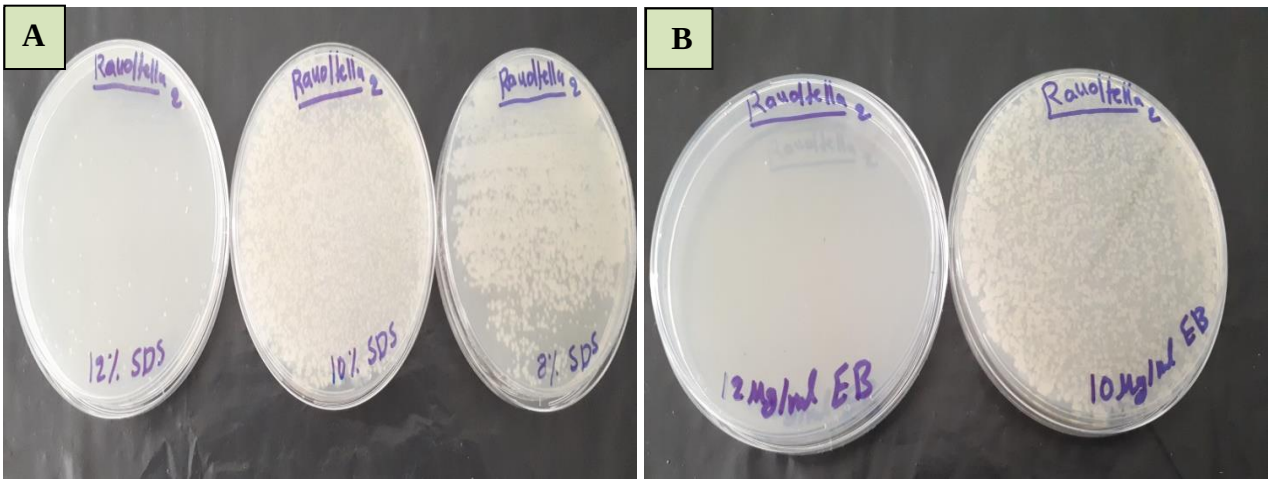



**Figure (5.13)** Plasmid curing of *R.ornithinolytica* in medium suplemented with different concentration **of A- SDS and B- E.B.**

## 5.9 Metal resistant genes

To survive in hostile conditions, bacteria have evolved heavy metal tolerance mechanisms through evolution (Aka and Babalola, 2017). *R.ornithinolytica* isolated from Tanjaro River's water had a high level of resistance to selected heavy metals, and it is clear from the results that showed good absorption/ adsorption potential. In the bioremediation processes, heavy metal resistance genes are of great importance, metal resistance determinants were initially found on bacterial plasmids.

Moreover, heavy metal resistance bacterial strains (HMRB) bearing multiple heavy metal-resistant genotypes and phenotypes could be more promising in bioremediation applications in complex environments (Das *et al.*, 2016).

Bacterial resistance to heavy metals is a complex process, the mechanisms of which are main; transportation, biosorption, and co-metabolism/ redox, which are determined by many genes on the genetic level. For instance, *czcA* (cadmium, zinc, and cobalt efflux pump), *chrB* efflux protein have been found for the transportation of chromium, *pbrT* which is responsible for the biosorption of Pb, *pcoD* - copper efflux pump (Nies, 2003; Jin *et al.*, 2018). The occurrence of heavy metal tolerance genes in *Raoultella* sp. isolated from wastewater samples is depicted in (Figure 5.14).

The PCR results revealed that *R. ornithinolytica* Figure (5.14-A) contains five genes out of the six selected metal resistant genes which are *(pbrT, chrB, nccA, iroN,* and *czcA)* that are responsible for (Pb biosorption, Cr efflux, Ni/Co efflux protein, iron uptake and Co/Zn/Cd efflux) that amplifying (448, 450, 1141,667 and 320 bp) genes respectively, *pcoD* gene was absence which responsible for copper efflux, which may be the reason behind that *R. ornithinolytica* has the lower resistance for copper in compare to the other metals, (Zagui *et al.,* 2020) suggested that copper is widely used in hospitals especially in surfaces for preventing biofilm formation and healthcare-associated infections.

Tanjaro river is almost far away from any hospital that may be the reason behind low copper concentration in the water and low resistance, while *R. planticola* (Figure (5.14-B) have a lower resistance and metal removing ratio in comparison to *R. ornithinolytica* which may be due to the presence of three genes out of six (*pcoD, pbrT, czcA*), however in a study done by Koc *et al.*,(2013) he found that *R. planticola* was resisted to each of copper, iron, lead, manganese, and nickel. Although determining the resistance phenotype is critical for clinical isolates, tolerance to antimicrobial drugs, even when below the resistance/susceptibility breakpoints, may provide a selective advantage for the organism in the environment.

Previous studies in which the occurrence of HMTG in bacteria from hospitalized wastea were evaluated found high occurrences of *czcA* tolerant genes in different bacterial species (Zagui *et al.,* 2020) which corroborates the results of the current investigation but this disagreed with the results of (Adekanmbi *et al.,* 2019) in which chromium-zinc–copper resistance genes *czcA,* were not detected in any of the isolates, while copper resistance genes, pcoA were detected in *Bacillus stratosphericus, chrB* encoding chromium resistance were detected in *Proteus mirabilis* and *Klebsiella oxytoca*. In the Ganges river, India, a high abundance of HMTG conferring tolerance to copper, iron, cobalt, and others metals were detected in water and sediments, being associated with pollution by wastewater and diffuse sources (Reddy and Dubey, 2019).

Multiple heavy metal-resistant phenotypes were identified with a higher rate of resistance and bioremediation potential among the HMRB strains in this study, was not reported in previous studies. Although there were inconsistencies between heavy metal-resistant phenotypes and genotypes, as only 5 metal resistant genes detected in *R. ornithinolytica* but phenotypically show resistance to the eight selected metals, this HMRB strain potentially provide a gene pool for future genetic methods to metal bioremediation.

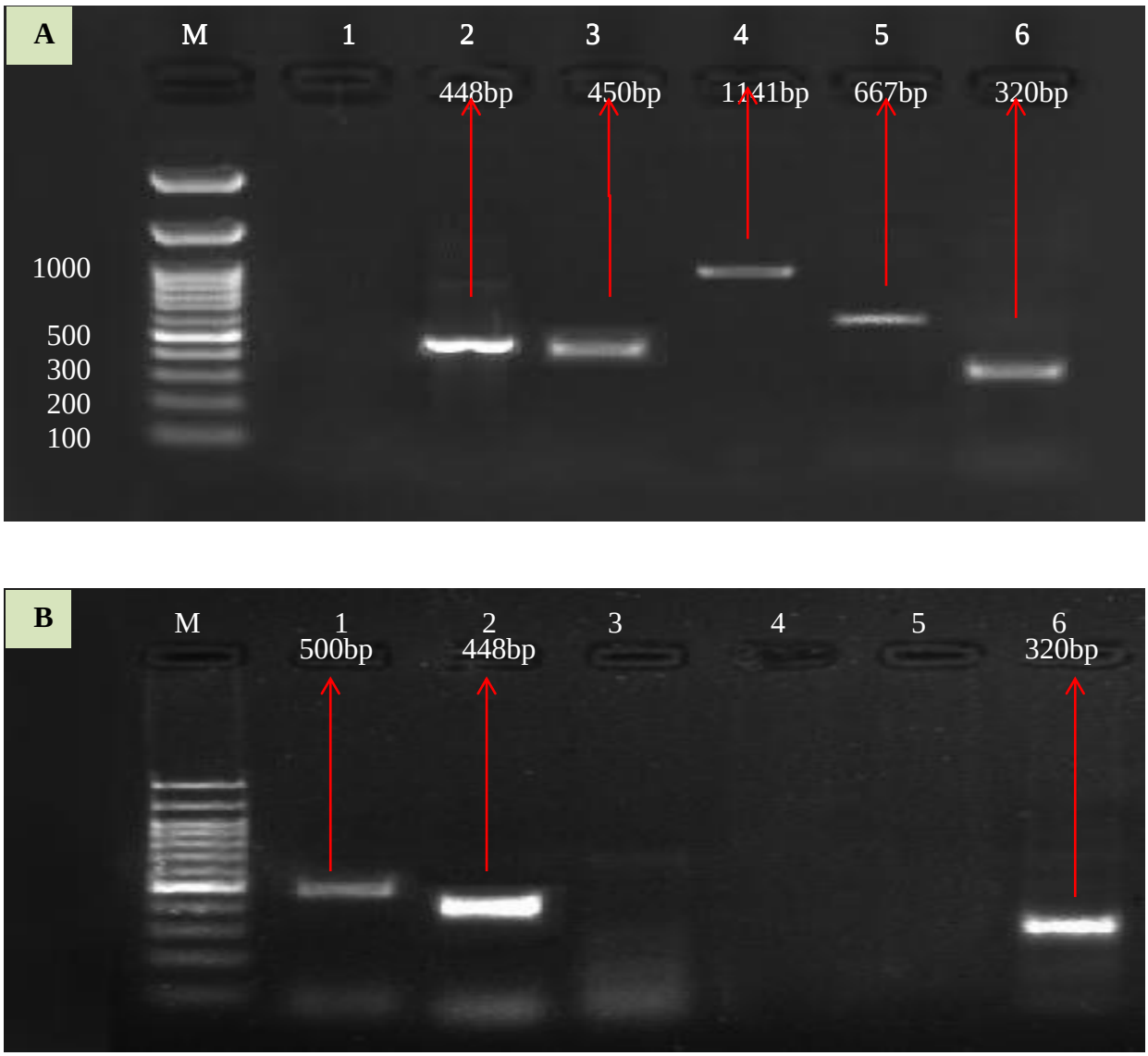



**Figure (5.14)** Agarose gel electrophoresis of metal resistant genes in *Raoultella sp.* **A-** *R. ornithinolytica***; B-** *R . planticola.* M= DNA ladder (100bp); lanes **1** *pcoD* gene 500bp,**2** *pbrT* gene 448 pb,**3** *chrB* gene450 pb ,**4** *nccA* gene 1141 pb, **5** *iroN* gene 667 pb, **6** *czcA* gene 320 bp.

## 5.10 Field emission scanning electron microscopy (FESEM) and energy dispersive X-ray spectroscopy (EDS) analysis

The high uptake isolate (of the eight metal ions), were selected for characterization, and identification before and after metal exposure, cell of the isolate was examined by field emission scanning electron microscope (FE-SEM) to detect any change in the morphology of the cells as a result of metal exposure, normal *R. ornithinolytica* without metal stress (control) were compared with metal stress to see the surface changes in bacteria due to metal stress.

The results SEM images showed in (figure 5.15- A and B). FE- SEM of *R. ornithinolytica* showed that they exist as aggregate short rods or as single cells in untreated culture (control) some dividing cells were found in the fields under the microscope, while the

SEM results of the cells cultured in L.B medium containing different heavy metal separately revealed changes in the bacterial cell size and the morphology in comparison to the control cells.

Generally, when the bacterial cells grow under metal stress they aggregate and stack on top of each other making curvature or dent appearance, this agreed with (Sodh *et al.,* 2020) who observed deformation in the bacterial cell wall when grows under stress of Cd and Cr, in which they became densely packed with a lot of aggregation and roughness in compare with the control cells.

(Chowdhury *et al.,* 2011) revealed three different types of changes in the cell size and surface morphology in comparison the control cells, when cells grow in the presence of Cd in the medium; the area/volume ratio decrease making the cells to be more elongated and produce a filamentous appearance reaching a length of 4.487µm (figure 5.15-C), same findings was documented by Chakravarty and Banerjee (2008) who observed cell surface modifications from smooth to the rough surface and membrane indentations in the presence of metal ions, also the growth of *Acidiphilium symbioticum* in Cd supplemented medium cause cells elongation, this was in agreement with (Afzal *et al.,* 2017) who documented that Ni and Co were adsorbed to the cell wall of *Klebsiella variicola* and change it by creating pores in the cell wall.

The cells in Pb rich medium clearly show the adsorption and the accumulation of Pb particles on the cell surface ( figure 5.15-D) with the decrease in the cell size to the nanoscale, same results obtained by (Liu *et al.,* 2019) who observed significant accumulation on the surface of Lactic Acid Bacteria treated with lead ions.

(Figure 5.15-E) represent the bacterial cells grow in a medium supplemented with copper (Cu), the morphology of the cell changes to resemble a fuzzy coat around the outer surface, which could be due to additional polysaccharide secretion by the cell, which can reduce the surface area of contact between the cell and metal thereby preventing further uptake, same changes were observed by (Chowdhury *et al.,* 2011) which may explain the reason behind the low resistance and uptake ratio of Cu by *R. ornithinolytica,* also (Vicentin *et al.,* 2018) demonstrated that the nature of the exopolysaccharides and their potential for metal adsorption may be linked to the capacity of metal removal.

In the case of bacterial growth in the presence of eight selected metals( multi-metal growth) the cell produce a high rate of aggregation that makes the cell distinguish difficult, with the appearance of crakes on the cell wall as in (figure 5.15-G).

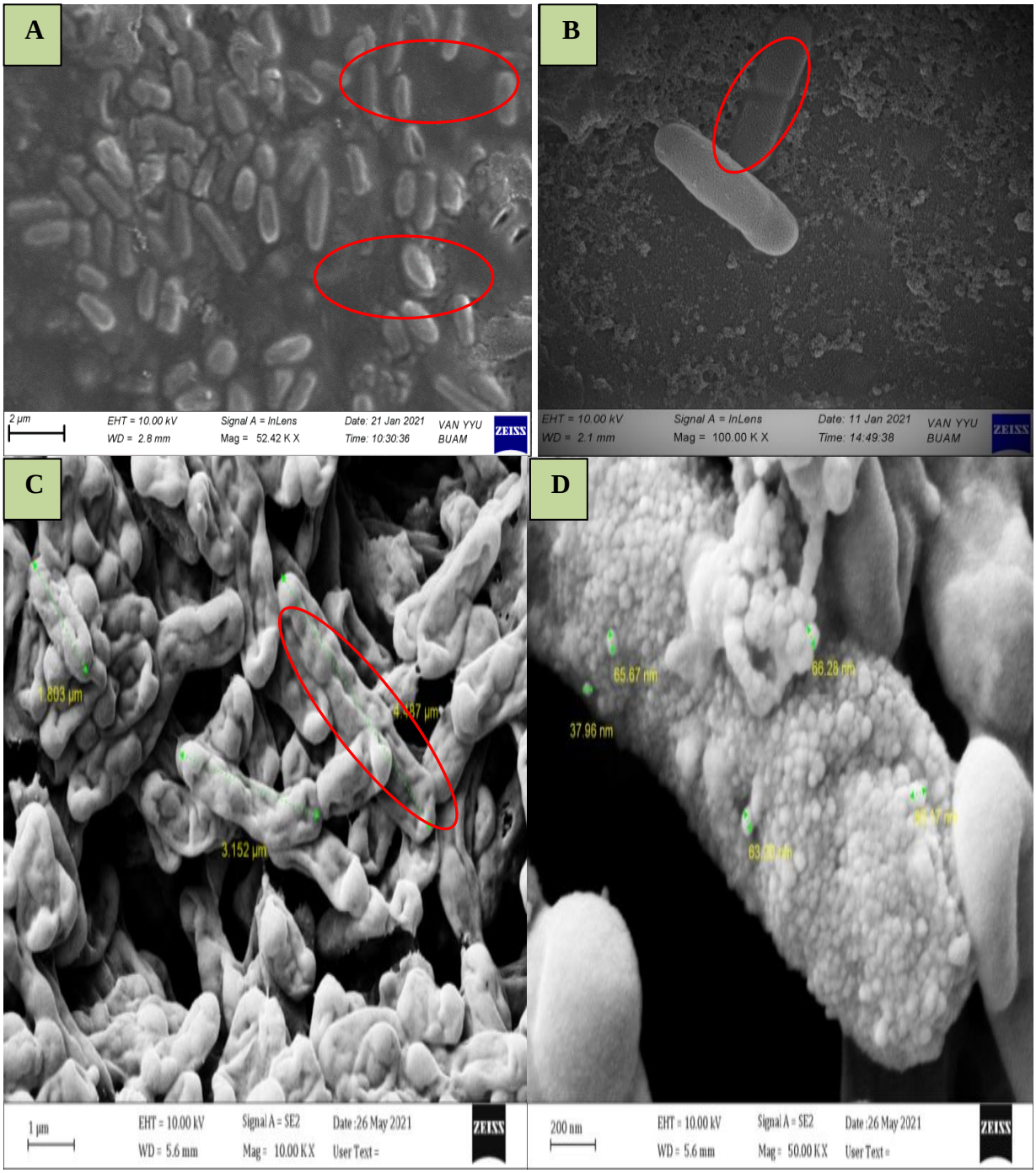



**Figure (5.15)** Field emission scanning electron microphage of *R. ornithinolytica* showing the effect of metal stress on the cell morphology and dimension in the **A& B-** absence of metal (control); and the presence of **C-** Cd; **D-** Pb; **E-** Cu; **F-** Cr; **G**; the presence of multi metals.

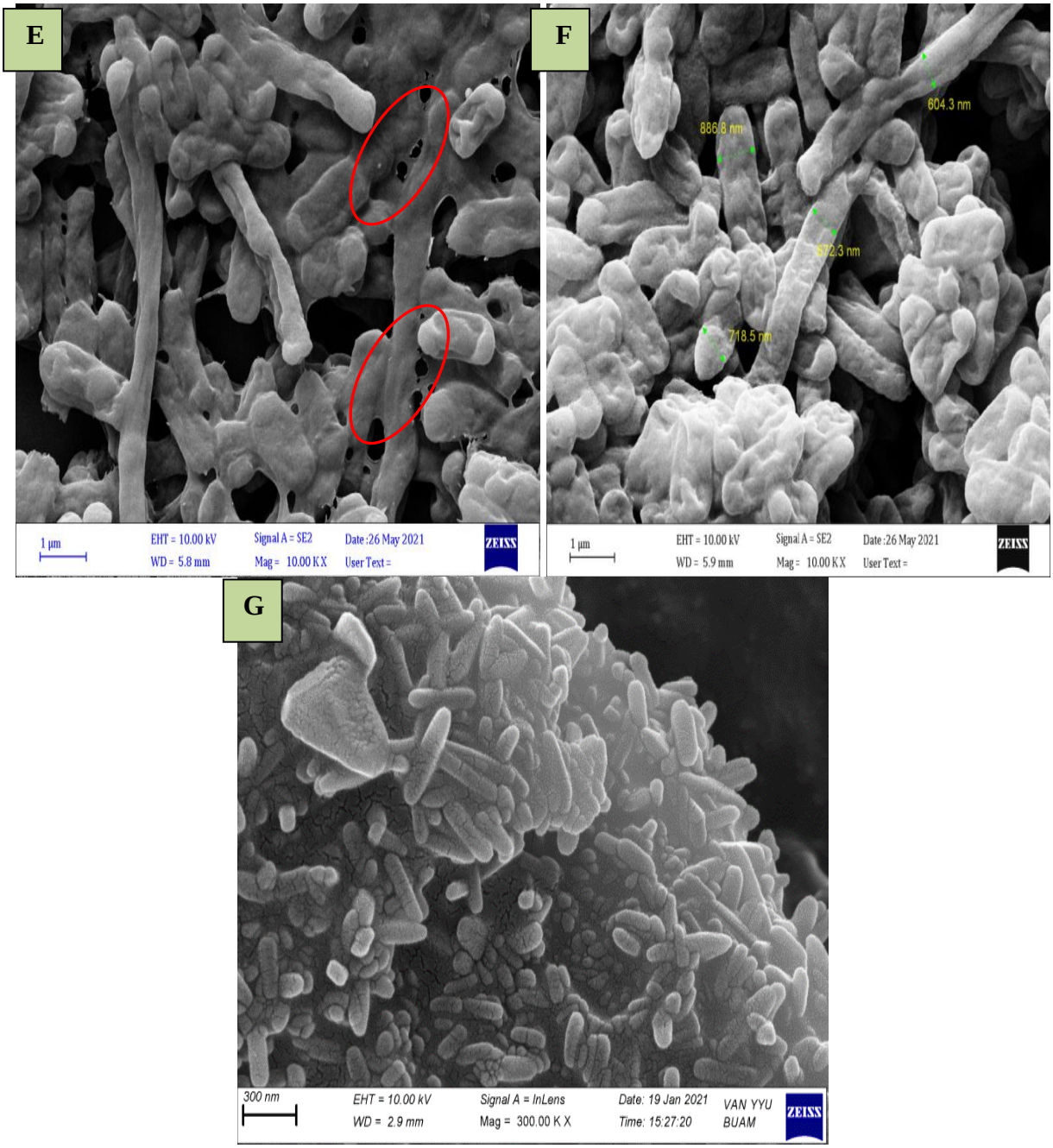



**Figure (5.15)** Field emission scanning electron microphage of *R. ornithinolytica* showing the effect of metal stress on the cell morphology and dimension in the **A& B-** absence of metal ( control); and the presence of **C-** Cd; **D-** Pb; **E-** Cu; **F-** Cr; **G**; the presence of multi metals.

On the other hand, energy dispersive X-ray spectroscopy (EDS) analysis was carried out to confirm the presence of different metals besides the other constituent groups of the bacterial cell wall (figure 5.16). EDS spectral images gave visible evidence of binding metal ions on the cell wall of bacterial cells which clearly showed that Cd, Pb, and Cr ions were adsorbed on the surface with different rate of binding for different metals. Among the metals, lead was found in major proportion in the cell wall with a weight percentaged15.4wt% (figure 5.16-C), in comparison to the other metals, this confirms the higher rate of Pb reducing from the medium by the bacteria that contain *pbrT* genes which responsible for the lead adsorption to the cell wall, and Cu have the minimum amount 0.1wt%,

However, there was little weight percentage of other elements; this was in agreement with the results of (Liu *et al.,* 2019) that may be due to the fact that bacteria's cell walls contain polysaccharides as fundamental building blocks with ion-exchange characteristics, as well as proteins and lipids, which provide a variety of functional groups capable of binding to heavy metals.

These functional groups, such as amino, carboxylic, sulfhydryl, phosphate, and thiol, have different metal binding affinity and selectivity, making them less competitive than lead (Al-Garni ,2005). This finding was agree with (Syed and Chinthala 2015) when study the metal biosorption by *Bacillus sp.* that record higher rate of lead biosorption and lower rate for copper, but results of the present study was disagree with those of (Akhter *et al.,* 2017) who detect the presence of chromium in major proportion in the cell wall and manganese was found in low proportion.

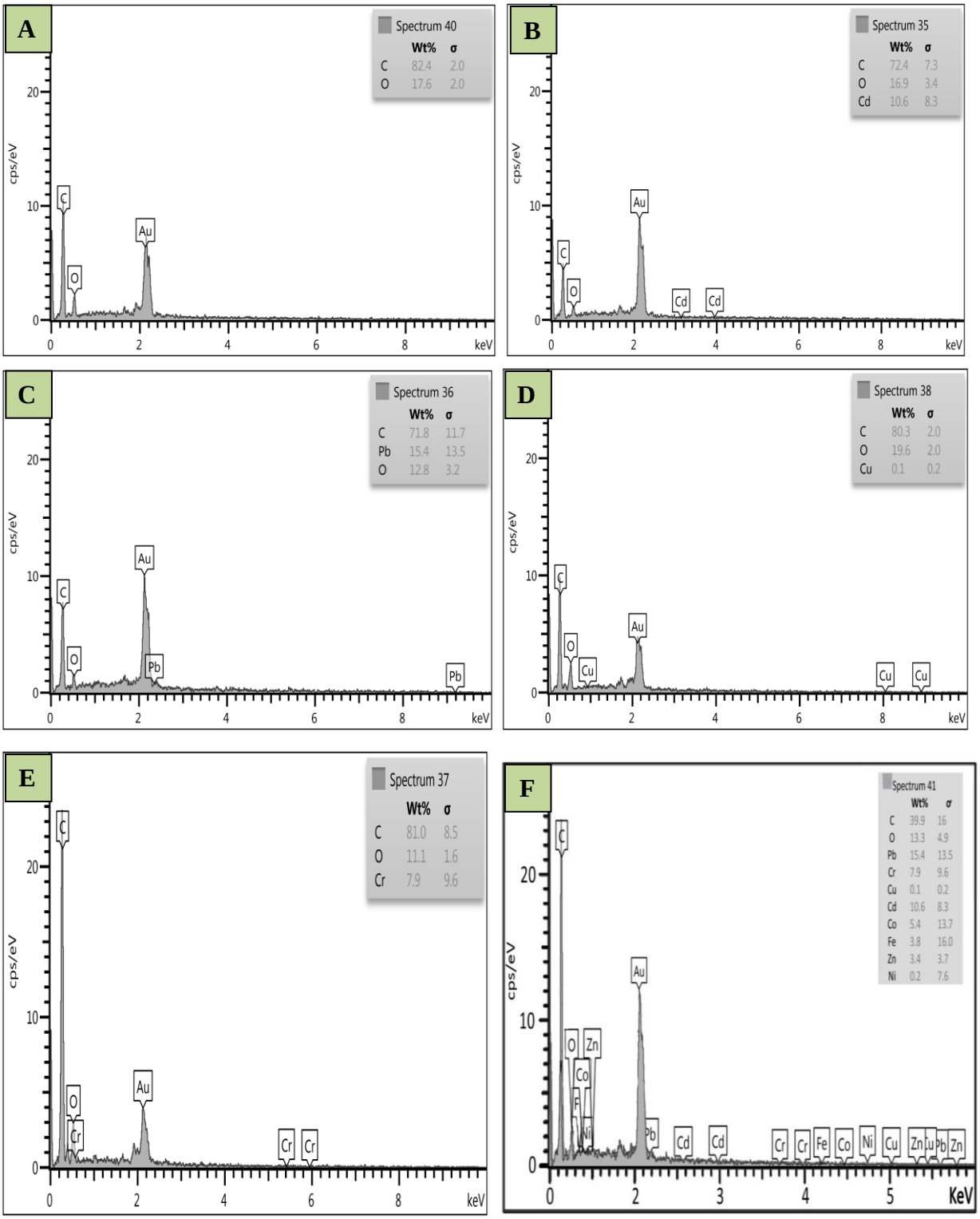



**Figure (5.16)** Energy dispersive X-ray spectroscopic (EDS) analysis for elemental composition on the cell surface of *R. ornithinolytica* **A-** without metal loading (control) **B-** Cd; **C-** Pb; **D-** Cu; **E-** Cr; **F-** presence of multi metals.

## 5.11 Localization and distribution of heavy metals in *R. ornithinolytica*

All the bacterial isolates have the ability to grow in the presence of the selected metals, however; *R. ornithinolytica* showed the maximum tolerance toward the eight metals with different uptake values, these differences in the uptake may be due to the difference in mechanisms by which the bacteria can tolerate and uptake different heavy metals.

To investigate the mechanisms and localization of adsorbed metal particles within the cells transmission electron microscope TEM was used (Upadhyay *et al.*, 2017) which provided an insight into the intra-cellular accumulation of heavy metals, each of the control and treated cultures were examined.

The TEM images (Figure 5.17) showed that the many electron-dense granules were found, mostly on cell walls and cytoplasmic membrane, Kim *et al.*, (2007) suggested that those electron-dense granules were the heavy metal complexes with the substances binding heavy metals in the bacterial cell. *R. ornithinolytica* perform different mechanism to uptake different types of metals, these difference may be due to differences in the cell wall structures, as well as the production of metal binding proteins (metalloproteins) same results was obtained by (Oladipo, 2018) who demonstrated that cell wall structure of microbes was a key factor in heavy metal uptake. In Pb, Zn and Co uptake cells the granules are mainly found on the cell wall and cell membrane that make cell surface adsorption the candidate mechanism (Figure 5.17-C, G,H), While Cd, Ni, Cr, Cu and Fe were accumulated inside the cell (Figure 5.17-B, F,I) ; same finding was reported by (Qurbani and Hamzah 2020) who worked on metal uptake by *Comamonas* from Tanjaro River, and (Vicentin *et al.*, 2018 ) who reported the accumulation of Cu and Zn within the cells of *Cupriavidus necator* strain.

Only few studies report the participation of *Raoultella sp.* in the metal uptake from the environment.

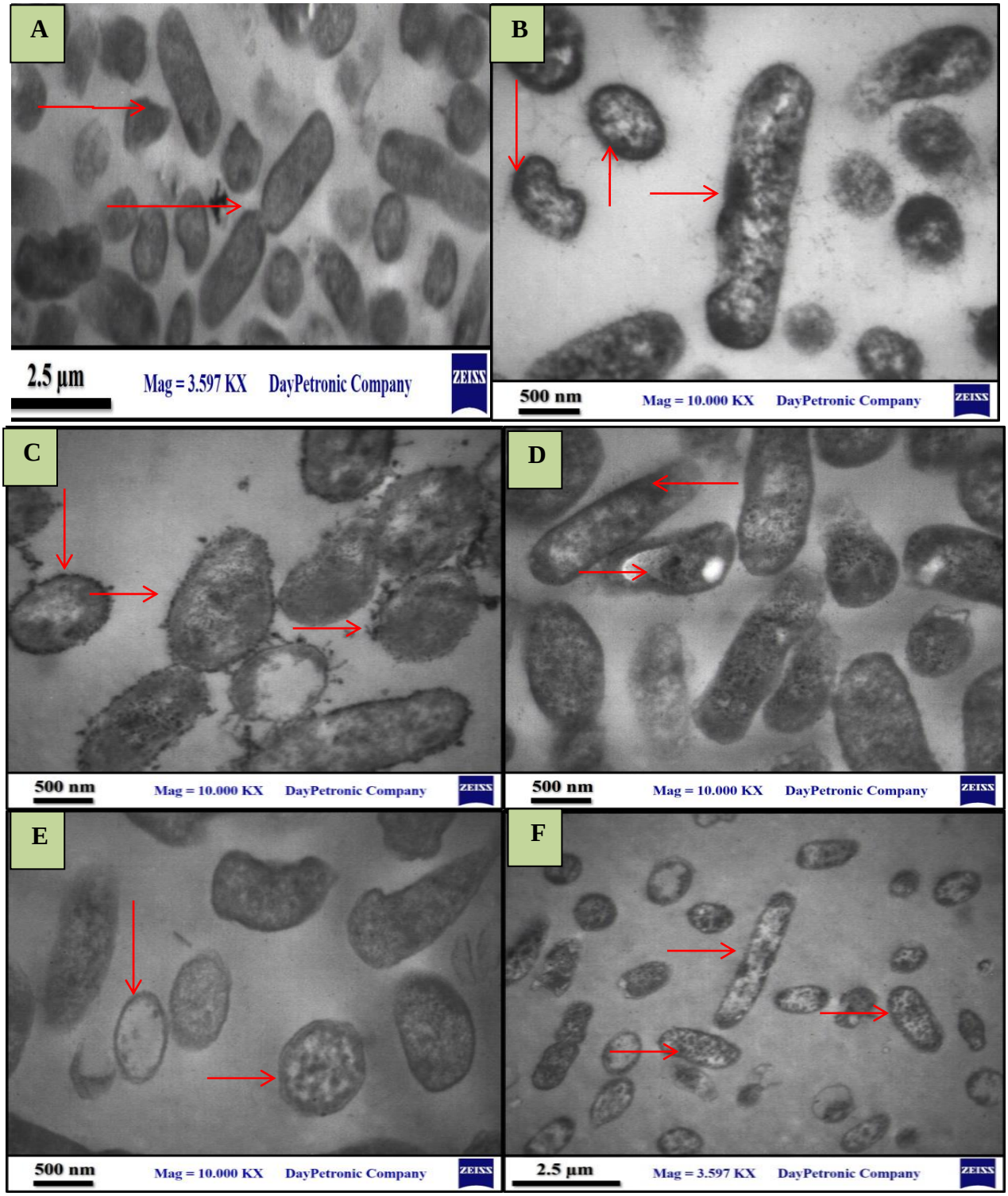



**Figure (5.17)** Transmission electron micrograph of *R. ornithinolytica* . cultured with different heavy metals **A-** (control) without any metals; **B-** Cd; **C-** pb; **D-** Cu; **E-** Cr; **F-** Ni; **G-** Zn; **H-** Co; **I-** Fe.

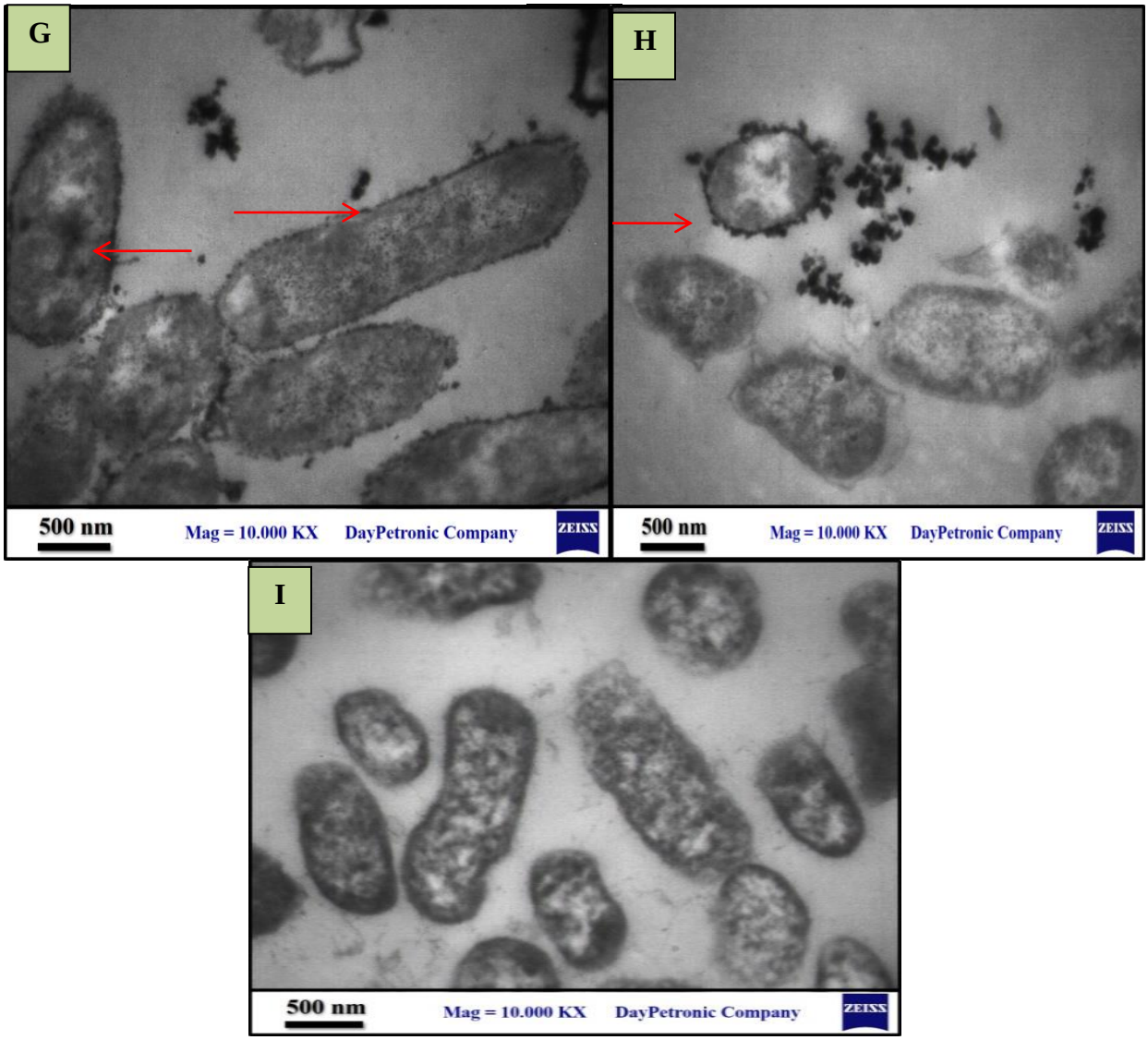



**Figure (5.17)** Transmission electron micrograph of *R. ornithinolytica* . cultured with different heavy metals **A-** (control) without any metals; **B-** Cd; **C-** pb; **D-** Cu; **E-** Cr; **F-** Ni; **G-** Zn; **H-** Co; **I-** Fe.

# Conclusions

# Conclusions

1- Physicochemical analysis of Tanjaro water showed that some water parameters (Total hardness, Alkalinity, Nitrate, and Sulfate) exceed the allowable ranges of drinking water stated by WHO and EPA.

2- Overall, our results showed that Cadmium, Lead, Chromium, and Nickel were present in high concentrations in the water samples, while Co, Cu, Fe and Zn were found within the normal range of WHO for drinking and lifstock.

3- Indigenous bacteria could provide new information about the diversity of the species, as well as their role in removing heavy metal from the contaminated area.

4- Fourty (40) metal resistant bacterial isoltaes were isolated from Tanjaro River; the selected bacterial isolates were highly heavy metal tolerance and uptakes metal.

5- For the first time in Iraq and Kurdistan region, *R. ornithinolytica* isolated from metal polluted Tanjaro River, indicating that the river contaminated by heavy metals, and can providing promising candidates for practical heavy metal bioremediation applications.

6- *Raoultella ornithinolytica*, *Bacillus safensis* and *Leucobacter chromiiresistens*, showed considerable tolerance ability against studied heavy metals with maximum resistance for lead ion. Also it has the ability to remove all the eight metals selected in this study with the exception of Cu.

7- *R. ornithinolytica* have the ability to remove lead from the medium to a range reach 89% which make it effective agent for lead uptake from lead contaminated sites.

8- Multiple heavy metal resistance genotypes and phenotypes were found in all the sequenced HMRB genomes, indicating that bioremediation using bacteria isolated *in situ* may be more efficient.

# Recommendations

# Recommendation

1- People must pay greater attention to environmental issues in order to avoid pollution, which is now prevalent and will continue to deteriorate in the future. Environmental protection laws must be enforced, and more environmental regulations must be implemented.

2- Treatment plant units should be established to treat wastewater before discharging to the environment.

3- Suffecient solid waste management is nessesary for protect Tanjaro River from pollution.

4- Further experiments needed to be conducted to determine the potential of bacterial strains in this study for heavy metal removal, as different culture conditions and medium may affect the bioremediation capability greatly.

5- More studies should be carried out on the metal resistance isolated strain to evaluate their resistance mechanisms.

6- Further studies are recommended on *R. ornithinolytica* to clean up the environment at the site.

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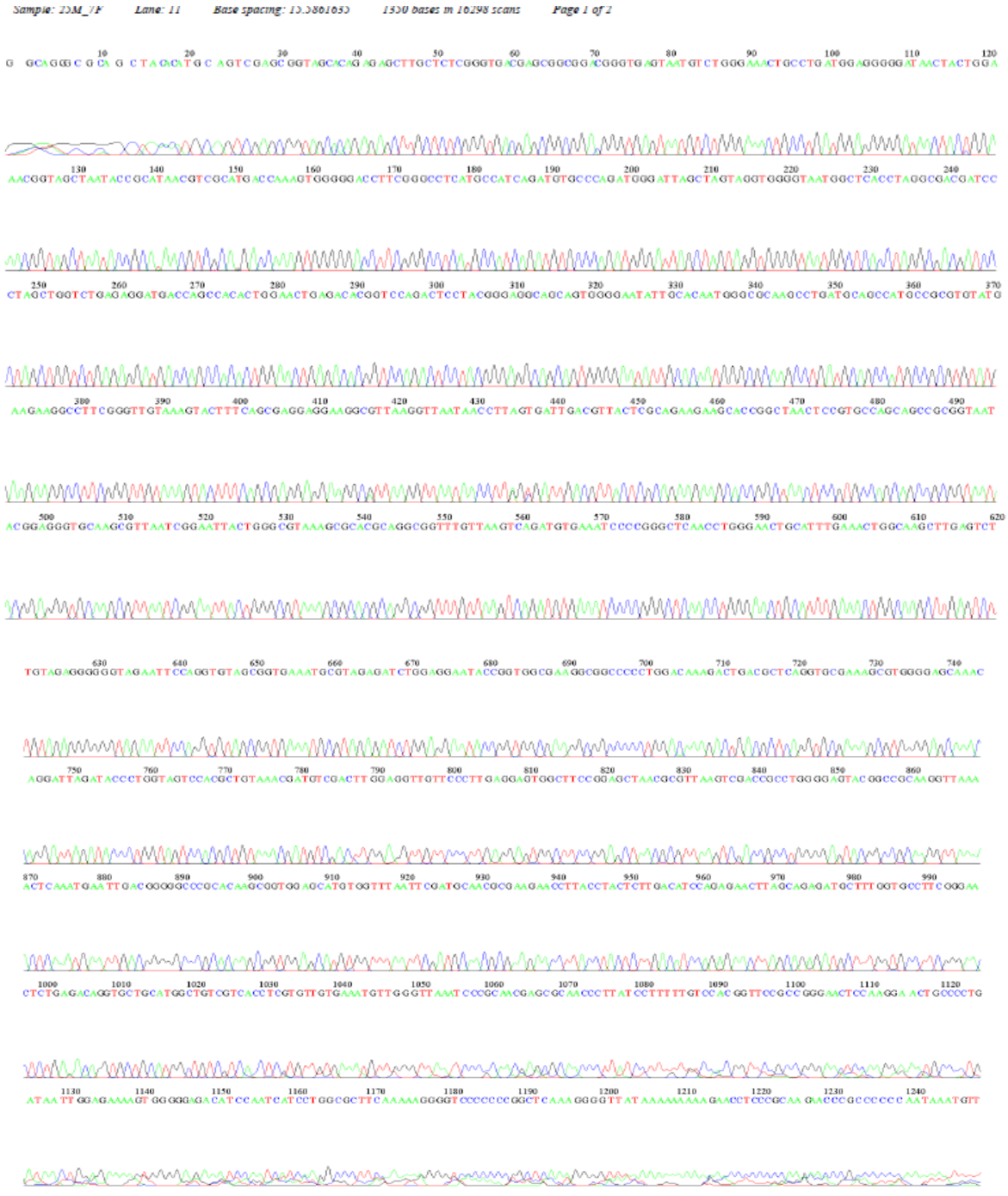
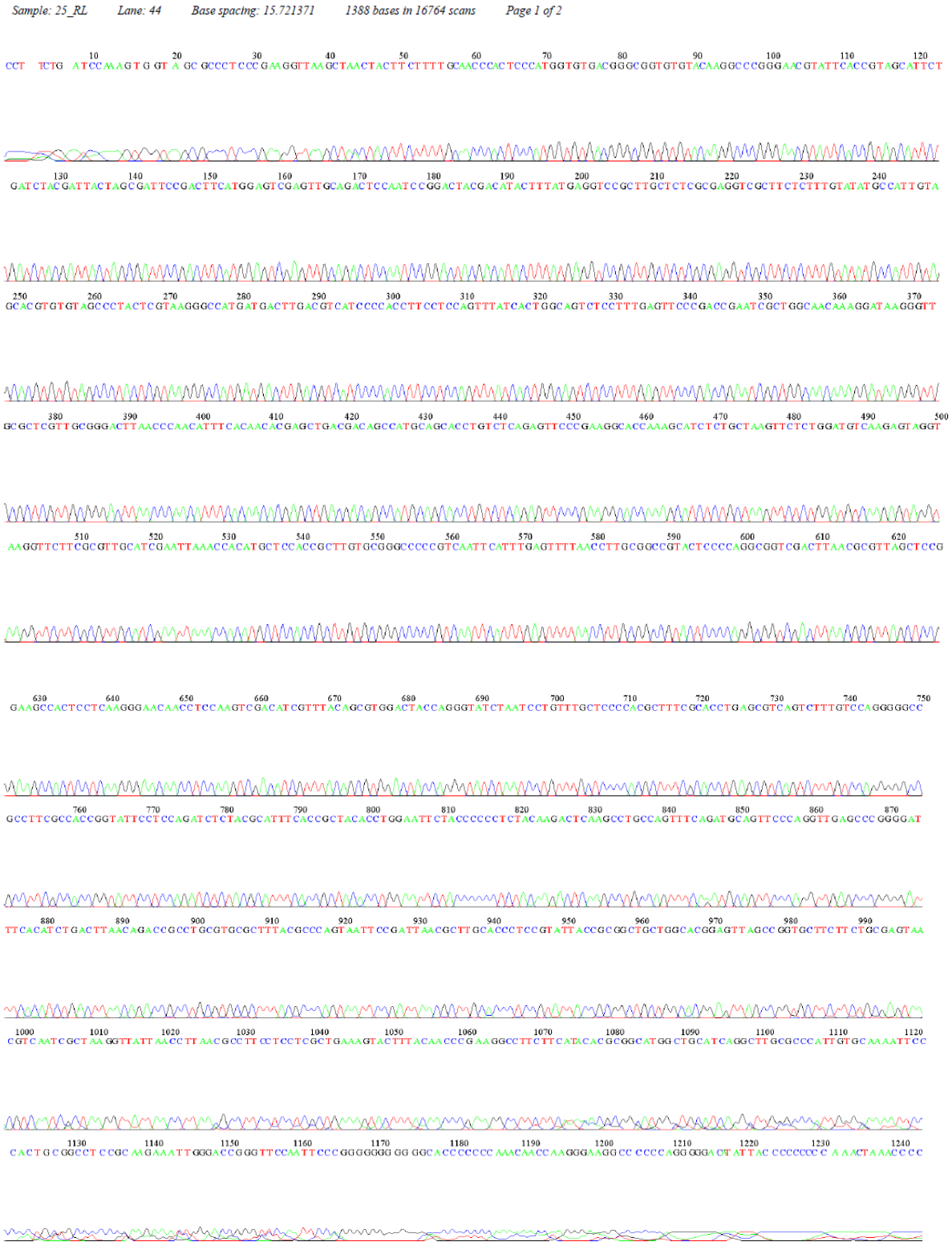
Appendix (1): Electropherograms and sequences of *Raoultella ornithinolytica* 16S rRNA forword primer.



Appendix (2): Electropherograms and sequences of *Raoultella ornithinolytica* 16S rRNA reverse primer.



(3)Alignment 16S rRNA sequences of *Raoultella* sp. submitted to NCBI using Clustal Omega

```
Raoultella.planticola-MZ447097              GTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCA---CTCCCAT     57
Raoultella.ornithinolytica-MZ447120         -GTGGTAGCGCCCTCCCGAAGGTTAAGCTAACTACTTCTTTTGCAACCCA---CTCCCAT     56
Raoultella.terrigena-NR_114503.1            ------------------------------------------------------------     0
Raoultella.ornithinolytica-NR_114502.1      ------------------------------------------------------------     0
Raoultella.planticola-NR_024996.1           -GGGTAATG--GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC     57
Raoultella.planticola-NR_119279.1           -GGGTAATG--GCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCAC     57
Raoultella.planticola-NR_113701.1           ------------------------------------------------------------     0
Raoultella.ornithinolytica-NR_044799        ------------------------------------------------------------     0
Raoultella.ornithinolytica-NR_114736.1      ------------------------------------------------------------     0
Raoultella.electrica-NR_125461              ------------------------------------------------------------     0

Raoultella.planticola-MZ447097              GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTC--------     109
Raoultella.ornithinolytica-MZ447120         GGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTC--------     108
Raoultella.terrigena-NR_114503.1            ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
Raoultella.ornithinolytica-NR_114502.1      ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
Raoultella.planticola-NR_024996.1           ACTGGAACTGAG-------ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT     110
Raoultella.planticola-NR_119279.1           ACTGGAACTGAG-------ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT     110
Raoultella.planticola-NR_113701.1           ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
Raoultella.ornithinolytica-NR_044799        ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
Raoultella.ornithinolytica-NR_114736.1      ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
Raoultella.electrica-NR_125461              ----------------------------------------GGAGGCAGCAGTGGGGAATAT     21
                                                                                     * **** *

Raoultella.planticola-MZ447097              TGATCTACG----------ATTACTAGCGATTCCGACTTCATGGAGT-------CGAGTT     152
Raoultella.ornithinolytica-MZ447120         TGATCTACG----------ATTACTAGCGATTCCGACTTCATGGAGT-------CGAGTT     151
Raoultella.terrigena-NR_114503.1            TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
Raoultella.ornithinolytica-NR_114502.1      TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
Raoultella.planticola-NR_024996.1           TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     170
Raoultella.planticola-NR_119279.1           TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     170
Raoultella.planticola-NR_113701.1           TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
Raoultella.ornithinolytica-NR_044799        TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
Raoultella.ornithinolytica-NR_114736.1      TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
Raoultella.electrica-NR_125461              TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTT     81
                                            **  * * *               *** ** ***  *  *** **        ** ***

Raoultella.planticola-MZ447097              GCAGACTCCAATCCGGACTACG-------ACATAC--------TTTATGAGGTCCGCTTG     197
Raoultella.ornithinolytica-MZ447120         GCAGACTCCAATCCGGACTACG-------ACATAC--------TTTATGAGGTCCGCTTG     196
Raoultella.terrigena-NR_114503.1            GTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAACCTTAGCGATTGACGTTA     141
Raoultella.ornithinolytica-NR_114502.1      GTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAACCTTAGCGATTGACGTTA     141
Raoultella.planticola-NR_024996.1           GTAAAGTACTTTCAGCGAGGAGGAAGGCGTTAAGGTTAATAACCTTAGCGATTGACGTTA     230
Raoultella.planticola-NR_119279.1           GTAAAGTACTTTCAGCGAGGAGGAAGGCRTTAAGGTTAATAACYTTRGTGATTGACGTTA     230
Raoultella.planticola-NR_113701.1           GTAAAGTACTTTCAGCGAGGAGGAAGGCNTTAAGGTTAATAACCTTNGNGATTGACGTTA     141
Raoultella.ornithinolytica-NR_044799        GTAAAGTACTTTCAGCGAGGAGGAAGGCATTAAGGTTAATAACCTTAGTGATTGACGTTA     141
Raoultella.ornithinolytica-NR_114736.1      GTAAAGTACTTTCAGCGAGGAGGAAGGCRTTAAGGTTAATAACCTTRGYGATTGACGTTA     141
Raoultella.electrica-NR_125461              GTAAAGTACTTTCAGCGAGGAGGAAGGCGTTGAGGTTAATAACCTCATCGATTGACGTTA     141
                                            * * * * *  ** *      *                     *      *    **

Raoultella.planticola-MZ447097              CTCTCGCGAGGTCGCTTCTCTTTGTATATG-------CCATTGTAGCACGTGTGTAGCCC     250
Raoultella.ornithinolytica-MZ447120         CTCTCGCGAGGTCGCTTCTCTTTGTATATG-------CCATTGTAGCACGTGTGTAGCCC     249
Raoultella.terrigena-NR_114503.1            CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     201
Raoultella.ornithinolytica-NR_114502.1      CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     201
Raoultella.planticola-NR_024996.1           CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     290
Raoultella.planticola-NR_119279.1           CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     290
Raoultella.planticola-NR_113701.1           CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     201
Raoultella.ornithinolytica-NR_044799        CTCGCAGAAGAAGC-CCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     200
Raoultella.ornithinolytica-NR_114736.1      CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     201
Raoultella.electrica-NR_125461              CTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAA     201
                                            *** *   **         **   *   **       **   ***  ***   *  **

Raoultella.planticola-MZ447097              TACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGC     310
Raoultella.ornithinolytica-MZ447120         TACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGC     309
Raoultella.terrigena-NR_114503.1            GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTCTGTCA-----     249
Raoultella.ornithinolytica-NR_114502.1      GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTCTGTTA-----     249
Raoultella.planticola-NR_024996.1           GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTTTGTTA-----     338
Raoultella.planticola-NR_119279.1           GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTTTGTTA-----     338
Raoultella.planticola-NR_113701.1           GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTTTGTTA-----     249
Raoultella.ornithinolytica-NR_044799        GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTCTGTTA-----     248
Raoultella.ornithinolytica-NR_114736.1      GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTCTGTTA-----     249
Raoultella.electrica-NR_125461              GCG-----TTAATCGGAATTACTGGGCGTAAAGCGCACGCAGG--CGGTCTGTTA-----     249
                                                       *   ** *** * *** *  * ***       * ** * * *

Raoultella.planticola-MZ447097              AGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAG---------GATAAGGGTTG     361
Raoultella.ornithinolytica-MZ447120         AGTCTCCTTTGAGTTCCCGACCGAATCGCTGGCAACAAAG---------GATAAGGGTTG     360
Raoultella.terrigena-NR_114503.1            AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATCCGAAACTGGCAGGCTAGA     309
Raoultella.ornithinolytica-NR_114502.1      AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGA     309
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Raoultella.planticola-NR_024996.1          AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTTGA     398
Raoultella.planticola-NR_119279.1          AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTTGA     398
Raoultella.planticola-NR_113701.1          AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAAGCTTGA     309
Raoultella.ornithinolytica-NR_044799       AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGA     308
Raoultella.ornithinolytica-NR_114736.1     AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGA     309
Raoultella.electrica-NR_125461             AGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTGAAACTGGCAGGCTTGA     309
                                           ****   * ***  ****          **** ***            *  * *

Raoultella.planticola-MZ447097             CGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAG--     419
Raoultella.ornithinolytica-MZ447120        CGCTCGTTGCGGGACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAG--     418
Raoultella.terrigena-NR_114503.1           GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     369
Raoultella.ornithinolytica-NR_114502.1     GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     369
Raoultella.planticola-NR_024996.1          GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     458
Raoultella.planticola-NR_119279.1          GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     458
Raoultella.planticola-NR_113701.1          GTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     369
Raoultella.ornithinolytica-NR_044799       GTC-TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     367
Raoultella.ornithinolytica-NR_114736.1     GTT-TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAVAGATCTGGAGGA     368
Raoultella.electrica-NR_125461             GTT-TGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA     368
                                                ** * ***   **      **  *        **  **   * **   ** **

Raoultella.planticola-MZ447097             -CACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCA     478
Raoultella.ornithinolytica-MZ447120        -CACCTGTCTCAGAGTTCCCGAAGGCACCAAAGCATCTCTGCTAAGTTCTCTGGATGTCA     477
Raoultella.terrigena-NR_114503.1           ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     428
Raoultella.ornithinolytica-NR_114502.1     ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     428
Raoultella.planticola-NR_024996.1          ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     517
Raoultella.planticola-NR_119279.1          ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     517
Raoultella.planticola-NR_113701.1          ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     428
Raoultella.ornithinolytica-NR_044799       ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     426
Raoultella.ornithinolytica-NR_114736.1     ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     427
Raoultella.electrica-NR_125461             ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGA-CGCTCAGGTGCGAAAGCGTGG     427
                                             *** **  *  **    *        *****  *    *** ** *        **

Raoultella.planticola-MZ447097             AGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCG     538
Raoultella.ornithinolytica-MZ447120        AGAGTAGGTAAGGTTCTTCGCGTTGCATCGAATTAAACCACATGCTCCACCGCTTGTGCG     537
Raoultella.terrigena-NR_114503.1           GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTAAACG-------     472
Raoultella.ornithinolytica-NR_114502.1     GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTAAACG-------     472
Raoultella.planticola-NR_024996.1          GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTAAACG-------     561
Raoultella.planticola-NR_119279.1          GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTAAACG-------     561
Raoultella.planticola-NR_113701.1          GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTAAACG-------     472
Raoultella.ornithinolytica-NR_044799       GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTA-ACG-------     469
Raoultella.ornithinolytica-NR_114736.1     GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTA-ACG-------     470
Raoultella.electrica-NR_125461             GGAGCAAACAGGA---------TTAGATACCCTGGTAGTCCACGCTGTA-ACG-------     470
                                            *** *   * *          **  **    *   *   ** ***  *

Raoultella.planticola-MZ447097             GGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTT     598
Raoultella.ornithinolytica-MZ447120        GGCCCCCGTCAATTCATTTGAGTTTTAACCTTGCGGCCGTACTCCCCAGGCGGTCGACTT     597
Raoultella.terrigena-NR_114503.1           -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     525
Raoultella.ornithinolytica-NR_114502.1     -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     525
Raoultella.planticola-NR_024996.1          -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     614
Raoultella.planticola-NR_119279.1          -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     614
Raoultella.planticola-NR_113701.1          -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     525
Raoultella.ornithinolytica-NR_044799       -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     522
Raoultella.ornithinolytica-NR_114736.1     -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     523
Raoultella.electrica-NR_125461             -------ATGTCGACTTGGAGGTTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTT     523
                                                  *     * *    *** *  ***** **     ** **   **   **  **

Raoultella.planticola-MZ447097             AACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAAGTC------------     646
Raoultella.ornithinolytica-MZ447120        AACGCGTTAGCTCCGGAAGCCACTCCTCAAGGGAACAACCTCCAAGTC------------     645
Raoultella.terrigena-NR_114503.1           AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     585
Raoultella.ornithinolytica-NR_114502.1     AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     585
Raoultella.planticola-NR_024996.1          AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     674
Raoultella.planticola-NR_119279.1          AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     674
Raoultella.planticola-NR_113701.1          AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     585
Raoultella.ornithinolytica-NR_044799       AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     582
Raoultella.ornithinolytica-NR_114736.1     AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     583
Raoultella.electrica-NR_125461             AAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCC     583
                                           **  **   **   ** **     ** *****  * ***    * *

Raoultella.planticola-MZ447097             ----------------GACATCGTTTACAGCGTGGACT-----ACCAGGGTATCTAATCC     685
Raoultella.ornithinolytica-MZ447120        ----------------GACATCGTTTACAGCGTGGACT-----ACCAGGGTATCTAATCC     684
Raoultella.terrigena-NR_114503.1           CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT     645
Raoultella.ornithinolytica-NR_114502.1     CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT     645
Raoultella.planticola-NR_024996.1          CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT     734
Raoultella.planticola-NR_119279.1          CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT     734
Raoultella.planticola-NR_113701.1          CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCT     645
Raoultella.ornithinolytica-NR_044799       CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGC-AAGAACCTTACCTACTCT     641
Raoultella.ornithinolytica-NR_114736.1     CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGG-AAGAACCTTACCTACTCT     642
Raoultella.electrica-NR_125461             CGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGG-AAGAACCTTACCTACTCT     642
                                                           *  * *****   **  *        *  *   ** *** **

Raoultella.planticola-MZ447097             TGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGC     745
Raoultella.ornithinolytica-MZ447120        TGTTTGCTCCCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGC     744
Raoultella.terrigena-NR_114503.1           TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     696
Raoultella.ornithinolytica-NR_114502.1     TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     696
Raoultella.planticola-NR_024996.1          TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     785
Raoultella.planticola-NR_119279.1          TGACATCC--AGRGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     785
Raoultella.planticola-NR_113701.1          TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     696
Raoultella.ornithinolytica-NR_044799       TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     692
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Raoultella.ornithinolytica-NR_114736.1      TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     693
Raoultella.electrica-NR_125461              TGACATCC--AGAGAACTTAGCAG-------AGATGCTTTGGTGCCTTCGGGAACTCTGA     693
                                            **    *          ** ***         *  *****       *    **  *

Raoultella.planticola-MZ447097              CACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCT     805
Raoultella.ornithinolytica-MZ447120         CACCGGTATTCCTCCAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCT     804
Raoultella.terrigena-NR_114503.1            GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     753
Raoultella.ornithinolytica-NR_114502.1      GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     753
Raoultella.planticola-NR_024996.1           GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     842
Raoultella.planticola-NR_119279.1           GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     842
Raoultella.planticola-NR_113701.1           GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     753
Raoultella.ornithinolytica-NR_044799        GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     749
Raoultella.ornithinolytica-NR_114736.1      GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     750
Raoultella.electrica-NR_125461              GACAGGTG---CTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCG     750
                                             ** ***    ** **   ** *   **  **        *  **      **   ***

Raoultella.planticola-MZ447097              CTACAAGACTCAAGCTTGCCAGTTTCAAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCA     865
Raoultella.ornithinolytica-MZ447120         CTACAAGACTCAAGCCTGCCAGTTTCAGATGCAGTTCCCAGGTTGAGCCCGGGGATTTCA     864
Raoultella.terrigena-NR_114503.1            CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGGTTCGGCCGGGAACTCA     804
Raoultella.ornithinolytica-NR_114502.1      CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGATTCGGTCGGGAACTCA     804
Raoultella.planticola-NR_024996.1           CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGGTCCGGCCGGGAACTCA     893
Raoultella.planticola-NR_119279.1           CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGGTCCGGCCGGGAACTCA     893
Raoultella.planticola-NR_113701.1           CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGGTNCGGCCGGGAACTCA     804
Raoultella.ornithinolytica-NR_044799        CAACGAGCGCAACCCTTATCCTTT---------GTTGCC-GCGATTCGGTCGGGAACTCA     799
Raoultella.ornithinolytica-NR_114736.1      CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGATTCGGTCGGGAACTCA     801
Raoultella.electrica-NR_125461              CAACGAGCGCAACCCTTATCCTTT---------GTTGCCAGCGATTTGGTCGGGAACTCA     801
                                            * ** **    *  * * *  **         *** ** *          ****  ***

Raoultella.planticola-MZ447097              CATCTGACTTAA-----CAAAC--CGCCTGCGTGCGCTTTACGCCCAGTAATTCC---GA     915
Raoultella.ornithinolytica-MZ447120         CATCTGACTTAA-----CAGAC--CGCCTGCGTGCGCTTTACGCCCAGTAATTCC---GA     914
Raoultella.terrigena-NR_114503.1            AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     864
Raoultella.ornithinolytica-NR_114502.1      AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     864
Raoultella.planticola-NR_024996.1           AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     953
Raoultella.planticola-NR_119279.1           AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     953
Raoultella.planticola-NR_113701.1           AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     864
Raoultella.ornithinolytica-NR_044799        AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     859
Raoultella.ornithinolytica-NR_114736.1      AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     861
Raoultella.electrica-NR_125461              AAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCC     861
                                             *   ****         * **         *** *  * *** * *** **

Raoultella.planticola-MZ447097              TTAACGCTT--GCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTC     973
Raoultella.ornithinolytica-MZ447120         TTAACGCTT--GCACCCTCCGTATTACCGCGGCTGCTGGCACGGAGTTAGCCGGTGCTTC     972
Raoultella.terrigena-NR_114503.1            TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     917
Raoultella.ornithinolytica-NR_114502.1      TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     917
Raoultella.planticola-NR_024996.1           TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC    1006
Raoultella.planticola-NR_119279.1           TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC    1006
Raoultella.planticola-NR_113701.1           TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     917
Raoultella.ornithinolytica-NR_044799        TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     912
Raoultella.ornithinolytica-NR_114736.1      TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     914
Raoultella.electrica-NR_125461              TTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAA-------GAGAAGCGACC     914
                                            ***    *   **  *   ***  ***   ***   *   *        *         *

Raoultella.planticola-MZ447097              TTCTGCGAGTAACGTCAATCGCTAAGGT-ATTAACCTTAATGCCTTCCTCCTCGCTGAAA    1032
Raoultella.ornithinolytica-MZ447120         TTCTGCGAGTAACGTCAATCGCTAAGGTTATTAACCTTAACGCCTTCCTCCTCGCTGAAA    1032
Raoultella.terrigena-NR_114503.1            TCGCGAGAGCAAGCGGACCTCATAAAGT--------ATGTCGTAGTCC-------GGATC     962
Raoultella.ornithinolytica-NR_114502.1      TCGCGAGAGCAAGCGGACCTCATAAAGT--------ATGTCGTAGTCC-------GGATT     962
Raoultella.planticola-NR_024996.1           TCGCGAGAGCAAGCGGACCTCATAAAGT--------ATGTCGTAGTCC-------GGATT    1051
Raoultella.planticola-NR_119279.1           TCGCGAGAGCAAGCGGACCTCATAAAGT--------ATGTCGTAGTCC-------GGATT    1051
Raoultella.planticola-NR_113701.1           TCGCGAGAGCAAGCGGACCTCATAAAGT--------ATGTCGTAGTCC-------GGATT     962
Raoultella.ornithinolytica-NR_044799        TCGCGAGAGCAAGCGGACCTCA-TAAGT--------ATGTCGTAGTCC-------GGATT     956
Raoultella.ornithinolytica-NR_114736.1      TCGCGAGAGCAAGCGGACCTCT-AAAGT--------ATGTCGTAGTCC-------GGATT     958
Raoultella.electrica-NR_125461              TCGCGAGAGCAAGCGGACCTCT-AAAGT--------ATGTCGTAGTCC-------GGATT     958
                                            *   * *** **    *       * **         *   *   ***         **

Raoultella.planticola-MZ447097              GTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCC    1092
Raoultella.ornithinolytica-MZ447120         GTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCC    1092
Raoultella.terrigena-NR_114503.1            GGAGTCTGCAACTCG-------ACTCCGTGAAGTCGGAATCGCTAGTAA----------T    1005
Raoultella.ornithinolytica-NR_114502.1      GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1005
Raoultella.planticola-NR_024996.1           GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1094
Raoultella.planticola-NR_119279.1           GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1094
Raoultella.planticola-NR_113701.1           GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1005
Raoultella.ornithinolytica-NR_044799        GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    999
Raoultella.ornithinolytica-NR_114736.1      GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1001
Raoultella.electrica-NR_125461              GGAGTCTGCAACTCG-------ACTCCATGAAGTCGGAATCGCTAGTAA----------T    1001
                                            * * * * **** **        ** * *  *  *** ** ***

Raoultella.planticola-MZ447097              CATTGTGCAAAATTCC--CACTGCTGCCTCCCGAAGGAATCTGGACCGGGTCTCAATTCC    1150
Raoultella.ornithinolytica-MZ447120         CATTGTGCAAAATTCC--CACTGCGGCCTCCGCAAGAAATTGGGACCGGGTTCCAATTCC    1150
Raoultella.terrigena-NR_114503.1            CGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1063
Raoultella.ornithinolytica-NR_114502.1      CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1063
Raoultella.planticola-NR_024996.1           CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1152
Raoultella.planticola-NR_119279.1           CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1152
Raoultella.planticola-NR_113701.1           CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1063
Raoultella.ornithinolytica-NR_044799        CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1057
Raoultella.ornithinolytica-NR_114736.1      CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1059
Raoultella.electrica-NR_125461              CGTAGATCAGAATGCTACGGTGAATACGTTCCCGGGCCTTGTACACACCGC--CCGTCAC    1059
                                            * * *  ** *** *          * * *    *   *    **   *   *  *  *
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Raoultella.planticola-MZ447097              AGGGTGGCTGGGCATCCCCCCAAACACCTAGGGATCGTCGCCCA---GGGGAGCCTTACC     1207
Raoultella.ornithinolytica-MZ447120         CGGGGGG-----------------------------------------------------     1157
Raoultella.terrigena-NR_114503.1            ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCG--------------     1107
Raoultella.ornithinolytica-NR_114502.1      ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCG--------------     1107
Raoultella.planticola-NR_024996.1           ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCG--------------     1196
Raoultella.planticola-NR_119279.1           ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC     1210
Raoultella.planticola-NR_113701.1           ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC     1121
Raoultella.ornithinolytica-NR_044799        ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTAACCTTCGGGAGGG-CGCTTACC     1114
Raoultella.ornithinolytica-NR_114736.1      ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTACTTAACCTT-CGGGAGGGCGCTTACC     1116
Raoultella.electrica-NR_125461              ACCATGGGAGTGGGTT--GCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACC     1117
                                                 **

Raoultella.planticola-MZ447097              C-        1208
Raoultella.ornithinolytica-MZ447120         --        1157
Raoultella.terrigena-NR_114503.1            --        1107
Raoultella.ornithinolytica-NR_114502.1      --        1107
Raoultella.planticola-NR_024996.1           --        1196
Raoultella.planticola-NR_119279.1           AC        1212
Raoultella.planticola-NR_113701.1           AC        1123
Raoultella.ornithinolytica-NR_044799        AC        1116
Raoultella.ornithinolytica-NR_114736.1      AC        1118
Raoultella.electrica-NR_125461              AC        1119
```

Appendix (4): Example of maximum allowable concentration of selected water quality variable for different uses.

| Use | | Human consmuption | | | | Aquatic life | |
|---|---|---|---|---|---|---|---|
| variables | WHO | EU | Iraq | Kurdistan region* | USA | WHO | EU |
| pH | <8.0 | >6.5and < 9.5 | 6.5-8.5 | 7.71 | 6-9 | 6-9 | 6-9 |
| TDS | 600 | 1000 | 1000 | | 500 | ------- | ----- |
| Nitrate (mg $l^{-1}$) | 50 | 50 | 50 | 66.049 | ----- | ------ | ---- |
| chloride | 250 | 250 | 350 | 4.816 | 350 | ------- | ---- |
| hardness | 500 | 500 | 500 | 156.77 | 150-500 | ------- | ---- |
| Alkalinity | 200 | 200 | 200 | 161.805 | ------ | ----- | ----- |
| $SO_4$ | 250 | 250 | 200 | ------ | 250 | ----- | ----- |
| ECμS $cm^{-1}$ | 600 | 2500 | 1500 | 297.83 | ----- | ----- | ----- |
| Cd ppm | 0.003 | 0.005 | 0.005 | 0.059 | 0.005 | 0.002 | ---- |
| Pb ppm | 0.01 | 0.05 | 0.01 | 0.038 | 0.015 | 0.001-0.007 | ----- |
| Cr ppm | 0.05 | 0.025 | 0.05 | ------ | 0.1 | 0.02-0.0020 | ------ |
| Cu ppm | 2 | 2 | 1 | 0.222 | 1 | 0.002-0.004 | 0.005-0.1 |
| Zn ppm | 3 | ---- | 3 | 0.340 | 5 | 1.1-3 | 0.03-2 |
| Ni ppm | 0.02 | 0.02 | 0.02 | 0.187 | 0.02 | 0.02 | 0.02 |
| Co ppm | 0.1 | 0.1 | ------ | ----- | ----- | --- | ---- |
| Fe ppm | 0.2 | 0.2 | 0.3 | 0.226 | 0.3 | ---- | ---- |

* Overall mean values of Kurdista region Parameters (Aziz and Abdulwahid, 2012)

## الخلاصة

تعتمد المعالجة البيولوجية على الميكروبات التي تعيش بشكل طبيعي في البيئة في ظل ظروف بيئية مثالية لتفكيك الملوثات ؛ هذه الميكروبات لا تشكل أي خطر على الناس في الموقع أو في المجتمع. خلال هذه الدراسة تم عزل بكتيريا تتحمل المعادن الثقيلة بشكل طبيعي من نهر تانجارو الواقع جنوب غرب مدينة السليمانية ، وتم تقييم قدرتها على امتصاص (الكادميوم ,الرصاص,النحاس ,الكروم ,النيكل ,الزنك ,الكوبالت والحديد) باستخدام جهاز مطياف الانبعاث البصري البلازمي المقترن بالحث.

كانت نتائج التحليلات الفيزيائية والكيميائية لعينات مياه تانجارو على النحو التالي: درجة الحرارة ١١,٩ - ٣١ درجة مئوية ، ودرجة الحموضة ٦,١-٨,٦٤ والتي تميزت بالانحياز نحو الجانب القلوي للحياد ، التوصيل الكهربائي ٥٢٥-٩٢٨ مايكرو سيمنز.سم $^{-1}$ ، إجمالي المواد الصلبة الذائبة ٢٦٨- ٤٦٤ مغ .لتر $^{-1}$ ، العسرة الكلية ٢٣٢-٤٨٥ مغ. لتر$^{-1}$ ، القلوية ١٢٢-٣٢٤,٣ مغ. لتر$^{-1}$ ، وقيم الأكسجين المذاب بين ٣- ٧,٧٥ مغ. لتر$^{-1}$ ، في حين تراوحت قيم المتطلب الحيوي للاوكسجين من ٣٦الى ١٢٠ مغ. لتر$^{-1}$ ، تركيز أيون الكلوريد ١٣,٢ - ٧٧,٩ مغ. لتر$^{-1}$ نترات تراوحت بين ١٩,٥٢ - ٤٨,٥٥ مغ.لتر$^{-1}$ ، تركيز كبريتات٢١,١٦ - ٣٣٦,٦٦ مغ .لتر$^{-1}$.

من بين المعادن الثقيلة التي تم تحليلها والمؤخوذة من نهر تانجارو ، كان لأيونات الرصاص أعلى تركيز ، بينما كان لأيونات الزنك والكادميوم أدنى تركيز. وكانت التراكيز كما في الترتيب التالي: Pb> Cr> Fe> Ni> Co> Cu> Zn> Cd مع تركيزات قصوى من ٠,٠٨٦ و ٠,٠٧٣ و ٠,٠٧١ و ٠,٠٦٨ و ٠,٠٥١ و ٠,٠٥٦ و ٠,٠٣١ و ٠,٠٢٤ مغ. لتر$^{-1}$. على التوالي.

تم عزل أربعين بكتريا مقاومة للمعادن تنمو على وسط مدمج معادن ثقيلة والذي اشتمل على بكتريا سالبة الجرام ٢٣ (٥٧,٥٪) و ١٧ (٤٢,٥٪) بكتريا موجبة الجرام. كشف التعرف الجزيئي على أساس 16S rRNA أن العزلات تنتمي إلى فصائل، Moraxellaceae، Morganellaceae، Enterococcaceae، Microbacteriaceae، Enterobacteriaceae، Pseudomonadaceae ، و Aeromonadaceae.

بناءً على قيم اقصى تركيز محتمل (MTC)، أظهرت السلالات المعزولة مستويات مختلفة من المقاومة بتراكيز تتراوح من ١٠-٤٣٠ مغ. لتر$^{-1}$. أظهرت جميع العزلات البكتيرية قدرات عالية للتحمل ضد الرصاص والحديد بينما لوحظ الحد الأدنى من التحمل ضد الكادميوم والزنك.

اظهرت العزلات المختلفة انماطا مختلفة لمقاومة واحدة او اكثر من المعادن الثقيلة, كل من *Leucobacter chromiiresistens* - LC15T و *Bacillus safensis* - BS16L كانت قادرة على التوالي على تحمل Cd(٨٠, ٩٠), Pb (١٦٠, ٢٥٠), Cr (١٠٠,٢١٠), Ni (٩٠, ١١٠), Co (١٦٠-١٧٠) مغ. لتر$^{-1}$.من بين العزلات البكتيرية التي تم فحصها كانت ، *R. ornithinolytica* - RO40LCH هي الأفضل من حيث معدل MTC واختزال المعادن الثقيلة ، وأظهر اقصى تحمل لـ Co , Cr , Pb,Cd و Fe (١٢٠ ، ٤٣٠ ، ٢٣٠، ٢١٠ ، ٣٤٠) مغ. لتر$^{-1}$ على التوالي.

*R. ornithinolytica* أظهر أعلى قدرة على إزالة المعادن المختاره في الدراسة الحالية باستثناء النحاس بنسبة (٦٧ ، ٨٩ ، ٦٣,٤ ، ٥٥,٦ ، ٥٦,٥ ، ٦٥ ، ٦١,٩٪) لكل من Cd ، Pb و Cr و Ni و Zn و Co و Fe على التوالي. تتأثر هذه المعدلات بظروف بيئية مختلفة (درجة الحرارة ، ودرجة الحموضة ، وفترات الحضانة) ، وأثبتت النتائج أن ٣٥ درجة مئوية كانت درجة الحرارة المثلى لامتصاص الكادميوم والرصاص والزنك والفسفور ، مما أدى إلى تحسين الامتصاص

من ٤٥ إلى ٦٧٪ ، ٦٥ إلى ٨٩٪ ، ٥٥ إلى ٥٦,٥٪ ، ومن ٥٠ إلى ٦٥٪ لكل من الكادميوم والرصاص والزنك والحديد والكوبالت على التوالي ، بينما كانت ٢٥ درجة مئوية هي الأمثل لامتصاص كل من الكروم ,النحاس والنيكل. تشير نتائج تباين الأس الهيدروجيني في هذه الدراسة إلى أن الرقم الهيدروجيني في النطاق ٧-٨ هو الأمثل لمعظم المعادن المختارة ، باستثناء الكوبالت والنيكل ، كان الرقم الهيدروجيني ٥ هو الأمثل لامتصاصها وزادت نسبة الامتصاص من ٦٥ إلى ٨٤٪ و ٥٥,٦ إلى ٧٣ ٪ لكل من كوبالت ونيكل على التوالي. يعزز التغيير في وقت الحضانة معدل الامتصاص من ٨٩ إلى ٩٥ ، ٣٦,٤ إلى ٤٥ و ٥٥,٦ إلى ٦٤٪ للرصاص ، والنحاس ، والنيكل على التوالي بعد ١٨ ساعة من الحضانة.

أشار استخلاص البلازميد لـ *R. ornithinolytica* بواسطة كل من سلفات دوديسيل الصوديوم SDS و بروميد الإيثيديوم E.B إلى أن قدرة *R. ornithinolytica* على النمو في وجود معادن ثقيلة مختلفة تم ترميزها بالبلازميد وتفقد هذه القدرة بعد معالجة البكتيريا بـ ١٢٪ SDS أو ١٠ ميكروغرام / مل E.B. تم اختيار ستة جينات مقاومة للمعادن لتحديد بعض الجينات المسؤولة عن المقاومة (*czcA* و *pcoA* و *chrB* و *pbrT* و *nccA* و *iroN*) ، أشارت نتائج PCR إلى أن *R. ornithinolytica* يحتوي على خمسة جينات من أصل ستة ( *pbrT*، *chrB*، *nccA*، *iroN* و *czcA*) ،وعدم وجود *pcoD* وهو الجين المسؤل عن ازالة النحاس من الوسط ، في حين أن *R. planticola* يحمل فقط ثلاث جينات ( *pcoD*، *pbrT*، *czcA*) من الجينات المسؤلة عن المقاومة للمعادن.

اظهر المسح المجهري الإلكتروني (SEM) لنتائج *R. Ornithinolytica المعرضة* للمعادن الثقيلة في الاستزراع الأحادي تغييرات في حجم الخلية البكتيرية وشكلها مقارنة بخلايا التحكم ، وفي حالة النمو البكتيري الناتج بعد التعريض للمعادن الثقيلة مجتمعة (نمو متعدد المعادن)انتجت الخلية نسبة عالية من التراكم الذي يجعل من الصعب تمييز الخلية ، مع ظهور تشققات على جدار الخلية.أعطت صور مطيافية تشتت الطاقة بالأشعة السينية EDS دليلاً مرئيًا على ارتباط أيونات معدنية على جدار خلية الخلايا البكتيرية والتي أظهرت بوضوح أن أيونات الكادميوم والرصاص والكروم تم امتصاصها على السطحبدرجات ارتباط مختلف للمعادن المختلفة.

أظهر المجهر الإلكتروني الناقل (TEM) آليات مختلفة لازالة المعادن وتوطين جزيئات المعدن الممتز داخل الخلايا ، لامتصاص الرصاص والزنك والكوبالت ، الامتزاز على سطح الخلية هو الآلية المرشحة ، بينما تراكم الكادميوم والنيكل والحديد داخل الخلية.(Bioaccumulation)

البكتيريا المعزولة في الدراسة الحالية وخاصة *R. ornithinolytica* يمكن استخدامها كوسيلة بيولوجية صديقة للبيئة لمعالجة وإزالة السموم من المعادن من البيئات الملوثة. تشير دراسات قليلة فقط إلى مشاركة *Raoultella sp*. في امتصاص المعادن من البيئة ، هذه هي الدراسة الأولى حول عزل وتصنيف *R. ornithinolytica* المقاوم للمعادن من المياه الملوثة بالمعادن في العراق والدول المجاورة.

حكومة أقليم كوردستان
وزارة التعليم العالي و البحث العلمي
جامعة السليمانية
كلية العلوم

# المعالجة الحيوية لبعض المعادن الثقيلة بواسطة البكتيريا المقاومة المعزولة من نهر تانجارو داخل مدينة السليمانية – اقليم كوردستان – العراق

اطروحة مقدمة الى مجلس كلية العلوم في جامعة السليمانية كجزء من متطلبات نيل
شهادة دكتورا فلسفة في علوم الحياة
(الاحياء المجهرية البيئية الجزيئية)
من قبل

**ليلى ابراهيم فقي صالح**

(بكالوريوس في علوم الحياة / جامعة السليمانية /٢٠٠٢)

(ماجستير في علوم الحياة / جامعة السليمانية /٢٠١٣)

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# پوختە

زیندەچارە پشت دە بەستی بە بوونی زیندەوەری سروشتی لە ژینگە دا کەوا بەرگری ئەوتۆیان هەیە بەرامبەر پیسکەرەکان. لە ماوەی ئەم تۆژینەوەیە ، کە لە مانگی یەک تا مانگی دەی ٢٠١٩ بۆ ردەوام بوو. نزیکەی ٤٠ جۆر لە بەکتریای سروشتی جیاکرانەوە و دەستنیشانکران کەوا بەرگری ئەوتۆیان هەیە بەرامبەر بە ٨ جۆری دیاریکراوی کانزای قورس (Pb،Cd ، Co، Zn، Ni، Cr،Cu ، و Fe) هەیە، کەوا لە هەردوو جۆری گرام-پۆزەتیڤ و گرام-نیگەتیڤ بوون، بە ڕێژەی ٢٣(٥٧.٥٪) و١٧ (٤٢.٥ ٪) بەدوای یەکدا.

هەروەها توانای ئەو بەکتریایانە بۆ لابردنی ئەو کانزایانە لە ناوەندەکە، بە بەکارهینانی ئامیری inductively coupled plasma-optical emission spectrometry، دیاریکرا.

شیکاریی بۆ کومەڵێک لە تایبەتمەندییە فیزیایی و کیمیاییەکان کرا، لەوانە: پلەی گەرمی، پەیتی ئایۆنی هایدرۆجین، ئاستی گەیاندنی تەزووی کارەبا، بری ئۆکسجینی تواوە، پەیتی هەریەک لە ئایونی نیترات ، کلۆر، و سەلفەیت.

لە ئەنجامی شیکردنەوەی کانزاکان دەرکەوت کەوا ڕیژەی pb، بەراورد بە ڕێژەی کانزاکانی دیکە، بەرزترین ئاستی تۆمارکرد.

: Pb > Cr > Fe > Ni > Co > Cu > Zn > Cd

بە شیوەیەکی گشتی بە پیی شیکردنەوەی 16*srRNA*، بەکتریاکان لە خێزانی Moraxellaceae،Bacillaceae ، Enterobacteriaceae، Microbacteriaceae، Enterococcaceae،Morganellaceae ، and Aeromonadaceae،Pseudomonadaceae . بوون.

ڕیژەی بەرگری بەکتریا لە نیوان ١٠ – ٤٣٠ PPM بوو.

هەموو بەکتریا جیاکراوەکان ئاستی بەرهەڵستیی بەرزیان هەبوو دژی Pb و Fe بەڵام نزمترین ئاستی بەرهەڵستیی دژی Cd، Zn تۆمارکرا.

بەکتریا بە ئاستی جیاواز بەرهەڵستیی جیاوازیان بەرامبەر کانزای جیاواز نواند, - *Leucobacter chromiiresistens C15T*و *Bacillus safensis* - BS16L توانای بەرگەگرتنی Cd(٨٠،٩٠)، Pb (١٦٠،٢٥٠)، Cr (١٠٠،١٢٠) Ni (٩٠،١١٠) و Co (١٦٠،١٧٠)یان هەبوو ، بەدوای یەکدا.

بەڵام لەناو بەکتریا جیاکراوەکاندا بەکتریای RO40LCH - *Raoultella ornithinolytica* لەڕووی بڕی بڕگری (MTC) و ڕێژەی کەمکردنەوەی کانزا قورسەکانەوە باشترین بوو، هڕوەها بەرگەگرتنی بۆ (Cr، Pb، Cd ، Co, Fe(١٢٠،٤٣٠، ٢٣٠، ٢١٠،٣٤٠) ppmنیشاندا، بەدوای یەکدا.

لەسەر بنەمای بەرهەڵستیی، *R. ornithinolytica* هەڵبژێردرا جونکە بەرزترین ئاستی بەرهەڵستیی هەبوو، هەروەها بەرزترین ئاستی لابردنی هەموو کانزاکان لە ناوەندەکە.

دواتر شیکاریی بۆ ئەم بەکتریایە کرا بۆ دیاریکردنی ئەوەی ئایا ئەو بەرهەڵستییە بە هۆی پلازمید یان کرۆمۆسۆمەوەیە. *دەرهێنانی پلازمید، بە بەکارهێنانی SDS وE.B ، ئەنجامدرا. دواتر ئەو جینانەی بەرپرسن لە بەرهەڵستیی لە بەکتریای R. ornithinolytica* دا، دیاریکران کە ئەمانە بوون: ( *iroN، nccA، pbrT، chrB، pcoA،czcA*).

ئەنجامەکانی مایکرۆسکۆپی ئەلیکترۆنی ڕووماڵکەر( *Scanning electron microscopy*) سەبارەت بە چاندنی *R. ornithinolytica* لەسەر ناوەندی خۆراکیی کە کانزای قورسی جیا بەجیا تێدا بوو، نیشانیدا کە قەبارە و شێوەی خانەکانی، بەراورد بە هی کۆنترۆڵ، گۆڕاون. گەشەی بەکتریا لە بوونی ستریسی بەهۆی هەر هەشت کانزا هەڵبژێردراوەکە پێکەوە

بووەهۆی ئەوەی کە خانەکانی بەکتریاکە پێکەوە تۆپەڵ ببن و جیاکردنەوەیان ئاسان نەبێت لەبەر شەقبوونی دیواری خانەکان. وێنەکانی Energy dispersive X-ray spectroscopy EDS بەڵگەی بینراوی پێماندا سەبارەت بە نووسانی ئایۆنی کانزاکانەوە بە دیواری خانەی بەکتریاکانەوە، کە بە روونیی نیشانیدا کانزاکانی Cd، Pb، و Cr بە ڕێژەی جیاجیا بە ڕووەکەیانەوە نووساون.

مایکرۆسکۆبی ئەلیکترۆنی تێپەڕ (TEM) Transmission electron microscopy میکانیزمی جیاوازی بۆ پێگەگرتنی کانزاکان بەڕووی خانەکانەوە، بۆ هەریەکەیان بە جیاواز، نیشاندا؛ (Pb, Zn و Co) بۆ ڕووی دەرەوە و (Cd, Ni و Fe) بۆ کەڵەکەبوون لەناو خانەدا.

ئەنجامەکان دەریانخست کە بەکتریا جیاکراوەکانی ئەم لێکۆڵینەوەیە، بەتایبەتیی R. ornithinolytica، دەتوانێ وەکو چارەسەری ژینگەدۆستانەی گونجاو بۆ پیسبوون و ژەهراویبوونی ژینگە بە کانزاکان، بەکاربهێنرێن. تەنها چەند لێکۆڵینەوەیەک دەریانخستووە کە Raoultella sp توانای وەرگرتن و کۆکردنەوەی کانزاکانی لە ژینگەوە هەیە، ئەمە یەکەم لێکۆڵینەوەیە بۆ جیاکردنەوە و دیاریکردنی R. ornithinolytica بەرهەڵستکار بۆ کانزاکان لە ئاوی بەکانزا پیسبووی ئێراق و وڵاتانی دراوسێوە.

حکومەتی هەرێمی کوردستان
وەزارەتی خوێندنی باڵا و توێژینەوەی زانستی
زانکۆی سلێمانی
کۆلێجی زانست

# زیندەچارە بۆ هەندێک کانزای قورس بە بەکتریای بەرهەڵستکاری جیاکراوە لە رووباری تانجەرۆ شاری سلێمانی- هەرێمی کوردستان- عێراق

**تێزی دکتۆرایە**
**پێشکەشکراوە بە ئەنجومەنی کۆلێجی زانست لە**
**زانکۆی سلێمانی وەک بەشێک لە پێداویستیەکانی بەدەست**
**هێنانی بڕوانامەی دکتۆرای فەلسەفە لە**
**(بایۆلۆجی)**
**مۆلیکیولەرمایکرۆ بایۆلۆجی ژینگەیی**

**لە لایەن**
**لیلی ابراهیم فقئ صالح**

بەکالۆریۆس لە زانستی بایۆلۆجی ٢٠٠٢ ، زانکۆی سلێمانی
ماستەر لە زانستی بایۆلۆجی ٢٠١٣ ، زانکۆی سلێمانی

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