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# Resistance to antibiotics and ability to tolerate heavy metals in bacteria isolated from Razi industrial wastewater treatment plant and effluent of refinery units in Isfahan, Iran

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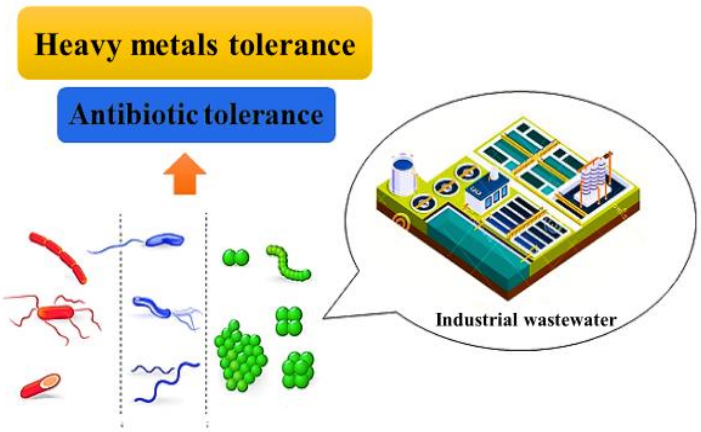
**Graphical Abstract**

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**ABSTRACT**

Industrial activities present a significant threat to the environment and natural ecosystems like water and soil due to the release of toxic metals. This article primarily concentrates on the identification and isolation of bacteria, with the goal of effectively eliminating pollutants from industrial wastewater. In order to achieve this goal, the study was conducted to assess the ability of bacterial strains to tolerate copper (Cu) and zinc (Zn), as well as their antibiotic resistance and ability to tolerate elevated metal concentrations. The resistance of the isolates to various metals and antibiotics were assessed using the minimum inhibitory concentration (MIC) values and disc diffusion (DD) method, respectively. The technique of colony PCR was employed to determine the identity of the bacteria that were separated. Resistance to multiple antibiotics was assessed, including Penicillin, Sulfamethoxazole, Tetracycline, Erythromycin, Amoxicillin, Cefoxitin, Streptomycin, Chloramphenicol, Vancomycin, Gentamycin, Cephalothin, Rifampicin, and Novobiocin. In the current investigation, a total of 5 bacteria with a positive gram stain and 7 bacteria with a negative gram stain were identified. The study found that the effluent from the wastewater treatment plant in Razi industrial town showed resilience to copper ions, especially at a concentration of 7mM. The effluent wastewater from the refinery unit exhibited the greatest level of tolerance towards zinc, with a concentration as high as 6mM. The rise in copper and zinc levels in industrial wastewater treatment plants causes microorganisms to develop resistance to these heavy metals. The study of Gram-positive resistant bacteria conducted in this research focused on the examination of their susceptibility to zinc and copper. Notably, *Staphylococcus hominis* displayed resistance to a majority of the antibiotics evaluated. However, *Kocuria rosea* demonstrated sensitivity to all antibiotics. *Agrobacterium fabrum* exhibited susceptibility to all antibiotics as opposed to other Gram-negative bacteria resistant to zinc and copper. The findings of this study indicated that some strains displayed a degree of resistance to both antibiotics and heavy metals. The presence of heavy metals in bacteria isolated from a wastewater treatment plant exhibited the capability to restrict antibiotic resistance.

## 1. Introduction

The expansion of industrial activities, the growth of urban areas, and a significant increase in population are the reasons behind the increase in pollution levels. The natural environment is further contaminated by heavy metals due to human activities such as mining and the unrestricted release of untreated industrial effluents (Tytła, 2019; Li et al., 2022; Türkmen et al., 2022). Heavy metal contamination has been

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connected to the participation of different industrial activities like paint and resin factories, textile facilities, gum production, tanning, metal smelting, and plating operations (Heidarzadeh et al., 2020; Das and Poater, 2021; Long et al., 2021). The previously mentioned practices lead to the release of large amounts of heavy metals into the environment, which raises concerns about the potential dangers involved in these situations. 1) The pollutants analyzed in this study are responsible for the decline of various ecological habitats, including, though not exclusively, soil, groundwater, and surface water (Raymond et al. 2011; Tchounwou et al., 2012; Alengebawy et al., 2021; Uroko and Njoku, 2021). In addition, the presence of these pollutants negatively affects the animal and plant species that live in the same environment. The described consequences can be demonstrated by providing examples of disturbances happening within food chains (Sabry, Ghozian, and Abou-Zeid, 1997; Fillali et al., 2000; Spain and Alm, 2003). The presence of microorganisms that can resist heavy metals is significant for environmental pollution, as they play a crucial role in cleaning up these areas (Sabry, Ghozian, and Abou-Zeid, 1997; Verma et al., 2001; Sharma, 2022). The treatment of wastewater with various components is often seen as a difficult task, with challenging financial and technical obstacles. The limited applicability and high costs associated with various techniques used to isolate heavy metals, such as chemical precipitation, adsorption, ion exchange, and solvent extraction, require significant upfront investment and ongoing operational expenses (Hammaini et al., 2007; Vidu et al., 2020; Pande et al., 2022). The application of microorganisms as biological agents to absorb heavy metals is being recommended as a feasible and possibly more efficient option for removing harmful substances and recovering industrial wastewater (Dixit et al., 2015; Pham et al., 2022; Tripathi et al., 2023). The challenges linked to current methodologies emphasize the benefits of investigating and adopting different approaches, specifically using microorganisms to enhance industrial and environmental sustainability (Timmis et al., 2017). The identification of bacteria that exhibit resistance to heavy metals plays a crucial role in addressing environmental pollution and ultimately expediting the process of environmental remediation (González Henao and Ghneim-Herrera, 2021; Fawwaz et al., 2022). Bacterial organisms as cofactors require exact measurements of heavy metals in the appropriate amounts. However, microorganisms still activate different protective mechanisms through specific pathways to enhance the removal of harmful effects when they encounter higher concentrations of these metals. Therefore, microorganisms are able to survive due to these adaptive responses (Nies, 1992; Spain and Alm, 2003; Chandrangsu, Rensing and Helmann, 2017). Various theories have been suggested in relation to the phenomenon of microbes developing resistance to metal elements. The primary cause of this resistance is attributed to the significant factors of being initially exposed to toxic metals and continuously exposed to environmental pollutants, both of which lead to harmful consequences (Fashola, Ngole-Jeme, and Babalola, 2016; Yazdankhah, Skjerve, and Wasteson, 2018; Larsson and Flach, 2022). Bacterial plasmids frequently harbor resistance genes against the heavy metal toxins, including but not limited to $Cu^{2+}$, $Pb^{2+}$, $Zn^{2+}$, $Hg^{+}$, and $Co^{2+}$. The divalent cations $Cd^{2+}$, $Ni^{2+}$, and $Ag^{+}$ are being considered. Occasionally, mechanisms linked to the emergence of resistant organisms are regulated by chromosomal genes (Silver and Ji, 1994; Spain and Alm. 2003; El-Deeb, 2009; Bukowski et al., 2019; Galetti et al., 2019). The existence of metals in the surroundings puts pressure on organisms, leading to the evolution and improvement of various microbial defense mechanisms that counteract the harmful impacts of heavy metals (Xu et al., 2017; Briffa, Sinagra, and Blundell, 2020; Vats, Kaur, and Rishi, 2022). There are different mechanisms that function to obstruct the cellular separation. These mechanisms include active transport, effluent pumps, detoxification enzymes, and reduced sensitivity to metal ions, all acting as cellular targets (Igiri et al., 2018). Antibiotics have proven to be an effective means of managing the growth of harmful bacteria. However, the widespread existence of antibiotic resistance in these microorganisms presents significant challenges and consequently weakens the efficacy of this previously effective tool (Coque et al., 2023). Numerous studies have demonstrated that the presence of non-antibiotic substances within the environment, such as disinfectants and heavy metals, possesses the capacity to facilitate the emergence of antibiotic resistance. The co-occurrence of genetic markers associated with resistance to both heavy metals and antibiotics on plasmids and transposons has been extensively documented in scientific literature (Russell and Chopra, 1990; Wales and Davies, 2015; Bischofberger et al., 2020). The academic community is interested in exploring the correlation between bacterial metal tolerance and antibiotic resistance. The presence of heavy metal resistance poses a significant risk to human well-being and ecosystems. It is important to monitor heavy metal presence in diverse environments. In case of increased heavy metal levels, urgent prevention measures are crucial to reduce harmful effects. The remediation of metal-contaminated sites involves reducing pollutant concentrations and establishing clean environmental conditions, known as concentration and decontamination processes. The presence of heavy metals in the surrounding environment as contaminants poses a detrimental effect not only on the proliferation of metal-resistant bacteria but also on the augmentation of antibiotic resistance (Dickinson et al. 2019; Khaira, Yusuf, and Khan, 2022). The objective of this study is to isolate and identify bacteria for the purpose of mitigating pollutants from industrial wastewater. In order to attain the desired objective, a comprehensive investigation was carried out to find out the degree of resistance exhibited by bacterial strains towards copper and zinc. Moreover, the study was carried out to assess the levels of resistance to antibiotics and the tolerance to heavy metals.

## 2. Materials and methods

### 2.1. Sampling and physico-chemical characterization

The main objective of this study was to examine samples of wastewater treatment effluent obtained from Razi industrial wastewater and the effluent produced by refinery units in Isfahan. Also, to analyze the existence of bacteria in these samples. After collection, the wastewater samples were obtained and then moved into sterilized one-liter glass containers. The aforementioned bottles were transferred to the research laboratory of Falavarjan University located in Isfahan, Iran, by keeping the bottles in close proximity to ice. The magnitudes of the biochemical oxygen demand (BOD), chemical oxygen demand (COD), electrical conductivity (EC), pH, and temperature were expeditiously evaluated. Furthermore, the concentrations of heavy metals, namely zinc and copper, were assessed using an atomic absorption spectrophotometer (Tahmors Pour and Kermanshah, 2005; Salehi, Akhtari, and Akhavan Sepahi, 2017; Yosofi, Almasi, and Mousavi, 2017).

### 2.2. Isolation and identification of resistant bacteria to heavy metals zinc and copper

The strains isolated from the Razi industrial wastewater treatment plant and effluent of refinery units in Isfahan, Iran were initially characterized using biochemical tests, which were further confirmed through molecular analyses. The identification of the isolates involved a series of assessments, including the examination of their staining capacity, morphological characteristics, ability to produce pigment, mucoid attributes of colonies growing on nutrient agar plates, as well as additional biochemical methods. In order to separate bacteria that have resistance, unique colonies were grown on PHG II agar medium containing peptone, yeast extract, agar, and glucose, with an additional 0.1% sodium pyruvate supplement.5 mM of zinc and copper, were painstakingly sorted and subsequently purified using linear culture methodologies. The pure bacterial colonies were transferred to a medium consisting of peptone, yeast extract, agar, and sodium chloride, commonly referred to as LB medium. Subsequently, the Gram staining technique was employed to examine the microscopic characteristics of the bacteria. The identification of bacteria was accomplished using the Colony PCR technique in conjunction with an analysis of the 16S ribosomal RNA gene, as extensively documented in prior studies (Ybarra and Webb, 1999; Verma et al., 2001; Teitzel and Peresk, 2003; Nouri and Montazer Faraj, 2022).

### 2.3. Determination of MIC and MBC

The assessment of microorganism susceptibility to antimicrobial agents through the application of the minimum inhibitory concentration (MIC) has accrued significance in research and clinical practices. The quantification of metal content in the culture media was achieved through the utilization of different metal concentrations via the PHG II agar medium. Subsequently, colonies possessing radial resistance were cultured on agar plates. The experimental protocol involved exposing the plates to temperatures of 30 °C at 24 to 48 hours. Following this, the optimal concentration level was determined to effectively suppress the proliferation of bacteria. The technique utilized in the bacterial suspension generated a volume equivalent to one-half of the McFarland standard. The concentration succeeding the minimal inhibitory concentration (MIC) was subsequently evaluated utilizing the minimal bactericidal concentration (MBC) (Alboghobeish, Tahmourespour, and Doudi, 2014; Shahsanaei Goneirani et al., 2016; Marzan et al., 2017; Yektamanesh et al., 2021).

### 2.4. Susceptibility testing of isolates to antibiotics

The present study conducted susceptibility testing of bacteria that exhibited resistance to heavy metals via the Kirby-Bauer method (Disc diffusion) on Muller Hinton agar (MHA) medium. An evaluation was conducted to determine the effectiveness of various antibiotics (Pronadaisa Conda Co., Spain), specifically Penicillin, Sulfamethoxazole, Tetracycline, Erythromycin, Amoxicillin, Cefoxitin, Streptomycin, Chloramphenicol, Vancomycin, Gentamycin, Cephalothin, Rifampicin, and Novobiocin. In this procedure, the MHA agar plate was inoculated with a standardized concentration of the tested bacteria, followed by the application of paper disks containing a predetermined concentration of antibiotics onto the bacterial lawn. After a period of 18-24 h incubation, the measurement of the diameter of the inhibited growth zone surrounding the disk was conducted based on the millimeter. The assessment of zone diameter growth was conducted based on the guidelines provided by the National Committee for Clinical Laboratory Standards (NCCLS). The present study elucidated the antibiotic resistance and sensitivity of bacteria in terms of the millimeter diameter of the inhibition zones around each well.

The selection of antibiotics was based on the study of other researches and articles (Rajbanshi. 2008; Rahimzadeh Torabi et al., 2021; Tahmourespour. 2021; Edet, Bassey, and Joseph, 2023).

### 2.5. 16S rRNA gene PCR

The isolated bacteria were identified at the molecular level using gene sequencing of 16S ribosomal RNA (16S rRNA). The current study utilized the colony PCR approach to perform the polymerase chain reaction. In the current investigation, a discrete section of the purified colony was chosen and subjected to dissolution in 10 mL of sterile distilled water utilizing a sterile loop. The dissolved material was subsequently utilized as a template to execute the polymerase chain reaction (PCR). The contemporary investigation implemented a PCR solution with a terminal capacity of 25 µl, comprising diverse constituents specified in Table 1, except for the DNA template. The vials containing the prepared PCR mixture were loaded into a thermal cycler (Applied Biosystems, USA) and underwent the prescribed thermal cycling process mentioned in Table 1. The current study utilized the 16S rRNA gene and employed specialized universal primers listed in Table 2 for the analysis. For this purpose, the general primers OF BUN and OR BUN of Pishgam Company were used (Shahsanaei Goneirani et al., 2016; Yektamanesh et al., 2021).

**Table 1.** PCR program in this study.

| Phase | Temperature,°C | Time, s |
|---|---|---|
| The initial denaturation | 94 | 300 |
| Denaturation | 94 | 45 |
| Primer annealing | 58 | 45 |
| Extension | 72 | 45 |
| Final extension | 72 | 300 |

**Table 2.** The sequence of general primers in this study

| Primers | Sequence | Primer length |
|---|---|---|
| OF BUN | 5'- AGAGTTGATCCTGGCTCAG -3' | 19 N |
| OR BUN | 5'- -GGTTACCTTGTTACGACTT-3' | 19 N |

## 3. Results and discussion

### 3.1. Identification of bacterial strains

In the present research, a total of five strains of gram-positive bacteria and seven strains of gram-negative bacteria were identified. Tables 3 and 4 presented an illustration of the observable characteristics of the isolates, which demonstrated the most elevated level of bacteria resistance. According to the findings observed and identified Gram-positive and Gram-negative microorganisms of varying genera, which were identified as *Massilia, Aeromonas, Pseudomonas, Bacillus, Arthrobacter, Enterococcus, Kocuria, Salmonella, Pseudoxanthomonas, Sphingopyxis* and *Micrococcus.* The presence of copper-resistant strains of *Pseudomonas oleovorans* and *Arthrobacter agilis* 2 was observed in the wastewater treatment facility of Razi industrial town. The presence of copper-resistant strains of *Pseudomonas oleovorans* and *Arthrobacter agilis* 2 were observed in the wastewater treatment facility of Razi industrial town. The presence of zinc-resistant strains of *Pseudoxanthomonas japonensis, Pseudomonas agilis 2, Kocuria rosea, Bacillus toyonensis, Arthrobacter agilis* 1 and *Pseudomonas oleovorans* were observed in effluent of refinery unit. The zinc-resistant strains of *Pseudoxanthomonas japonensis* was detected in the effluent of refinery unit. effluent of refinery unit. The wastewater treatment facility of Razi industrial town revealed the existence of zinc-resistant strains of *Sphingopyxis bauzanensis, Massilia haematophila, Salmonella enterica* and *Aeromonas Veronii.*

### 3.2. The Zn and Cu resistant pattern of bacteria

The minimum inhibitory concentration and minimum bactericidal concentration of bacteria resistant to copper and zinc are illustrated in Tables 3 and 4, respectively. The current research findings reveal that the effluent of the wastewater treatment plant in Razi industrial town displayed the highest level of resistance towards copper ions, particularly at a concentration of 7mM. The wastewater samples collected from the refinery unit's effluent showed the highest level of zinc resistance, which reached 6mM.

**Table 3.** The results of MIC and MBC of resistant bacteria to copper in different type of the wastewater.

| Type of wastewater | Type of bacteria | Name of bacteria | Copper concentration, mM | |
|---|---|---|---|---|
| | | | MBC | MIC |
| Effluent of refinery unit | Gram-negative *Bacillus* | *Pseudoxanthomonas japonensis* | 4 | 3.5 |
| | Gram-negative *Bacillus* | *Pseudomonas agilis 2* | 5 | 4 |
| | Gram-positive Coccus | *Kocuria rosea* | 6 | 5 |
| Razi industrial town wastewater treatment plant | Gram-positive *Bacillus* | *Bacillus toyonensis* | 4 | 3.5 |
| | Gram-positive *Bacillus* | *Arthrobacter agilis 1* | 4 | 3 |
| | Gram-negative *Bacillus* | *Pseudomonas oleovorans* | 7 | 6 |
| | Gram-negative *Bacillus* | *Pseudomonas oleovorans* | 6 | 5 |
| | Gram-positive *Bacillus* | *Arthrobacter agilis 2* | 4 | 3.5 |

**Table 4.** The results of MIC and MBC of resistant bacteria to zinc in different type of the wastewater.

| Type of wastewater | Type of bacteria | Name of bacteria | Zinc concentration, mM | |
|---|---|---|---|---|
| | | | MBC | MIC |
| Razi industrial town wastewater treatment plant | Gram-negative *Bacillus* | *Sphingopyxis bauzanensis* | 5 | 4 |
| | Gram-negative *Bacillus* | *Massilia haematophila* | 6 | 5 |
| | Gram-negative *Bacillus* | *Salmonella enterica* | 4 | 3.5 |
| | Gram-negative *Bacillus* | *Aeromonas Veronii* | 6 | 5 |
| Effluent of refinery unit | Gram-negative *Bacillus* | *Pseudoxanthomonas japonensis* | 5 | 4 |

### 3.3. Physico-chemical properties

The physico-chemical parameters and amount of heavy metals in the wastewater are extensively covered in Table 5. The pH values recorded in the three examined wastewater treatment systems were as follows: 7.23 in effluent of refinery unit, and 7.33 in Razi industrial town wastewater treatment plant. The measurement of biochemical oxygen demand (BOD) and chemical oxygen demand (COD) were key parameters used in the assessment of water and sewage pollution levels. The present research revealed the quantitative values of BOD and COD in three different samples of wastewater undergoing treatment. Specifically, the recorded BOD values for the samples extracted from effluent of refinery unit and Razi industrial town wastewater treatment plant were 45 mg/L, and 299 mg/L, respectively. Meanwhile, the corresponding COD values were 168 mg/L, and 589 mg/l for the above-mentioned samples, respectively. The present study characterized the copper content in three distinct sources, namely effluent of refinery and Razi industrial town wastewater treatment plant. The results revealed that effluent of refinery exhibited a copper content of 0.288 mg/l, and Razi industrial town wastewater treatment plant registered 0.073 mg/L. While no observations of zinc concentration were made within Effluent of refinery unit. Notably, the zinc concentration within the wastewater treated during Razi industrial town treatment plant process was measured as 0.115 mg/L.

**Table 5.** The properties of the physico-chemical and biological wastewater

| Wastewater | BOD, mg/L | COD mg/L | EC, ds/m | pH | Temperature, ˚C | The amount of copper, mg/L | The amount of zinc, mg/L |
|---|---|---|---|---|---|---|---|
| Effluent of refinery unit | 45 | 168 | 513 | 7.23 | 19.3 | 0.288 | - |
| Razi industrial town wastewater treatment plant | 299 | 589 | 1365 | 7.33 | 17.9 | 0.073 | 0.115 |

Sign (-): The heavy metal content is less than the limit of detection units. P<0.001

### 3.4. The Resistance and sensitivity pattern of strains

Bacterial strains were classified according to their susceptibility by observing transparent zones ranging in size from 0 to 20 mm, as revealed in the present study. No growth halo was observed in bacterial strains that were identified as resistant. The results obtained revealed that a significant proportion of the bacterial strains exhibited sensitivity towards selected antibiotics while displaying resistance towards others. The Gram-positive resistant bacteria isolated to zinc and copper, *Staphylococcus hominis* to the most antibiotics were resistant, but *Kocuria rosea was* sensitive to all antibiotics. After *S. hominis*, *Bacillus toyonensis,* and *Arthrobacter agilis* bacteria showed sensitivity to most antibiotics (Fig. 1).

Among the antibiotics used against Gram-positive bacteria, the bacterial strains were sensitive to erythromycin, chloramphenicol, and tetracycline gentamicin. Among the Gram-negative resistant bacteria isolated to zinc and copper, *Agrobacterium fabrum* was sensitive to all antibiotics. After that, *Sphingopyxis bauzanensis* was sensitive to most antibiotics. The susceptibility of the bacterial strains towards different antibiotics such as erythromycin, chloramphenicol, streptomycin, tetracycline, and sulfamethoxazole was increased (Fig. 2).

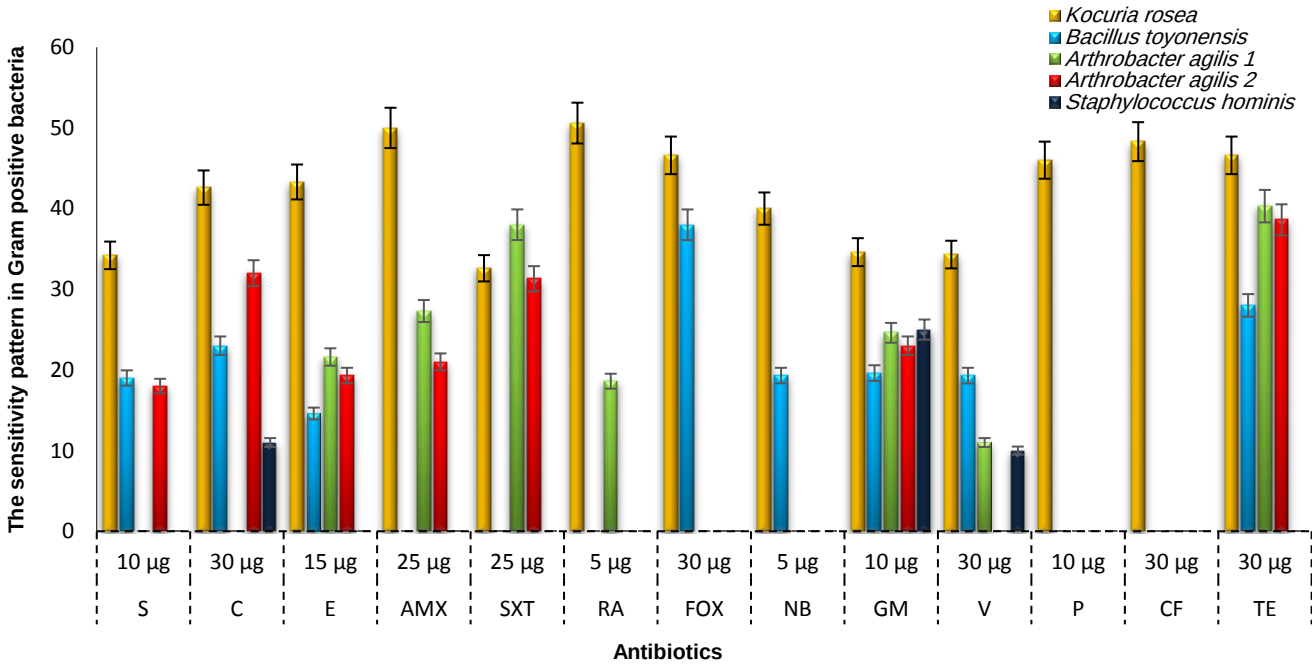


**Fig. 1.** Antibiogram results of Gram-positive resistant bacteria to zinc (Zn) and copper (Cu) by Kirby-Bauer method; (TE: Tetracycline, CF: Cephalothin, P: penicillin, V: Vancomycin, GM: Gentamycin, NB: Novobiocin, FOX: Cefoxitin, RA: Rifampicin, SXT: Sulfamethoxazole, AMX: Amoxicillin, E: Erythromycin, C: Chloramphenicol, S: Streptomycin; R: resistant, S: sensitive, I: intermediate; It should be noted that the numbers S, R and I was determined for each of the antibiotics on the basis of the information contained in (NCCLS). Note: the scale listed in the table is in millimeters numbers. ** Bacterial isolates: 1) *Kocuria rosea,* 2) *Bacillus toyonensis*, 3) *Arthrobacter agilis 1,* 4) *Arthrobacter agilis 2, 5) Staphylococcus hominis)*

The prevalence of copper and zinc was found to be significant in industrial wastewater. The investigation of antibiotic resistance and resistance to heavy metals in bacteria isolated from industrial wastewaters, specifically those contaminated with copper and zinc, offers great potential for enhancing industrial societies and mitigating environmental pollutants. The pattern of resistance was assigned by Minimum Inhibitory Concentration assay. In this study, the focus was on examining the occurrence of antibiotic resistance in a range of compounds, including Gentamycin, Novobiocin, Cefoxitin, Tetracycline, Cephalothin, penicillin, Vancomycin, Rifampicin, Sulfamethoxazole, Amoxicillin, Erythromycin, Chloramphenicol, Streptomycin. The outcomes of this investigation indicated that microbial organisms have undergone evolutionary processes and successfully acclimated to novel environmental circumstances. The current study revealed that the effluent originating from the wastewater treatment plant situated in Razi Industrial Town demonstrated noticeable resistance towards copper ions, specifically when subjected to a 7mM concentration. The wastewater samples obtained from the effluent of the refinery unit exhibited a notable degree of resistance to zinc, with a concentration as high as 6mM. Numerous studies have indicated a correlation between antibiotic resistance and metal tolerance within the natural environment (Najar et al., 2022; Knapp et al., 2017). The proximity of

genes associated with antibiotic resistance and heavy metal resistance on a plasmid suggests a likelihood of these genes being present in a transferable state (Bukowski et al., 2019; Li, Xia, and Zhang, 2017). The treatment of infectious diseases has been significantly hampered and burdened financially due to the prevalence of antibiotic-resistant pathogenic bacteria. The effective use of antibiotics should be prioritized in society, given the need to recognize the importance of exploring alternative antimicrobial (Callaway et al., 2021; Aslam et al., 2018; Spain and Alm, 2003). The bacterial strains *Pseudomonas oleovorans* exhibited a significantly elevated MIC value of 7mM, when compared to copper. Teitzel and Peresk (2003) reported the minimum inhibitory concentration of copper as 2 mM for the bacterium *Pseudomonas aeruginosa*, while also noting that the MIC ranged up to 6 mM for the same bacterial strain.

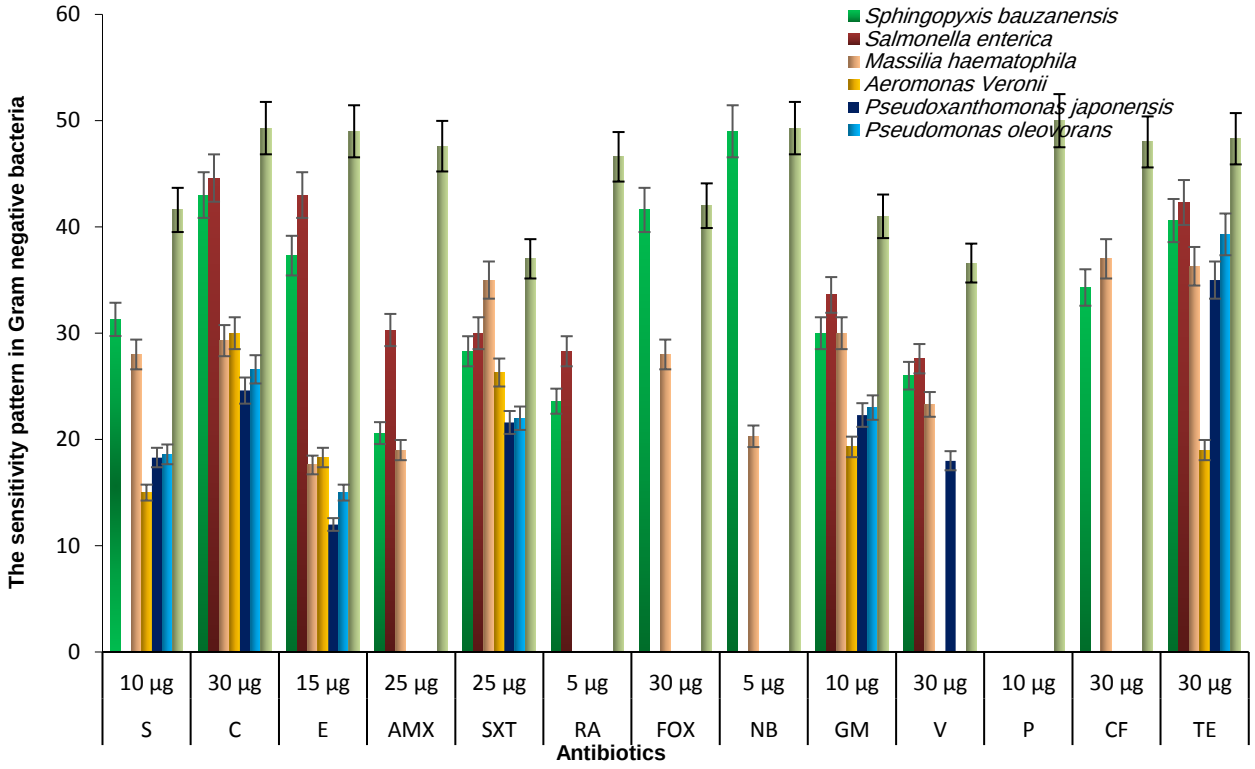


**Fig. 2.** Antibiogram results of Gram-negative resistant bacteria to zinc (Zn) and copper (Cu) by Kirby-Bauer method. (TE: Tetracycline, CF: Cephalothin, P: penicillin, V: Vancomycin, GM: Gentamycin, NB: Novobiocin, FOX: Cefoxitin, RA: Rifampicin, SXT: Sulfamethoxazole, AMX: Amoxicillin, E: Erythromycin, C: Chloramphenicol, S: Streptomycin; R: resistant, S: sensitive, I: intermediate It should be noted that the numbers S, R and I was determined for each of the antibiotics on the basis of the information contained in (NCCLS). Note: the scale listed in the table is in millimeters numbers. ** Bacterial isolates: 1) *Sphingopyxis bauzanensis,* 2) *Salmonella enterica,* 3) *Massilia haematophila,* 4) *Aeromonas Veronii,* 5) *Pseudoxanthomonas japonensis,* 6) *Pseudomonas oleovorans,* 7) *Agrobacterium Fabrum*)

Santo, Morais, and Grass (2010) reported the isolation of 22 bacterial species exhibiting resistance to copper on copper metal surfaces. The isolated bacteria were assessed for their minimum inhibitory concentrations, which ranged from 0.755 µg/mL. The findings of this study indicated that effluent of Razi industrial town wastewater treatment plant, had exhibited the most substantial resistance against copper ions at the concentration of 7mM. The maximum level of resistance to zinc, amounting to 6mM, was observed in the wastewater samples obtained from effluent of refinery unit. The bacterial strain *Pseudoxanthomonas japonensis* exhibited a substantially higher minimum inhibitory concentration of 6mM compared to zinc metal. Nasrazadani, Tahmourespour, and Hoodaji (2010) conducted investigations on specimens of industrial effluent. According to their findings, the maximum level of minimum inhibitory concentration of zinc in various samples of wastewater reported distinct values. Specifically, one sample exhibited MIC levels of 1, 2, and 3 mM, while another sample had higher levels of 0.5, 4, 5, and 6 mM. Researchers conducted a study investigating the presence of microbial-induced corrosion (MIC) in wastewater originating from a battery factory. The research findings revealed that isolated bacterial strains contained a concentration of 3 mM/L of zinc metal (Dinu, Anghel, and Jurcoane, 2011). The present study aimed to evaluate the susceptibility of isolated bacteria to several standard antibiotics by comprehensively examining the resistance exhibited by the bacterial population. In present study, the capacity of Gram-positive bacteria to withstand zinc and copper enabled them to develop antibiotic resistance, particularly noticeable in *Staphylococcus hominis*. Contrarily *S. hominis*, *Kocuria rosea* displayed susceptibility to all tested antibiotics. After careful consideration and thoughtful analysis, it has been determined that, *Bacillus toyonensis*, and *Arthrobacter agilis* bacterial strains exhibited a notable susceptibility to the majority of antibiotics tested, as depicted in Fig 1. The sensitivity of the bacterial strains against erythromycin, chloramphenicol, tetracycline, and gentamicin, which are antibiotics commonly employed to combat Gram-positive bacteria, was observed. *Agrobacterium fabrum* exhibited sensitivity to all antibiotics, despite being one of the Gram-negative bacteria that showed resistance to both zinc and copper. It was observed that *Sphingopyxis bauzanensis* was susceptible to most antibiotics. The bacterial strains increased susceptibility to a variety of antibiotics, such as erythromycin, chloramphenicol, streptomycin, tetracycline, and sulfamethoxazole. In a separate investigation, the resistance of five strains of *Staphylococcus epidermidis* bacteria to 16 different antibiotics and seven heavy metals, including zinc and copper, was evaluated through research (Resheg Al-Sa'ady et al., 2014). Tahmourespour conducted an examination of the heavy metal and antibiotic resistance patterns of effluent bacterial isolates. Bacteria exhibiting resistance to heavy metal were extracted from effluents and subjected to the determination of their Minimum Inhibitory Concentration (MIC). The MIC values for $Cu^{2+}$, $Pb^{2+}$, $Cd^{2+}$, and $Zn^{2+}$ were determined to be 4, 8, 12, and 24 mM/L, respectively. The majority of the isolates exhibited resistance to $Cd^{2+}$, $Pb^{2+}$ and $Zn^{2+}$ ions, as well as the heavy metal resistant isolates indicated high resistance to several antibiotics such as, Carbencilin, Vancomycin, Ampicilin, Cefalothin, Clindamycin and Penicillin. The resistant isolates were *Staphylococcus* sp. and *Klebsiella oxytoca* ATHA1 (Tahmourespour, 2021). Sinegani and Younessi have demonstrated, in their study, the occurrence of diverse patterns of antibiotic resistance among the bacterial isolates that are present in agricultural soils. Specifically, resistance to commonly used antibiotics such as Ampicillin, Amoxicillin, Vancomycin, Tetracycline, Doxycycline, and Streptomycin was observed. It is important to note that in addition to the aforementioned points, there are also other factors to be considered. The findings revealed a notable prevalence of co-resistance to both mercury and antibiotics among the Gram-negative strains, in addition to resistance to beta-lactam antibiotics, as well as to zinc, mercury, and nickel amongst the gram-positive strains (Sinegani and Younessi, 2017). The findings of other study indicated that the levels of Pb and Cd in sewage samples exceeded the established global thresholds. *Bacillus cereus* and *Salmonella enterica* are two

bacterial species that have been extensively studied in their research. The bacterial strain *S. enterica* serovar *Typhi* was observed to possess the greatest potency in terms of resistance to lead among all tested isolates. The Bacillus cereus isolate demonstrated significant levels of resistance towards both cefixime and penicillin (Sadeghabady et al., 2021).

**Table 6.** Other relative findings on antibiotics and heavy metals resistant bacteria.

| Polluted wastewater | Bacteria | Antibiotic | Metals | References |
|---|---|---|---|---|
| Sewage water of Casablanca, Morocco | *Ps. Fluorescens, Ps. Aeruginosa, Klebsiella pneumoniae, Proteus mirabilis,* and *Staphylococcus* sp. | - | Hg, Cd, Zn, Cu, | Filali et al., 2000 |
| Urban sewage and copper smeltery industrial wastewater | *Klebsiella, Moraxella* and *Escherichia coli* | Ofloxacine, Penicilline, Sulfometoxasole, Lincomycin, Kanamycin, Streptomycin, Clindamycin, Vancomycin, Cefradin and Neomycin | Pb, Ni, Cd, and Cu | Alboghobeish, Tahmourespour and Doudi, 2014 |
| The sewage treatment plant of Ahvaz, Iran | *Salmonella enterica serovar typhi* and *Bacillus cereus* | Cefixime and penicillin | Pb and Cd | Sadeghabady et al., 2020 |
| Natural Waters of a Mining Area in Mexico | *Pseudomonadaceae Enterobacteriaceae*, and *Staphylococcus* sp. | - | Cr, Zn, Cu, Ag, Hg, and Co | Escamilla-Rodríguez, Carlos-Hernández, and Díaz-Jiménez, 2021 |
| Sludge and Sewage in Iraq | *Pseudomonas* sp. | - | Hg, Cu, Ni, and Cd | Fawwaz Alfarras et al., 2022 |
| Sikkim's four hot springs, namely: Polok, Borong, Reshi, and Yumthang | *Geobacillus* species | - | Cu, Mn, Co, Zn, and Hg | Najar et al., 2022 |
| An open dumpsite soil (Lemna dumpsite) | *B. Subtilis, B. Cereus, C. Freundii, P. Aeruginosa, Enterobacter sp,* and *E. Coli* | Ciprofloxacin Streptomycin, Septrin, Gentamycin, Amoxicillin, Ceftriazone, Ciprofloxacin, Pefloxacin, Gentamycin and Augmentin | Fe, Ni, Cr, Cd, Pb, Mn, Co, Zn | Edet, Bassey, and Joseph, 2023 |

Despite this, the presence of certain isolates with higher susceptibility to antibiotics suggests that metal-resistant yet antibiotic-susceptible isolates could potentially be used to eliminate metals from contaminated wastewater in the future. The detection and removal of resistant genes in strains that have acquired resistance to both heavy metals and antibiotics can be greatly advantageous in the treatment of wastewater.

## 4. Conclusions

The results of this research showed that the bacteria isolated from Razi industrial wastewater treatment plant and Isfahan refinery units had high sensitivity and relative resistance to heavy metals and antibiotics. It is possible that in the future, the presence of heavy metals such as copper and zinc in the treatment of industrial wastewater and wastewater of refinery units will lead to the development of resistance in microorganisms in these environments.

## Nomenclature

| | |
|---|---|
| MIC | Minimum inhibitory concentration |
| MBC | Minimum bactericidal concentration |
| BOD | Biochemical oxygen demand |
| COD | Chemical oxygen demand |
| PCR | Polymerase chain reaction |
| EC | Electrical conductivity |
| 16SrRNA | 16S ribosomal RNA |
| NCCLS | National committee for clinical laboratory standards |
| LB | Luria-Bertani |

## Author Contributions

Neshat Etetali Ghanbarzadeh: Analysis, writing original draft, and investigation, methodology, formal analysis

Monir Doudi: Contributed to the design and implementation of the research and supervised the project

Ali Mohammad Ahadi: Analysis of the results and writing of the manuscript

Ladan Rahimzadeh Torabi: Writing original draft, analysis of results, review and editing

Mohammad Hossein Pazandeh: Formal analysis and verified the analytical methods

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## Conflict of Interest

The authors declare no conflict of interests.

## Data Availability Statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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