A synergistic effect of copper and nickel ions on the growth rates of *Pseudomonas aeroginosa* and *Staphylococcus aureus* isolates

Makia M. Al-Hejuje
Department of Biology , College of Science , University of Basrah, Basrah-Iraq.

Summary

Two isolates of bacteria : *Pseudomonas aeroginosa* and *Staphylococcus aureus* were exposed to different concentrations (25, 50 and 100 ppm) of copper and nickel ions (as single and mixed),and growth rate of these isolates were measured after different exposure periods of (0 , 2 , 4 , 24 and 72) hrs.

The results were showed that the effect of ions on the growth rate of these isolates were depending on ion type , concentration of ions in the medium , the nature of medium (solid or liquid) , the group of bacteria($Gram^{+ve}$ or $Gram^{-ve}$) , and the periods of exposure . Also , the results were showed a synergistic effect caused by the mixture of the two metals ions on the growth rate of these isolates , and this effect including an elongation in the lag phase , decreasing of colonies numbers and colonies diameter , and dead of bacteria according to the ions concentration in the medium .

Introduction

Some novel transition metal such as Cu^{2+} and Ni^{2+} were discharged into the environment from different sources such as : sewage , industrial and agricultural effluents, municipal . Nickel is an important environmental inorganic pollutant , with allowed level under 0.04 ppm in human consumption water , higher concentration affect normal flora in ecosystem and are toxic for human beings (Rodriguez *et al* , 2006). Copper is known to have activity against bacteria and fungi , it's natural ability to reduce the bioburden of environmental microbes is exploited in water purification , paint and building material , and the textile industry (Mehtar *et al* , 2008) .

The activity of Cu^{2+} against Gram – positive cocci such as methicillin – resistant *Staphylococcus aureus* (MRSA) has been reported (Noyce *et al* , 2006). Sani *et al* (2001) found that the effects of Cu^{2+} toxicity on *Desulfovibrio desulfuricans* bacteria were observed in terms of inhibition in total cell protein , longer lag times , lower specific growth rates , and in some cases no measurable growth .

Bacteria have developed a variety of resistance mechanisms to counteract heavy metals effect . These mechanisms included , their interaction and adsorption to the microbial surfaces (Brown and Lester , 1979), the formation and sequestration of heavy metals in complexes , reduction of metal to less toxic species , and direct efflux of a metal out of the cell . In bacteria , efflux system such as cop system of *Pseudomonas syringae* the cop B and cop D genes are involved in the transport of Cu^{2+} across the membrane , while the products of the cop A and cop C genes are outer membrane proteins that bind Cu^{2+} in the periplasm , protecting the cell from Cu^{2+} . In *Staphylococcus aureus* and in other Gram - positive bacteria , there are another types of efflux systems found such : simple pump out toxic metal ions by using a phosphor-aspartate intermediate (Nies , 1999).

Because heavy metal ions weren't found as single in the environment , Munda and Hudnik (1986) were studied the uptake of Cu^{2+} and Ni^{2+} by using *Fucus vesiculosis* algae , they found that the uptake was decreased two times and Cu^{2+} dominated ten times over Ni^{2+} in the terms of accumulation , accumulation of Cu^{2+} was relative high . In different combinations Cu^{2+} displaced all the other metals and accumulated to the same extent as if applied singly .

There were many papers on the evaluation of the toxicity of a single metal on microorganisms , especially bacteria . However , there were very little information was available in the literature regarding the interaction effect between two metals . The aim of this study was to establish the *in –vitro* activity of Cu^{2+} and Ni^{2+} as single and as mixed together against *Pseudomonas aeroginosa* and *Staphylococcus aureus* isolates .

Materials and Methods

Pseudomonas aeroginosa and *Staphylococcus aureus* isolates were obtained from the microbiological research lab. (at Biology dep., College of Science , University of Basrah)from Jun. - Nov./ 2008.

Solid medium of nutrient agar was prepared for culturing and activation the above isolates for 24 hrs. ,then 1×10^{6} cell/ ml concentration of bacterial suspension was prepared by using Petroff - Hauser counting chamber (Quinn *et al* , 1998) .

One ml of this concentration was used as inoculum for each tube that containing 20 ml of nutrient broth medium which previously supplemented with one concentration (25 , 50 or 100 ppm) of copper as $Cu(NO_3)_2.3H_2O$ or nickel as $Ni(NO_3)_2.6H_2O$ singly or as mixed together .Triplicates were done for each treatment in addition to control treatment . These treatments were incubated at 37 ˚C .The growth rate (optical density (OD)at 600 nm) was measured after different exposure periods (0,2,4,24 and 72 hrs.).

Also , another experiment was carried out by using solid (nutrient agar) medium (15 ml/ Petri dish)which supplemented with the same previous concentrations as singly or as mixed together ,duplicate were done for each treatment in addition to control .Then ,0.02 ml of 1×10^2 cell/ ml bacterial suspension was spreading onto the Petri dish and incubated at 37 ˚C for different exposure periods (24 , 48 and 72 hrs.),and the appearance of colonies were observed after these periods.

Growth rate (GR)was calculated by the following equation : $GR= \Delta N/\Delta t$ Where Δt is the length of time during which the growth curve is linear , and ΔN is the increased amount of bacteria during Δt. Growth inhibition rate (GI%) is defined as : $GI\% = 100(1-GR_{tox} / GR_{ref})$, where GR_{tox} is the GR when bacteria are expoed to heavy metals and GR_{ref} is the GR of control . The interaction effect of metal was calculated according to Aoyama *et al* (1987), using percent inhibition rates obtained from metal tested individually as follow : $Pe = Pa + Pb(100 – Pa) / 100$ Where Pe is the expected additive effect of the combined metals , Pa is the inhibition rate due to heavy metal A alone , and Pb is the inhibition rate due to heavy metal B alone . The expected inhibition rate was then compared with the observed rate (Po).Synergitic and antagonistic were defined by whether the experimentally observed toxicity was greater or less than the expected , respectively .

Data were analyzed statistically by using the analysis of variance test (ANOVA test) and the means was compared by least significant differences test (RLSD test)

Results

The effect of copper ions (Cu^{2+}) on the growth rate of *P. aeroginosa* :

In liquid medium , an elongation(24 hrs.) in the lag phase were recorded at 25 or 50 ppm treatments , but yielded final growth rate equivalent to that in control treatment .In contrast , at 100 ppm treatment , there was slight growth was recorded even after a prolonged incubation of 72 hrs. , table.(1).

In solid medium , the colonies number at 25 ppm treatment was similar to that at the control treatment , but a significant decreasing($p <0.05$) in colonies number was showed at the 50 ppm treatment and no colony was appeared at the 100 ppm treatment , plate (1).

Table (1) :- Effect of copper ions on the growth of *P. aeroginosa* in liquid medium at different exposure periods, (mean ± SD) .

Exposure Period / Treatment	Optical Density at 600 nm				
	0 hrs.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Control	**0.003** ± 0.00	**0.008** ±0.002	**0.029** ± **O .006**	**0.352** ± 0.099	**0.713** ± 0.073
25 ppm Cu	**0.003** ± 0.00	**0.032** ± 0.005	**0.038** ±0.006	**0.314** ± 0.182	**0.682** ± 0.028
50 ppm Cu	**0.003** ± 0.00	**0.045** ± 0.007	**0.054** ± 0.008	**0.244** ± 0.020	**0.609** ± 0.039
100 ppm Cu	**0.003** ± 0.00	**0.078** ± 0.001	**0.075** ± 0.005	**0.082** ± 0.003	**0.084** ± 0.009

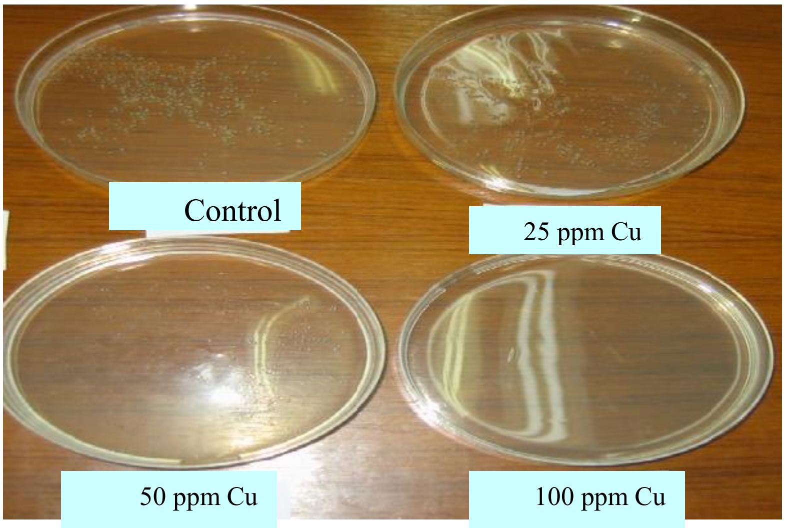


Plate (1) :- Effect of copper ions on the growth of *P. aeroginosa* on the solid medium after 72 hrs. of exposure periods .

The effect of nickel ions (Ni^{2+}) on the growth rate of *P . aeroginos*

In liquid medium , an elongation (24 hrs.)in the lag phase was appeared at 50 ppm treatment only , but yielded final growth rate equivalent to that at 25 ppm or control treatment . At 100 ppm treatment , there was no measurable growth was recorded along the experimental periods (72 hrs.) table (2) .

In solid medium , the colonies diameters were decreased significantly (p <0.05) at the 50 ppm treatment and the colonies diameter was smaller than those at the 25 ppm or at the control treatments . Whereas ,no colony was appeared at 100 ppm treatment along the experimental periods (72 hrs.), plate (2).

Table (2):- Effect of nickel ions on the growth of *P.aeroginosa* in liquid medium at different exposure periods (mean ± SD)

Exposure Period / Treatment	Optical Density at 600 nm				
	0 hrs.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Control	**0.003** ± 0.00	**0.008** ±0.002	**0.029 ± O .006**	**0. 352**± 0.099	**0.713** ± 0.073
25 ppm Ni	**0.003** ± 0.00	**0.009** ± 0.000	**0.022** ± 0.011	**0.309** ± 0.014	**0.669** ± 0.051
50 ppm Ni	**0.003** ± 0.00	**0.008** ± 0.001	**0.005** ± 0.001	**0.076** ± 0.010	**0.606** ± 0.049
100 ppm Ni	**0.003** ± 0.00	**0.003** ± 0.007	**0.007** ± 0.002	**0.007** ± 0.002	**0.005** ± 0.002

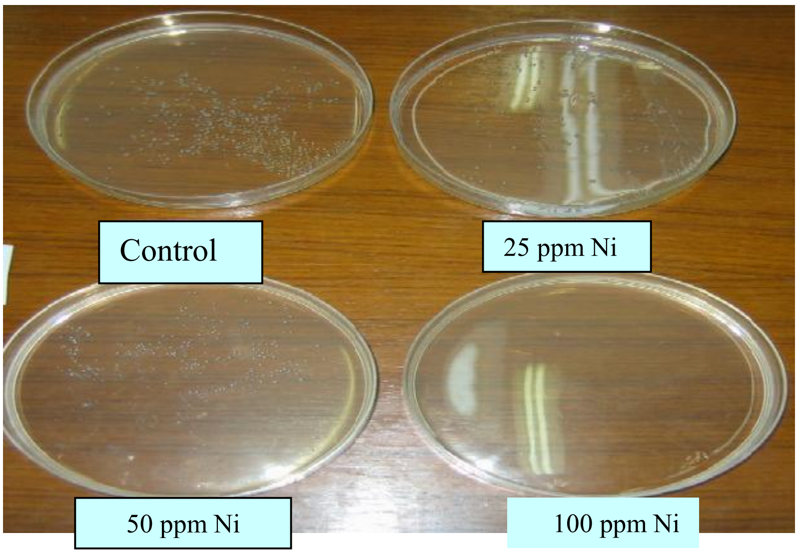
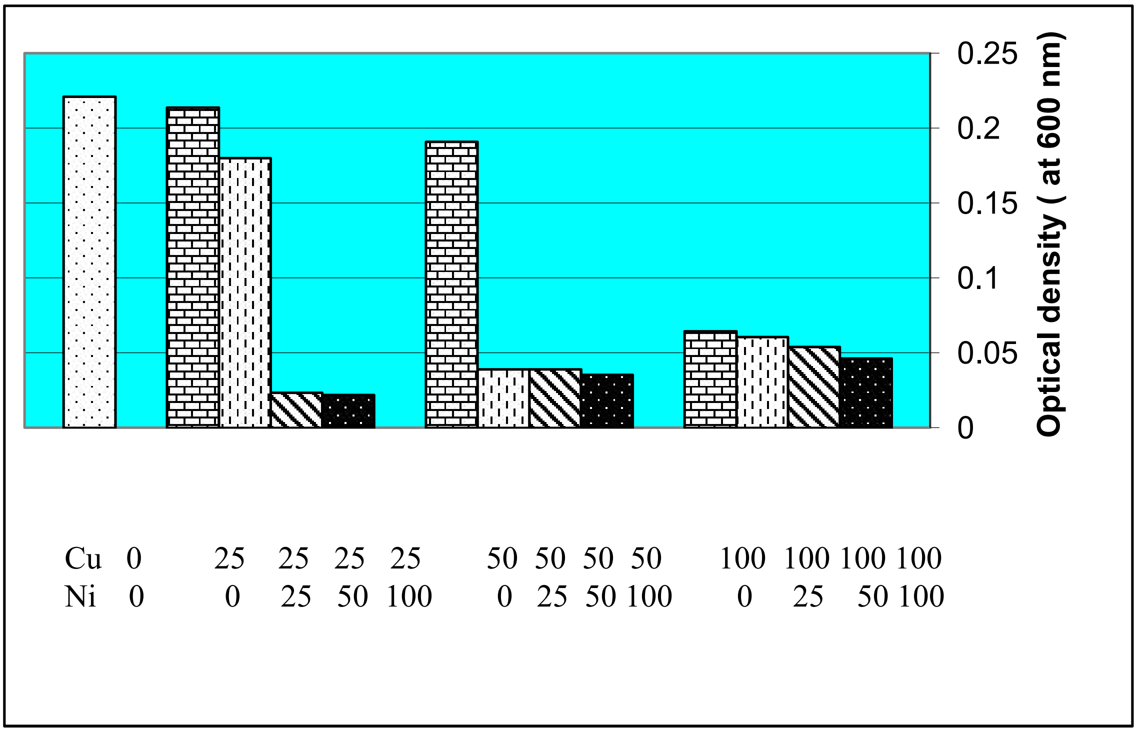


Plate (2):- Effect of nickel ions on the growth of *P. aeroginosa* on the solid medium after 72hrs. of exposure periods .

The combined effect of Cu^{2+} and Ni^{2+} on the growth rate of *P. aeroginosa*:-

In liquid medium , fig (1) showed that there was no significant decreasing (p> 0.05) in the growth rate of the above isolate at (25ppmCu + 25 ppm Ni) treatment as compared with the control treatment , whereas in solid medium a significant decreasing (p <0.05) in the colonies number were appeared at the same concentrations , plate (3 ,a) .

At (25 ppm Cu + 50 ppm Ni) treatment , rare growth was recorded in the liquid medium along the experimental periods, fig.(1) , but less number of small colonies were observed at the solid medium at the end of experiment , plate (3,a) At (25 ppm Cu+100 ppm Ni)treatment a synergistic effect was happened , and rare growth was appeared in liquid and no growth was appeared at the solid medium along the exposure periods .At the other treatments(50 ppm Cu+ 25 ppm Ni), (50 ppm Cu+50 ppm Ni), (50 ppm Cu+ 100 ppm Ni), (100 ppm Cu+ 25 ppm Ni), (100 ppm Cu+ 50 ppm Ni),and (100 ppm Cu+100 ppm Ni) treatments , there were rare growth were recorded in the liquid medium at all these treatments along the experimental periods fig. (1), while , in solid medium the (50 ppm Cu + 25 ppm Ni) treatment was showed an elongation in lag phase , and less number of smaller colonies (as compared with the control treatment) were appeared after 48 hrs.of the exposure periods , plate (3,b).



Fig**(1):- The combined effect of Cu^{2+} and Ni^{2+} on the growth of *P .aeroginosa* in liquid medium .**

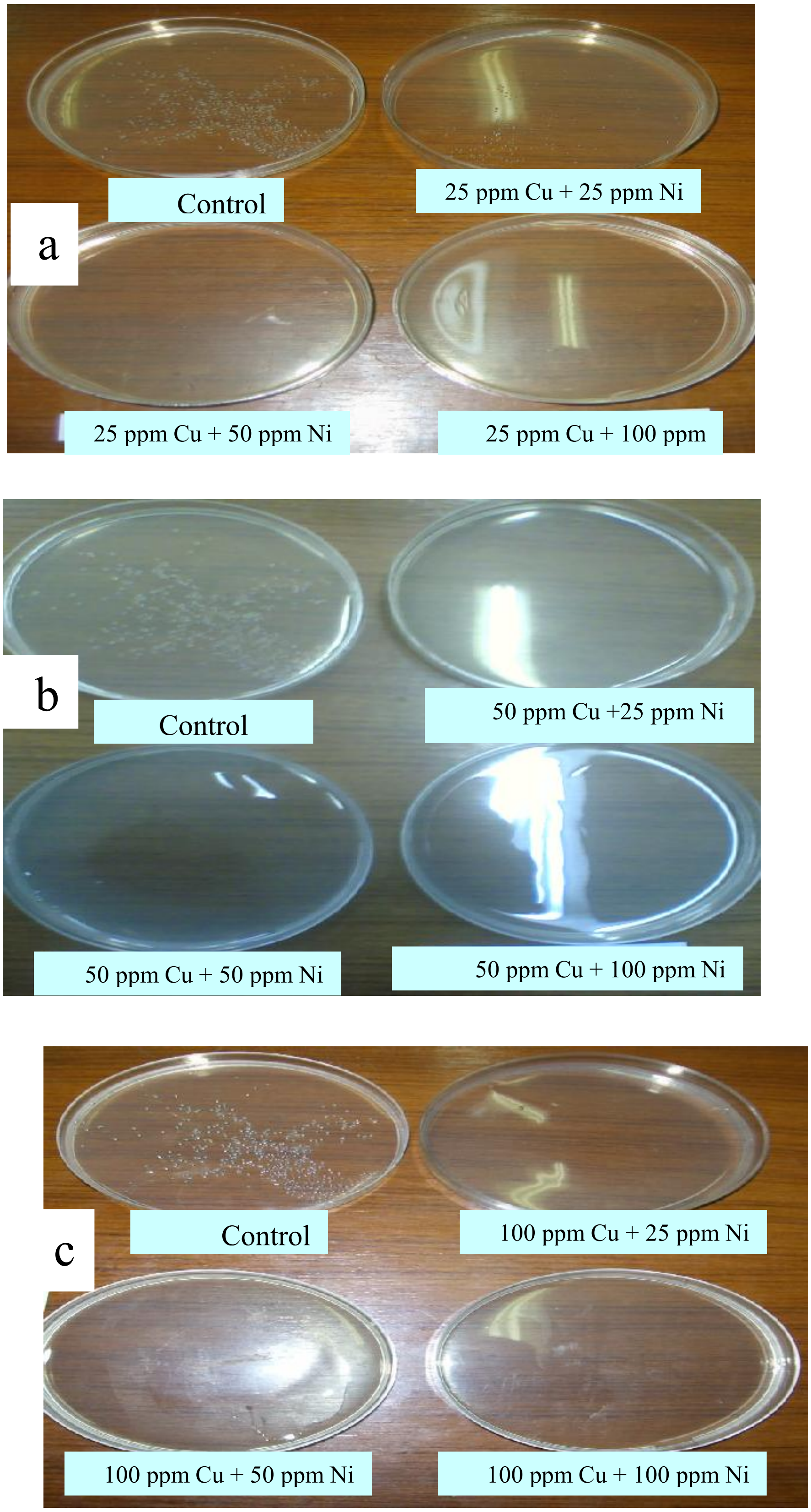


Plate (3):- The combined effect of copper(a : 25 ppm Cu , b : 50 ppm Cu , c : 100 ppm Cu) and nickel ions on the growth of *P. aeroginosa* on the solid medium after 72 hrs. of exposure periods .

The effect of copper ions (Cu^{2+}) on the growth rate of *S. aureus* :-

In liquid medium , the growth rate of ***S. aureus*** at 25 ppm of Cu treatment was similar to that at the control treatment , whereas a significant decreasing ($P<0.01$) was recorded in the growth rate of this isolate at 50 ppm Cu treatment , and no growth was recorded at 100 ppm Cu treatment along the experimental period , table(3) In solid medium , the colonies' number and diameter at both (25 ppm Cu and 50 ppm Cu) treatments ,were observed similar to that at the control treatment , while , no colony was observed at highest concentration (100 ppm Cu) along the experimental period , plate (4) .

Table(3):- Effect of copper ions on the growth of *S . aureus* in liquid medium at different exposure periods (mean ± SD) .

Exposure Period / Treatment	Optical Density at 600 nm				
	0 hrs.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Control	**0.003** ± 0.00	**0.011** ± 0.003	**0.038** ± **O .006**	**0.195** ± 0.025	**0.628** ± 0.016
25 ppm Cu	**0.003** ± 0.00	**0. 029** ± 0.001	**0.047** ±0.006	**0.249** ± 0.058	**0.627** ± 0.095
50 ppm Cu	**0.003** ± 0.00	**0.043** ± 0.003	**0.049** ± 0.004	**0.133** ± 0.019	**0.343** ± 0.091
100 ppm Cu	**0.003** ± 0.00	**0.075** ± 0.003	**0.078** ± 0.001	**0.081** ± 0.005	**0.077** ± 0.003

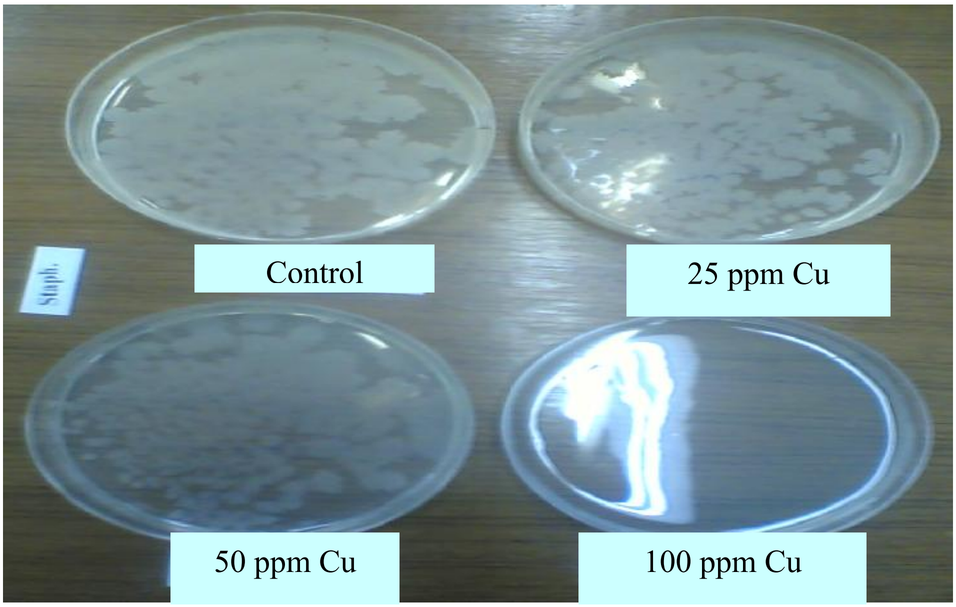


Plate (4) :- Effect of copper ions on the growth of *S. aureus* on the solid medium after 72 hrs. of exposure periods

The effect of nickel ions (Ni^{2+}) on the growth rate of *S. aureus* :-

In liquid medium , the growth rate of the present isolate was not affected when the nickel ions was added at low concentration (25 ppm) , but , when the nickel concentration was added at moderate (50 ppm) , an elongation in the lag phase of this isolate was recorded , and the growth rate was decreased significantly ($P<0.01$) at this treatment as compared with the control treatment .Slight growth was recorded at the highest concentration (100 ppm) of nickel along the experimental period , table (4).

In solid medium , heavy colonies number with small diameter were observed at the 50 ppm treatment as compared with the (25 ppm Ni) or with the control treatments , while , no colony was appeared at the highest concentration (100 ppm Ni) along the experimental period , plate (5) .

Table(4):- Effect of nickel ions on the growth of *S . aureus* in liquid medium at different exposure periods (mean ± SD).

Exposure Period / Treatment	Optical Density at 600 nm				
	0 hrs.	2 hrs.	4 hrs.	24 hrs.	72 hrs.
Control	**0.003** ± 0.00	**0.011** ± 0.003	**0.038 ± O .006**	**0. 195** ± 0.025	**0.628** ± 0.016
25 ppm Ni	**0.003** ± 0.00	**0.008** ± 0.002	**0.024** ± 0.001	**0.155** ± 0.028	**0.545** ± 0.031
50 ppm Ni	**0.003** ± 0.00	**0.008** ± 0.002	**0.015** ± 0.001	**0.016** ± 0.005	**0.243** ± 0.112
100 ppm Ni	**0.003** ± 0.00	**0.006** ± 0.001	**0.013** ± 0.003	**0.018** ± 0.002	**0.016** ± 0.003

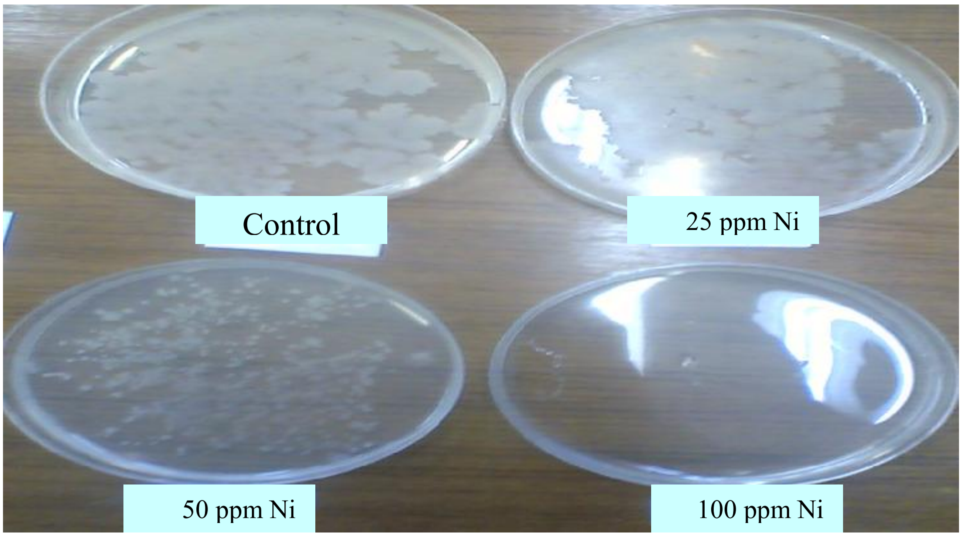
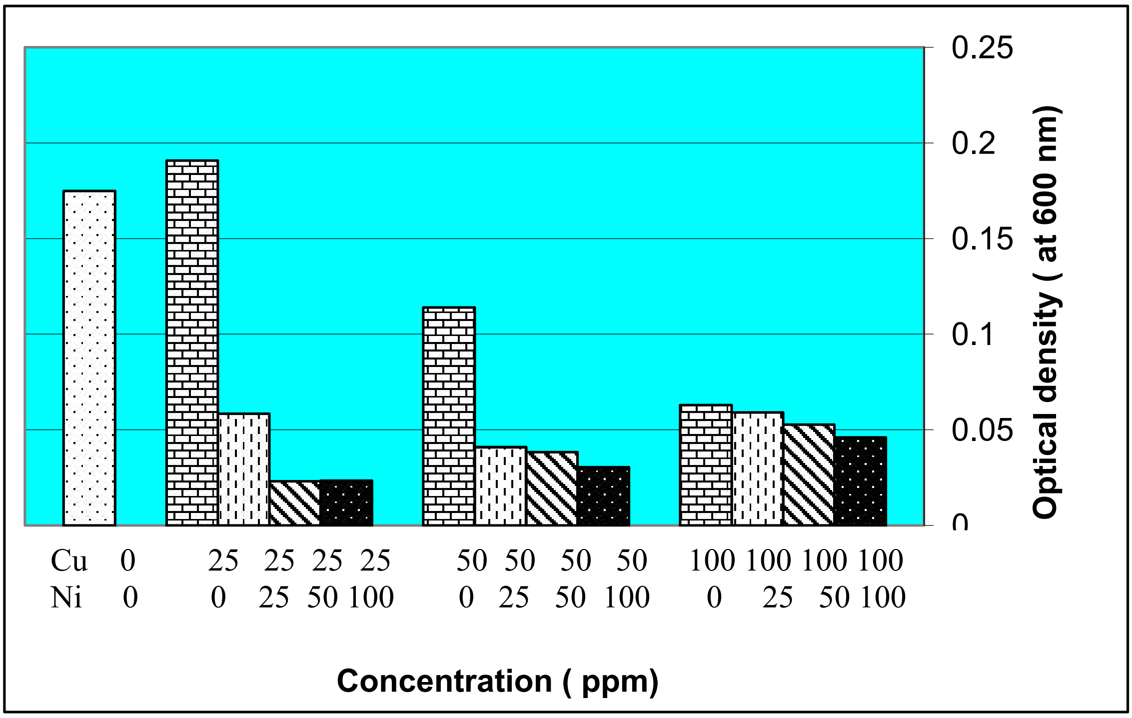


Plate (5) :- Effect of nickel ions on the growth of *S. aureus* on the solid medium after 72 hrs. of exposure periods .

The combined effect of Cu^{2+} and Ni^{2+} on the growth rateof *S.aureus* :-

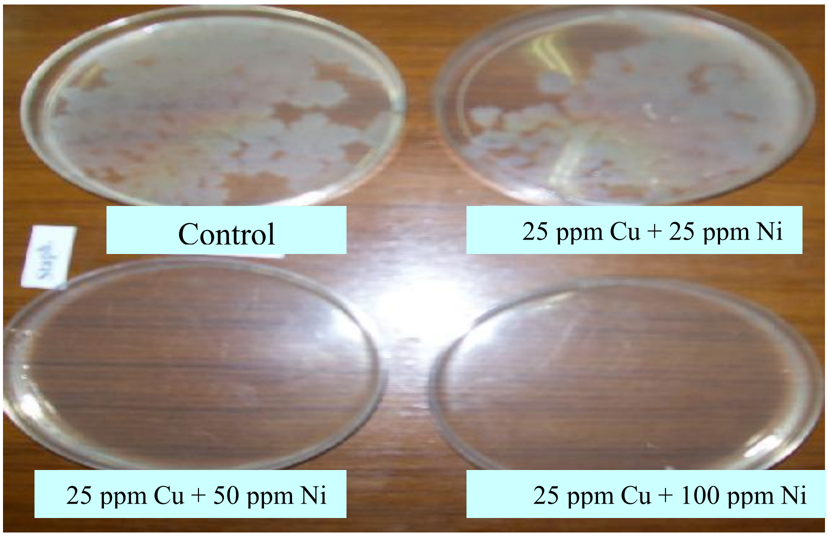
In liquid medium , there was an elongation in the lag phase of *S. aureus* isolate was recorded at (25ppm Cu + 25 ppm Ni) treatment , and the growth rate was decreased significantly ($P<0.01$)as compared with the treatments that supplemented with these concentration as singly , or as compared with the control treatment . There were slightly optical density was recorded at the other treatments that supplemented with concentrations more than the above such as (25 ppmCu + 50ppm Ni) , (25 ppm Cu +100 ppm Ni) , (50 ppm Cu +25 ppm Ni) , (50 ppm Cu + 50 ppm Ni) , (50 ppm Cu + 100 ppm Ni) , (100 ppm Cu +25 ppm Ni) , (100 ppmCu+ 50 ppm Ni) ,and (100 ppm Cu + 100 ppm Ni) treatments , fig. (2).



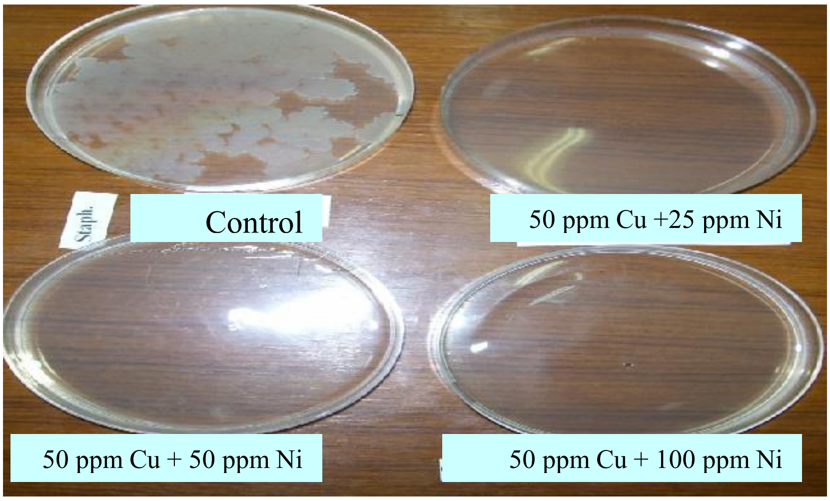
Fig(2):-The combined effect of Cu^{2+} and Ni^{2+} on the growth of *S. aureus* in liquid medium .

In solid medium , no growth was appeared at most treatment, which supplemented with mixed ions, along the experimental periods ,except two treatment , the first, (25 ppm Cu+ 25 ppm Ni) which was not affected ,and have colonies similar to that at the control treatment , and the second (50 ppm Cu + 25 ppm Ni) which record an elongation in the lag phase (48 hrs.), and have less number of smallest colonies as compared with the control treatment , plate (6).

a



b



c

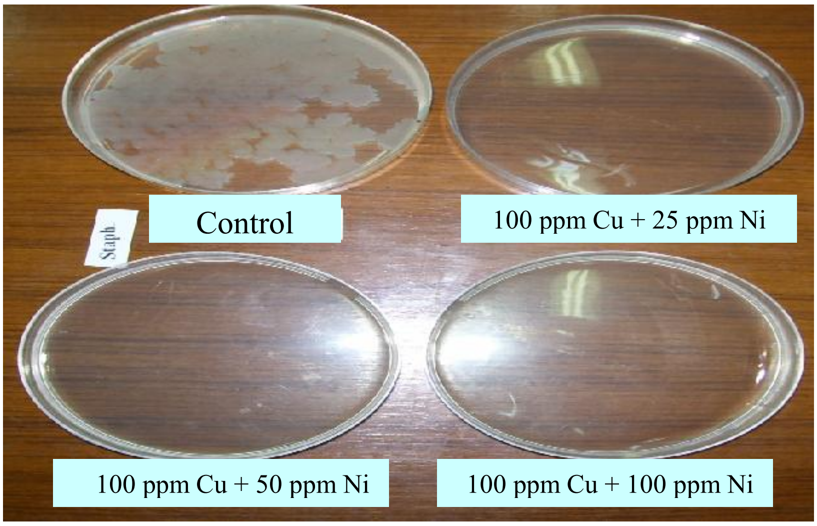


Plate (6):-The combined effect of copper (a : 25 ppm Cu , b : 50 ppm Cu , c : 100 ppm Cu) and nickel ions on the growth of *S .aureus* on the solid medium after 72hrs. of exposure periods.

Discussion

Microorganisms require some metals like Cu^{2+} and Ni^{2+} at low concentrations as essential micronutrients for vital cofactors for metalloproteins and certain enzymes , However , at higher concentration , it has been reported that these metals interact with nucleic acids and enzyme active sites , (Nies , 1999).

Microbial cell wall was provided with aminic , carboxylic , thiolic , phosphoric and sulphydrylic functional groups that can bind heavy metals ions (Converti *et al* , 2006) , histidine that found on the cell wall is able to bind Cu^{2+} because furnishes a bidentate site. Aminic and carboxylic groups can also interact bidentatelly with Cu^{2+} (Xue *et al* ,

1988) . Rodriguez *et al* (2006) found that the optimum residence time for Ni^{2+} adsorption by *P . aeroginosa* was 100 minutes , and they suggest that *P . aeroginosa* could have a lower number of wall binding sites to interact with Ni^{2+} , but with a stronger binding to this metal . The present study showed no growth appeared at the highest concentration of ions (100 ppm) , this may be due to the saturation of extracellular polymers binding sites which occurred at the highest metal concentrations , and this result was in agreement with the finding of Brown and Lester (1982) for nickel ions .

Also, the present result showed a variation in the response of each isolate to the heavy metals ions stress according to the bacterial species , this variation may be depending on the differences in the cell wall and membrane structures of these two different groups (Gram $^{+ve}$ and Gram $^{-ve}$ bacteria) . Churchill *et al* (1995) found that Gram $^{-ve}$ strains (*E. coli* K-12) was the most efficient at binding Cu , Cr and Ni , whereas Gram $^{+ve}$ strains (*Micrococcus luteus*) sorbed Co most efficiently .

Some treatments of the present study growen at the broth medium , but no colonies was appeared at the solid medium for the same ions concentrations , this was caused by the different in the state of medium (solid or broth) ,studies concerning effects of heavy metals on microorganisms have shown that the composition of the culture medium greatly influences the apparent toxicity of metal to microorganisms (Babich and Stotzky , 1978) . Our result was in agreement with the finding of Mitra (1984) who found that exposure of *E. coli* to 3μM Cd^{2+} results in 84%-95% of the cells losing their ability to form colonies on plates of nutrient agar , transfer of this cells to Cd^{2+} free liquid medium results in a recovery of colony – forming ability without significant synthesis of DNA.

An elongation in lag phase was appeared at the most present treatments , my opinion is that elongation due to selection for metal – resistant phenotypes . The present finding was in agreement with another studies (Higham *et al* , 1986 ; Nies , 1999) who found an elongation in lag phase of bacteria caused by metals ions stress .

The interaction between Cu^{2+} and Ni^{2+} ions has a synergistic effect on the growth of the present isolates , this result was in agreement with Lasheen *et al* (1990) who found a synergistic effect caused by compensation of Cu and Cd . Also , in agreement with Mehtar *et al* (2008) who tested *S. aureus* and *P . aeroginosa* against copper and its alloys , and they found that *P . aeroginosa* was inhibited by Brass(Cu 70% and Zn 30%) at 180 minutes ,while , inhibited by Cu alone at 270 minutes.

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التأثير المضاف لايونات النحاس والنيكل على معدلات النمو للعزلتين

Pseudomonas aeroginosa و *Staphylococcus aureus*

مكية مهلهل خلف الحجاج

قسم علوم الحياة ، كلية العلوم ، جامعة البصرة ، البصرة – العراق .

الخلاصة

عرضت العزلتين البكتيريتين *Staphylococcus aureus* و *Pseudomonas aeroginosa* إلى تراكيز مختلفة (25 ، 50 و 100 جزء بالمليون) من ايونات النحاس(Cu^{2+}) و النيكل (Ni^{2+}) (بصورة مفردة و خليط) . ثم قيست معدلات النمو لهاتين العزلتين بعد فترات تعريض مختلفة (0 ، 2 ، 4 ، 24 و 72 ساعة).

أظهرت النتائج إن تأثير الايونات على معدلات النمو للعزلتين كان معتمدا على : نوع الايون ، تركيزه في الوسط الزرعي ، طبيعة الوسط الزرعي (صلبا كان أم سائلا) ،المجموعة البكتيرية (موجبة لصبغة گرام أم سالبة لصبغة گرام) ، وفترة التعريض.

[redacted] كذلك أظهرت النتائج وجود تأثيرا مضافا على نمو العزلتين تسبب عن خلط المعدنين وهذا التأثير تضمن زيادة فترة الطور التمهيدي (lag phase) ، نقصان أعداد المستعمرات ، نقصان أقطار المستعمرات ، وموت البكتيريا اعتمادا على تركيز الايونات في الوسط الزرعي .