The efficacy of different antimicrobial metals at preventing the formation of, and eradicating bacterial biofilms of pathogenic indicator strains.

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Abstract

The emergence of multidrug resistant pathogens and the prevalence of biofilm-related infections, has generated a demand for alternative antimicrobial therapies. Metals have not been explored in adequate detail for their capacity to combat infectious disease. Metal compounds can now be found in textiles, medical devices, and disinfectants – yet, we know little about their efficacy against specific pathogens. To help fill this knowledge gap, we report on the antimicrobial and antibiofilm activity of seven metals; silver, copper, titanium, gallium, nickel, aluminum and zinc against three bacterial strains, *Pseudomonas aeruginosa, Staphylococcus aureus,* and *Escherichia coli*. In order to evaluate the capacity of metal ions to prevent the growth of, and eradicate biofilms and planktonic cells, bacterial cultures were inoculated in the Calgary Biofilm Device (MBEC™) in the presence the metal salts. Copper, gallium, and titanium were capable of preventing planktonic and biofilm growth, and eradicating established biofilms of all tested strains. Further, we observed that the efficacies of the other tested metal salts displayed variable efficacy against the tested strains. Further, contrary to the enhanced resistance anticipated from bacterial biofilms, particular metal salts were observed to be more effective against biofilm communities versus planktonic cells. In this study, we have demonstrated that the identity of the bacterial strain must be considered prior to treatment with a particular metal ion. Consequently, as the use of metal ions as antimicrobial agents to fight multidrug resistant and biofilm related infections increases, we must aim for more selective deployment in a given infectious setting.

Key Words: antibiofilm, antimicrobial, metals, biofilm, metal toxicity, metal tolerance

Background

The progression of bacterial resistance to antibiotics has led us to an era that urgently requires alternative antimicrobial therapies. Furthermore, recent knowledge regarding antibiotic efficacy has led to the realization that targeted antimicrobial strategies are required for use against chronic infections – such as those caused by biofilms - which are remarkable different from acute infections. Typically, more than half of infections are caused by organisms that are involved in surface-attached communities immersed in a self-produced hydrated extracellular polymer matrix, known as a biofilm [1]. This matrix has been observed to complicate wound healing by facilitating the transition between acute and chronic infections [2], and contaminate clinical surfaces and implanted medical devices such as catheters and endotracheal tubes [3]. The physiological changes characteristic of biofilms results in enhanced resistant to elimination by the host immune system and some antibiotics [4]. The use of modern antibiotics to treat infections caused by bacteria is now a multifactorial challenge given the threat of both multi-drug resistant bacteria and biofilm-related infections. As a consequence, the administration of metals to combat both threats has recently regained attention. Metal compounds can now be found in wound dressings [5], liquid formulations for hand-washing [6] impregnated into textiles such as socks [7], and on medical devices like catheters [8].

The antimicrobial properties of metals have been documented in many bodies of work [9] and continue to be the subject of investigation in an attempt to understand the mechanisms of metal toxicity and resistance [10–14]. Despite the wealth of literature committed to examining the antimicrobial activity of metals, less attention has been paid to determining the susceptibility of bacteria to metals within a defined set of conditions. While the minimal inhibitory concentrations, minimal bactericidal concentration, and minimal biofilm eradication concentrations for many metals have been determined, the lack of consistency between techniques, conditions and media have

resulted in difficulties when comparing the susceptibilities of bacterial strains to metal compounds. Additionally, present data on the antimicrobial properties of metals are inadequate, which is alarming, particularly since applications have expanded into industry, agriculture and healthcare [9].

Here we describe our observations from testing the antimicrobial and antibiofilm activity of seven different metals with demonstrated antimicrobial activity and utility (silver, copper, titanium, gallium, nickel, aluminum, and zinc) against three indicator strains, *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922). Chemically simulated wound media (CSWM) was used to provide a rich environment for bacterial growth, warranting that variation in susceptibility between the three strains was not a result of nutrient limitations in the growth media. In addition, this growth media provided an environment comparable to a wound infection – a clinical challenge where metals have a realized potential for utility. Experiments were designed to experimentally reproduce an acute wound infection by assessing both the prevention and eradication of biofilms as well as the susceptibility of planktonic cultures. Using the Calgary Biofilm Device (CBD)/MBEC^{TM}, the minimal biofilm bactericidal concentrations (MBBC), the minimal planktonic bactericidal concentrations (MPBC), and the minimal biofilm eradication concentrations (MBEC) were determined under the various metal challenges.

Methods.

Bacterial strains and culture media

Bacterial strains were stored at -70°C in Microbank™ vials as described by the manufacturer (proLab Diagnostics, Richmond Hill, ON, Canada). The three bacterial strains *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 were gifts from Dr. Joe J. Harrison (University of Calgary).

Throughout our studies – present and past – we have observed that the growth media chosen to culture bacterial cells is a significant factor that dictates the efficacy of the metal challenge. Hence, we selected a media that provides a rich environment to ensure robust bacterial growth in each strain. Chemically simulated wound media (CSWM), modified from [15] [50% bovine serum (66g/L): 50% peptone water (0.85% NaCL, 0.1g/L peptone)] was used for metal susceptibility testing throughout this work. For the dilution of metal working solutions, a 2X peptone water (0.85% NaCl, 0.2g/L peptone) solution was used.

Biofilm cultivation

In this work, all biofilms were cultivated using the Calgary Biofilm Device (CBD)/MBEC™ as described in [16,17] and by the manufacture's guidelines (Innovotech, Edmonton, AB, Canada). Following overnight growth of the pre-culture, colonies were suspended in CSWM and matched to a 1.0 McFarland standard. Next, the suspended cells were diluted 30 times in CSWM. In order to cultivate the biofilm, 150uL of the diluted inoculum was placed into a 96-well microtitre plate (Nunclon, VWR, International) followed by placement of the CBD lid, which contained 96 equivalent pegs. The CBD was placed on a gyrorotary shaker operating at 150rpm in a humidified incubator at 37°C for either 4hr or 24hr.

2.3 Stock and working metal solutions

Silver nitrate ($AgNO_3$), copper (II) sulfate ($CuSO_4$), titanium (III) chloride ($TiCl_3$), gallium (III) nitrate ($Ga(NO_3)_3 \bullet H_2O$), and nickel sulfate ($NiSO_4 \bullet 6H_2O$) were all obtained from Sigma-Aldrich (St. Louis, MO, USA). Aluminum sulfate ($Al_2(SO_4)_3 \bullet H_2O$) was obtained from Matheson Coleman and Bell (Norwood, OH, USA), and zinc sulfate ($ZnSO_4 \bullet 7H_2O$) was received from Fisher Scientific (Fair Lawn, NJ, USA). Stock solutions of $CuSO_4$, $TiCl_3$, and $Al_2(SO_4)_3 \bullet H_2O$ were made up to 1M, $ZnSO_4 \bullet 7H_2O$ was made up to 1.5M, $NiSO_4 \bullet 6H_2O$ to 2.5M, and $AgNO_3$ to 500mM in distilled and deionized (dd)H_2O. All stock metal solutions were stored in glass vials at 21°C for no longer than two weeks. No more than 30 minutes prior to experimental use, working solutions were made from stock metal solutions in equal amounts of CSWM and 2X peptone water (dilution factor of 2). In a 96-well plate (the challenge plate) serial dilutions of each metal, with a dilution factor of 2, were prepared; reservation of the first row served as a growth control (0.0mM metal salt).

Prevention of planktonic growth and biofilm formation

In order to assess the capability of the metal salts to prevent the growth of biofilms and planktonic cells, bacterial cultures were inoculated in the CBD in the presence of the metal salt. The CBD was then placed in a 37°C humidified incubator on a gyrorotary shaker at 150rpm for 4hr. This treatment provided the minimal planktonic bactericidal concentrations (MPBC) and the minimal biofilm bactericidal concentrations (MBBC). Overall evaluating if bacteria could establish a culture planktonically or as a biofilm in the presence of the metal salts.

Eradication of established biofilms

To evaluate the ability of the metal salts to eradicate established biofilms, a biofilm was first

cultivated on the pegged lid of the CBD for 24hr. The lid was then rinsed twice with 0.9% NaCl and placed into a 96-well microtitre plate containing two-fold serial dilutions of the metal salts. The plate was then incubated for 24hr in a humidified incubator at 37°C on a gyrorotary shaker at 150rpm. This treatment was used to determine the minimal biofilm eradication concentration (MBEC) of each metal salt.

Assessment of metal efficacy

To assess the susceptibility of planktonic and biofilm populations to the metal salts, the peg lids from both treatments were first rinsed twice in 0.9% NaCl. Subsequently, the biofilms were disrupted from the pegs by sonication using a 250HT ultrasonic cleaner (VWR, International) for 10 minutes into 200uL of Lysogeny Broth (LB) media [25 g/L] containing 0.1% Tween®20 and universal neutralizer (UN) [18] [0.5 g/L histidine (Sigma, USA), 0.5 g/L-cysteine (Sigma, USA), and 0.l g/L reduced glutathione (Sigma, USA) in (dd)H_2O]. To establish the MBBC and MBEC of the disrupted biofilm populations, 6 dilutions, with a dilution factor of 10, in 0.9% NaCl were performed. The samples were spot plated on tryptic soy agar plates in order to determine the viable cell numbers from the biofilm, and subsequently incubated overnight at 37°C. To determine the MPBC of the planktonic populations 8 serial dilutions, with a dilution factor of 10, were carried out into 96-well plates with 0.9% saline and UN. Similarly, spot plating the diluted samples onto TSA plates and incubating overnight at 37°C generated viable cell counts. The concentrations at which each metal salt gave rise to no viable microbial colonies were determined to be the MPBC, MBBC and MBEC.

Results

Various metal salts can prevent planktonic growth and biofilm formation

To determine the capacity of metal salts to prevent the formation of biofilms of the selected indicator strains, *P. aeruginosa* ATCC 27853, *S. aureus* ATCC 25923, and *E. coli* ATCC 25922, were grown for 4h in the presence of the metal salts. This approach gave rise to the minimal planktonic bactericidal concentration (MPBC) **(Fig. 1a)** and in parallel, the minimal biofilm bactericidal concentration (MBBC) **(Fig. 1b)**. In order for the biofilms to form in the presence of the metal ions, the planktonic cells would need to survive the metal concentrations long enough to permit attachment and expression of biofilm related genes. Therefore, this experiment measures both cell attachment and biofilm proliferation in the presence of metal salts.

For all three strains the MPBC **(Fig. 1a)** and MBBC **(Fig. 1b)** of Cu, Ga and Ti was reached within the tested concentrations. A lower concentration of Cu, as opposed to Ga, was needed to prevent *P. aeruginosa* attachment and growth **(Table 1)**. This was not observed for *E. coli*, in which a greater concentration of Ga, in comparison to Cu, was needed to attain the MBBC and MPBC **(Table 2)**. *S. aureus* biofilms were 4-fold more resistant to Ti than their planktonic counterparts indicated by the MBBC and MPBC **(Table 3)**. A 4-fold higher concentration of Cu was needed to prevent planktonic growth than the formation of biofilms in *P. aeruginosa* **(Table 1)**.

The metals Ag and Al were successful for preventing biofilm formation in *P. aeruginosa* and *E. coli* **(Fig. 1b),** however, only Al was capable of eliminating planktonic populations in these two strains following the concurrent 4hr metal exposure and incubation period **(Fig. 1a)**. Notably, the MBBC for Al was found to be 250-fold lower in *P. aeruginosa* when compared to *E. coli*. In addition, a greater concentration of Al was needed to reach the MPBC as opposed to the MBBC in *P. aeruginosa*. In the concentrations of Ag tested, little change in viable planktonic cells was observed

for *P. aeruginosa* and *E. coli* **(Fig 2a)**. The MPBC and MBBC for *S. aureus* were not reached within the concentrations of Al examined, however, a log decrease in biofilm formation and ~2 log decrease in planktonic cells was observed based on the reduction in viable cell numbers (**Fig. 2)**. Higher concentrations of Al were not explored due to the solubility of this metal in (dd)H_2O. Finally, in the presence of Ag the MPBC and MBBC for *S. aureus* were not reached within the concentrations tested. The addition of Ag at a concentration >500mM to the CSWM led to extensive precipitation; thus concentrations greater than 500mM could not be explored.

For *S. aureus,* only the MBBC was reached upon challenge with Ni **(Fig. 1b)**, while a 2-fold reduction in planktonic growth was observed **(Fig. 2a)**. This metal was incapable of inhibiting planktonic growth and biofilm formation in *P. aeruginosa* and *E. coli* **(Fig. 1)**. Zn could not prevent the formation of biofilms and planktonic cell growth in *P. aeruginosa*. Challenge with Zn or Ni resulted in a 1-log and 2-log reduction in planktonic **(Fig. 2a)** and biofilm viable cell numbers **(Fig. 2b)** respectfully, for *P. aeruginosa.* For *S. aureus,* the attachment of biofilms and planktonic growth was prevented upon incubation with Zn, yet only biofilm attachment was prevented in *E. coli.* Lastly, there was no observed reduction in planktonic or biofilm viable cell numbers after exposure of *E. coli* to Ni for 4hr **(Fig. 2)**.

Certain metal ions are capable of eradicating established biofilms

The eradication of biofilms by various metal salts was assessed in a similar manner as the prevention of biofilms, however to determine the concentration needed to eradicate an established biofilm, biofilms were established by incubating the inoculum in a CBD for 24hr. This was followed by exposure to serial dilutions (two-fold) of the metal salts for an additional 24hr. After metal exposure it was observed that Cu, Ag, Ga, Ti, and Al had the capacity to eradicate biofilms of all

three of the tested strains **(Fig. 3)**. Although the metal salts Ni and Zn were found to be effective at eradicating *S. aureus* and *E. coli* biofilms after 24hr metal exposure, *P. aeruginosa* biofilms were not eliminated - rather a 50% decrease in viable cell numbers was observed **(Fig. 4)**. A higher concentration of Ag, more so than any other metal, was needed to eradicate *S. aureus,* while the opposite was observed for *E. coli* **(Fig. 3)**.

Discussion

Numerous accounts of resistance from bacterial biofilms to conventional antimicrobials have been reported since the 1990's [1]. We are entering an era where our options to treat acute and chronic infections are limited. Consequently, alternative strategies to combat biofilm bacterial resistance and tolerance are being investigated [19–22]. Among these alternate strategies is the use of metal compounds as antimicrobial agents that are capable of disrupting growth and/or eradicating biofilms [9]. Despite their reemerging use, little effort has been directed toward comparing the susceptibility of bacteria, both as planktonic cells and biofilm communities, to metals under a defined set of conditions. Here, we demonstrate how a reproducible screening method was used to compare the susceptibility of bacterial strains to several metal salts. Chemically simulated wound media was used to provide a rich environment containing proteins, lipids, and a large variety of ions for promoting bacterial growth. The aim of this study was to provide a robust comparison of the efficacy of various metals against three defined indicator strains, namely *P. aeruginosa, S. aureus,* and *E. coli.*

Ag has been studied for its efficacy at disrupting and/or eliminating biofilms [23]. Contrary to such studies, the MPBC and MBBC for *S. aureus* were not reached in the concentrations tested in this work **(Fig. 1)**. Decreased antimicrobial susceptibility may be regarded as the most consequential phenotype of bacterial biofilms, and for many antimicrobial agents this concept holds true [24]. Despite this, data has suggested that under selected growth conditions residence within a biofilm does not always provide enhanced resistance against antimicrobials [25–27], and several of our observations support this. In fact, Ag was successful at preventing the formation of *P. aeruginosa* and *E. coli* biofilms **(Fig. 1b)**, however, this metal was incapable of inhibiting planktonic growth within these two strains **(Fig. 1a)**.

Cu(II) is known to increase intracellular levels of reactive oxidative species (ROS) [28–30],

catalyze hydroxyl radical formation [31], and target enzymes in the iron-sulfur dehydratase family [12]. Both Cu(II) and Ag(I) are thiophilic metals and share similar selectivity for biological donor ligands in the bacterial cell [9]. Yet, one key difference between the two metals is their biological function. Cu(II) is an essential metal for many cellular redox enzymes, while Ag(I) is a non-essential metal in which the precise manner of toxicity within all cell types still remains unclear. In this work, we found Cu to be effective for preventing biofilm attachment **(Fig. 1b)** and eradicating established biofilms **(Fig. 3)**. In addition, this metal was capable of preventing the growth of planktonic cells **(Fig. 1a)**, different from what was observed with Ag. In general, we determined that the tendency of Ag to precipitate in CSWM proved its efficacy as an antimicrobial agent against cells in either cellular state to be secondary to Cu. Nonetheless, the efficacy of Ag as an antimicrobial agent continues to be observed [32], and a substantial amount of effort has gone into developing silver-based materials [33].

Certain transition metals have a documented capacity to disrupt cellular donor ligands that coordinate the essential ion Fe(III) [9]. Destruction of [Fe-S] clusters may release additional Fenton-active Fe into the cytoplasm increasing intracellular ROS formation [11,14,34]. Ga(III) has been found to target solvent-exposed [Fe-S] clusters since many biological systems are unable to distinguish between Ga(III) and Fe(III) [35]. In fact, we observed that this metal was effective at inhibiting biofilm and planktonic cell growth in all three strains **(Fig. 1 and 3)**. The use of Ga as an antimicrobial agent is not novel, and in parallel with our data, the antimicrobial properties of this metal have been demonstrated both *in vitro* and *in vivo* against numerous organisms [36]. It should be noted however, that upon comparison to other bodies of work we observed that higher concentrations of Ga were needed to eliminate all three strains[10,37]. This observation provides insight into the influence of experimental conditions on biofilm and planktonic antimicrobial susceptibility. In fact, we have repeatedly observed that different media formulations give rise to exceedingly different tolerance

293 levels (unpublished data).

294 Al(III), like Ag(I), is also a non-essential metal in which the precise mechanism of cellular
295 uptake has yet to be determined. This metal was found to be effective at preventing the formation of
296 biofilms and planktonic cells in *P. aeruginosa* and *E. coli* **(Fig. 1)**. Contrary to this, Al was not
297 effective at preventing biofilm formation and planktonic cell growth in *S. aureus* in the
298 concentrations tested, however, a single-fold reduction in viable cell numbers was observed during a
299 4hr metal exposure **(Fig. 2b)**. Since the MBEC was reached for *S. aureus* in the presence of Al
300 during the 24hr incubation, we speculate that the mechanism of Al toxicity is subject to longer metal
301 exposure. *E. coli* was found to comply to the same trend based on the concentrations needed to reach
302 the MBBC and MBEC **(Table 2)**, again, a reflection into the requirement of prolonged metal
303 exposure for the efficacy of some metals [25].

304 Contrary to what was observed for Ag and Al, the biofilms of each indicator strain were found
305 to be less susceptible to Ti when compared to the planktonic cells **(Fig. 1)**. This was particularly
306 evident for *S. aureus*, in which there was a 4-fold increase in the concentration of Ti needed to
307 prevent the formation of a biofilm when compared to the concentration needed to eliminate the
308 planktonic cells **(Table 3)**.

309 The MBBC was reached upon the addition of Zn in *E. coli* and *S. aureus* in the concentrations
310 tested **(Fig. 1)**. For both strains the MBBC were found to be comparable to work completed in other
311 studies, in which biofilm growth was found to decrease by at least 50% upon exposure to $ZnSO_4$ [38].
312 *P. aeruginosa* was found to be tolerant to this metal salt within the concentrations tested since no
313 change in the growth of planktonic cells and biofilms were observed after 4hr and 24hr treatments
314 **(Fig 1 and 3).** Upon longer metal exposure *E. coli* and *S. aureus* biofilms were eradicated, again,
315 giving insight into the time-dependence of metal toxicity **(Fig. 3)**.

316 Ni, similar to Zn, was also observed to be less effective against all three strains. In *P.*
317 *aeruginosa* and *E. coli* no change in viable cell numbers were found upon Ni exposure. This metal
318 was only capable of preventing the assembly of a biofilm in *S. aureus* **(Fig. 1b)**. The results suggest
319 that a concentration well above 650mM may be needed to reach the MPBC for all three strains, the
320 MBBC for *P. aeruginosa* and *E. coli*, and the MBEC for *P. aeruginosa* in the conditions tested. Still
321 this would be problematic as at these concentrations the metal salts precipitate. Nonetheless, this does
322 not preclude the use of Ni and Zn as surface contact antimicrobials for certain infectious settings [9].
323 The literature suggests a variety of mechanisms responsible for metal toxicity, and it is likely
324 that each metal has different cellular targets and resultant toxicological effects [9]. Here, we observed
325 that a comparison between the seven metals gave rise to remarkably different efficacies vs three
326 bacterial species. Additionally, comparing the susceptibilities of the three strains to a even a single
327 metal revealed pronounced differences. Upon further analysis, we revealed that the planktonic and
328 biofilm cells of *P. aeruginosa* appeared to behave similarly with a 4hr metal exposure **(Fig 3a)**. This
329 trend was not observed for *E. coli* and *S. aureus*, in which the concentrations capable of inhibiting
330 growth were different between planktonic cells or those residing within a biofilm. The planktonic
331 cells of the Gram-negative strains demonstrated similar MPBCs fto Ti, Ag and Ni, however the
332 biofilms did not share these similarities **(Fig. 5a)**. Furthermore, differences were found in biofilm
333 susceptibility of *S. aureus* and *E. coli*, revealing the greatest degree of dissimilarity between the
334 MBBCs within the experimental conditions used in this study. Finally, upon biofilm establishment
335 followed by 24hr metal exposure, the biofilms of *S. aureus* and *E. coli* had similar MBECS,
336 particularly following Al, Cu, Zn and Ni addition **(Fig. 5b)**.
337
338

Conclusions

Based on the MPBC, MBBC and MBEC data generated in this study, Cu, Ti and Al were the most effective metals for preventing the formation of, and eradication *P. aeruginosa* biofilms. Meanwhile, Cu, Ti and Ga were the most efficacious metals against *S. aureus* and *E. coli* biofilms. From our observations in this study, Cu, Ti, and Ga were found to have extended activity against planktonic cell growth, the attachment of biofilms and biofilm proliferation. This leads us to conclude that Cu and Ti are the only metals that have reasonable broad-spectrum efficacy against the strains used in this study. However, an overarching theme of this study is that no metal should be considered a '*silver bullet'*. The study of metal resistance genes during the 1990's has revealed that specific resistance mechanisms exist for almost all metals studied to date [39]. With the ever-increasing use of metal ion formulations and nanoparticles as antimicrobials, we must heed to the history of the evolution of antibiotic resistance and aim for more responsible use of antimicrobial metals – a situational approach of the appropriate metal, at the appropriate concentration for a given infectious setting.

List of Abbreviations

CBD – Calgary Biofilm Device/MBEC™

MBBC – Minimal biofilm bactericidal concentration

MPBC – minimal planktonic bactericidal concentration

MBEC – Minimal biofilm eradication concentration

CSWM – Chemically simulated wound media

UN – Universal neutralizer

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

None.

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Authors' contributions

NG designed experimental methodology, conducted experiments, analysed the data and wrote the manuscript. JL designed experimental methodology, analysed data and contributed in writing the manuscript. RT, the corresponding author, contributed in writing the manuscript and provided additional research funding. All authors have read and approve the manuscript.

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Figure 1. The efficacies of different metals for preventing the growth of planktonic and biofilm bacterial populations. A) MPBCs and **B)** MBBCs of *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) in the presence of $AgNO_3$, $CuSO_4$, $TiCl_3$, $Ga(NO_3)_3 \bullet H_2O$, $NiSO_4 \bullet 6H_2O$, $Al_2(SO_4)_3 \bullet H_2O$ or $ZnSO_4 \bullet 7H_2O$. The bacteria were grown over a concentration range defined by 2-fold serial dilutions of each metal; viable cells were counted to determine the MPBC and MBBCs. Values are represented as the mean ± the SD n=3. #Note: all metal stock solutions were prepared at equal molar equivalents of metal molecule. Hence the concentrations found in this figure are reflective of the concentrations of metal and not the compounds themselves. Only the metal salts that were capable of preventing growth in the concentrations tested are shown.

Table 1. Metal concentrations required to prevent planktonic growth (MPBC), prevent biofilm growth (MBBC) and eradicate established biofilms (MBEC) in *P. aeruginosa* (ATCC 27853).*

Metal salt	MPBC (mmol L^{-1})†	MBBC (mmol L^{-1})†	MBEC (mmol L^{-1})‡
$AgNO_3$	>0.50	6.25×10^{-2}	1.56
$CuSO_4$	6.25	1.56	7.81
$TiCl_3$	1.95	1.95	0.98
$Ga(NO_3)_3 \bullet H_2O$	15.63	15.63	7.81
$Al_2(SO_4)_3 \bullet H_2O$	1.95	9.77×10^{-1}	7.81
$ZnSO_4 \bullet 7H_2O$	> 375	>375	> 250
$NiSO_4$	> 625	> 625	> 625

* Values represented as the median of n=3.
† Growth in the presence of metal salt for 4hr incubation.
‡ Establishment of biofilms for 24hr followed by growth in the presence of metal salt for 24hr.

Table 2. Metal concentrations required to prevent planktonic growth (MPBC), prevent biofilm growth (MBBC) and eradicate established biofilms (MBEC) in *E. coli* (ATCC 25922).[*]

Metal salt	MPBC (mmol L^{-1})[†]	MBBC (mmol L^{-1})[†]	MBEC (mmol L^{-1})[‡]
$AgNO_3$	> 10	1.56×10^{-1}	3.90×10^{-2}
$CuSO_4$	12.50	3.13	3.125
$TiCl_3$	1.95	9.77×10^{-1}	1.22
$Ga(NO_3)_3 \bullet H_2O$	31.25	31.25	7.81
$Al_2(SO_4)_3 \bullet H_2O$	250	125	4.88×10^{-1}
$ZnSO_4 \bullet 7H_2O$	> 650	23.44	2.93
$NiSO_4$	> 625	> 625	9.77×10^{-1}

[*] Values represented as the median of n=3.
[†] Growth in the presence of metal salt for 4hr incubation.
[‡] Establishment of biofilms for 24hr followed by growth in the presence of metal salt for 24hr.

Table 3. Metal concentrations required to prevent planktonic growth (MPBC), prevent biofilm growth (MBBC) and eradicate established biofilms (MBEC) in *S. aureus* (ATCC 25923).[*]

Metal salt	MPBC (mmol L^{-1})[†]	MBBC (mmol L^{-1})[†]	MBEC (mmol L^{-1})[‡]
$AgNO_3$	> 125	> 125	10.00
$CuSO_4$	12.50	12.50	3.13
$TiCl_3$	1.95	7.81	1.46
$Ga(NO_3)_3 \bullet H_2O$	15.63	7.81	15.63
$Al_2(SO_4)_3 \bullet H_2O$	> 250	> 250	9.77×10^{-1}
$ZnSO_4 \bullet 7H_2O$	23.44	1.46	2.20
$NiSO_4$	> 625	1.22	1.22

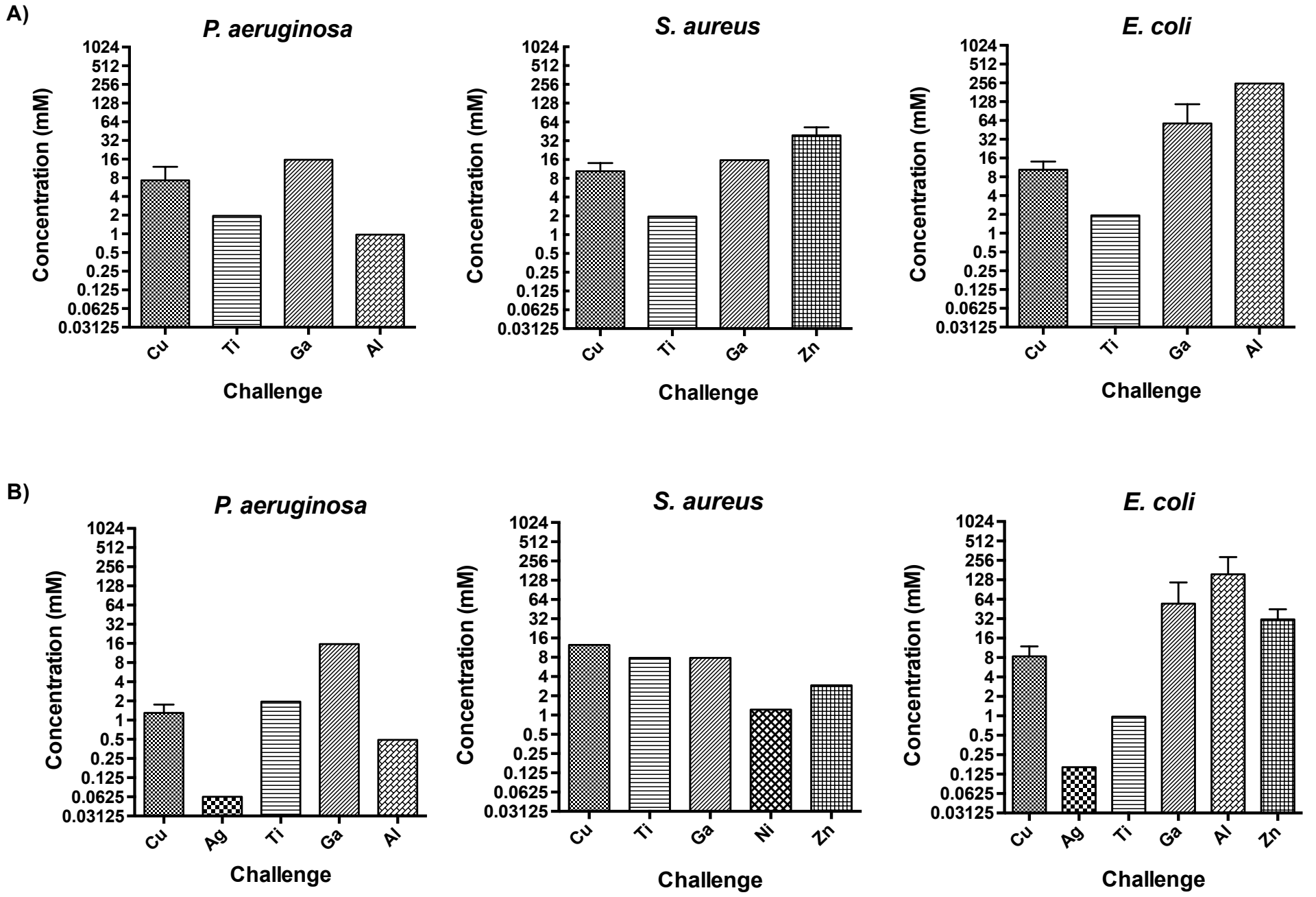
[*] Values represented as the median of n=3.
[†] Growth in the presence of metal salt for 4hr incubation.
[‡] Establishment of biofilms for 24hr followed by growth in the presence of metal salt for 24hr.

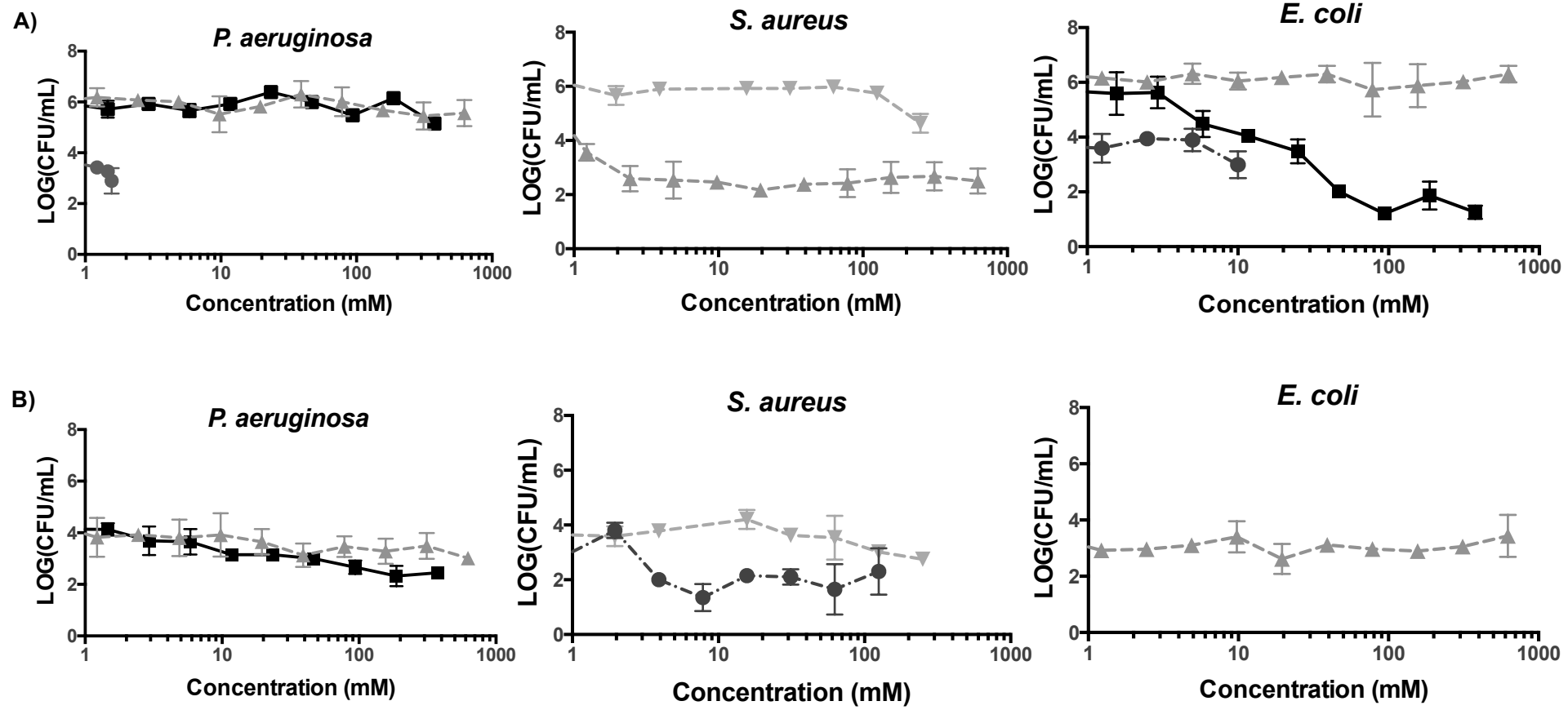
Figure 2. Growth tolerance of *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) to several metals. Within the concentrations tested, the metals that could not prevent the growth of planktonic cells are shown in **A)**, and those incapable of preventing biofilm growth are shown in **B)**. The CBD was inoculated with the bacteria in the presence of $AgNO_3$ (●), $NiSO_4 \bullet 6H_2O$ (▲), $Al_2(SO_4)_3 \bullet H_2O$ (▼) or $ZnSO_4 \bullet 7H_2O$ (□). The cells were exposed to serial dilutions (2-fold) of each metal for 4hr followed by viable cell counts. Values are represented as the mean ± the SD n=3. #Note: all metal stock solutions were prepared at equal molar equivalents of metal molecule. Hence the concentrations found in this figure are reflective of the concentrations of metal and not the compounds themselves.

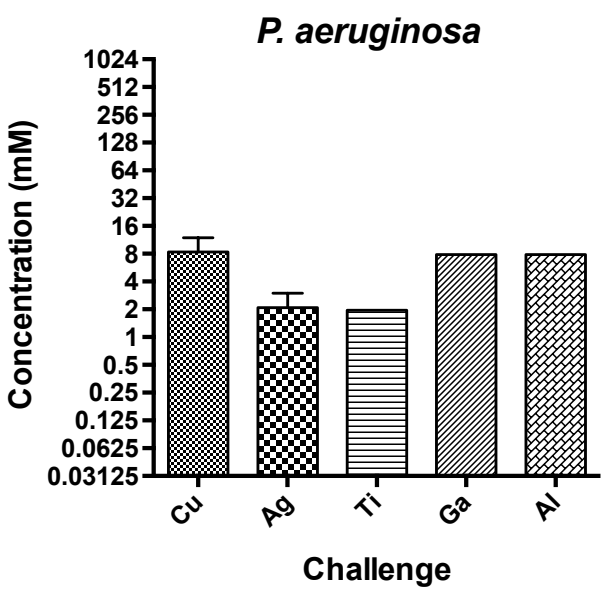
Figure 3. Ability of the metals to eradicate established biofilms. The MBECs of *P. aeruginosa* (ATCC 27853), *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922) in the presence of $AgNO_3$, $CuSO_4$, $TiCl_3$, $Ga(NO_3)_3 \bullet H_2O$, $NiSO_4 \bullet 6H_2O$, $Al_2(SO_4)_3 \bullet H_2O$ or $ZnSO_4 \bullet 7H_2O$. The CBD was inoculated in the absence of the metals salts and grown for 24hr. The established biofilms where then exposed to 2-fold serial dilutions of each metal; viable cells were counted to determine the MBEC. Values are represented as the mean ± the SD n=3. #Note: all metal stock solutions were prepared at equal molar equivalents of metal molecule. Hence the concentrations found in this figure are reflective of the concentrations of metal and not the compounds themselves. Only the metals that were capable of eradicating established biofilms in the concentrations tested are shown.

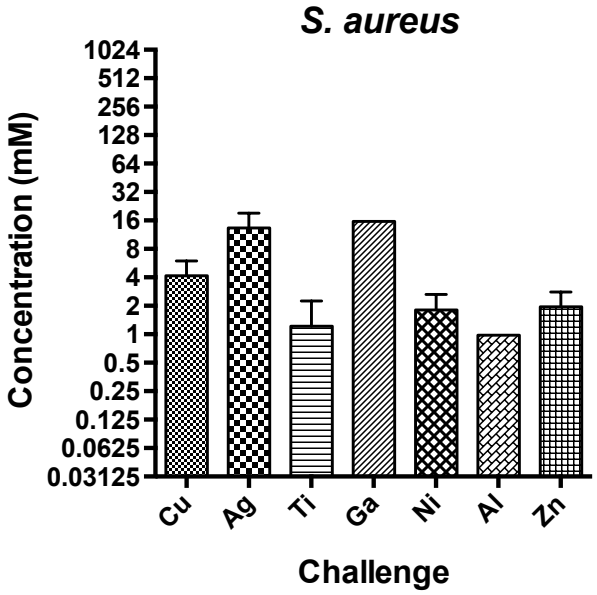
Figure 4. Biofilm eradication tolerance. Efficacy of $NiSO_4 \bullet 6H_2O$ (▲) and $ZnSO_4 \bullet 7H_2O$ (□) against *P. aeruginosa* ATCC 27853. The CBD was inoculated and incubated for 24hr in the absence of the metal challenges. The established biofilm was then treated with serial dilutions (2-fold) of the metal salts. Values are represented as the mean ± the SD, n=3. #Note: all metal stock solutions were prepared at equal molar equivalents of metal molecule. Hence the concentrations found in this figure are reflective of the concentrations of metal and not the compounds themselves.

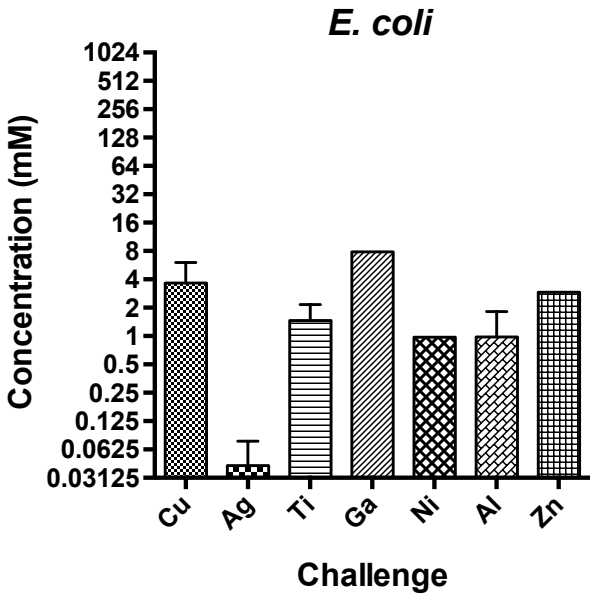
Figure 5: Heatmaps for the MPBC, MBBC and MBEC of the three bacterial strains tested. Analysis generated from the **A)** MPBC (planktonic), MBBC (biofilm) and **B)** MBECs (biofilm), in the presence of $AgNO_3$, $CuSO_4$, $TiCl_3$, $Ga(NO_3)_3 \bullet H_2O$, $NiSO_4 \bullet 6H_2O$, $Al_2(SO_4)_3 \bullet H_2O$ or $ZnSO_4 \bullet 7H_2O$. The metals that could not prevent and/or eradicate growth in the concentrations tested were included in the heatmaps and recorded as the maximum dilution tested. For precise concentrations refer to **Table 1 – 3.**

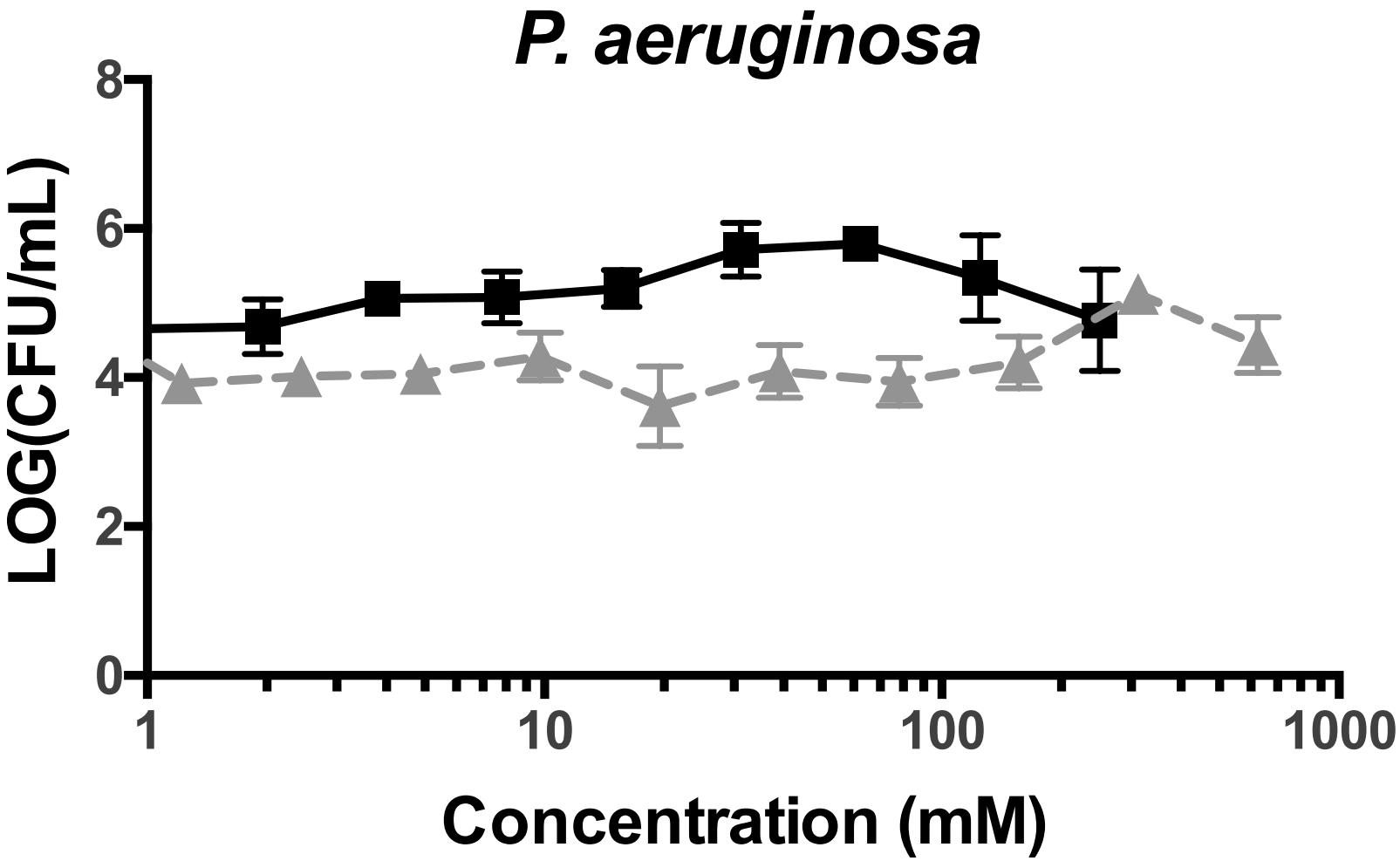

A)
P. aeruginosa
S. aureus
E. coli
Concentration (mM)
1024
512
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
Cu
Ti
Ga
Al
Zn
Challenge
B)
P. aeruginosa
S. aureus
E. coli
Concentration (mM)
Cu
Ag
Ti
Ga
Ni
Al
Zn
Challenge


A)
P. aeruginosa
S. aureus
E. coli
LOG(CFU/mL)
Concentration (mM)
B)
P. aeruginosa
S. aureus
E. coli
LOG(CFU/mL)
Concentration (mM)

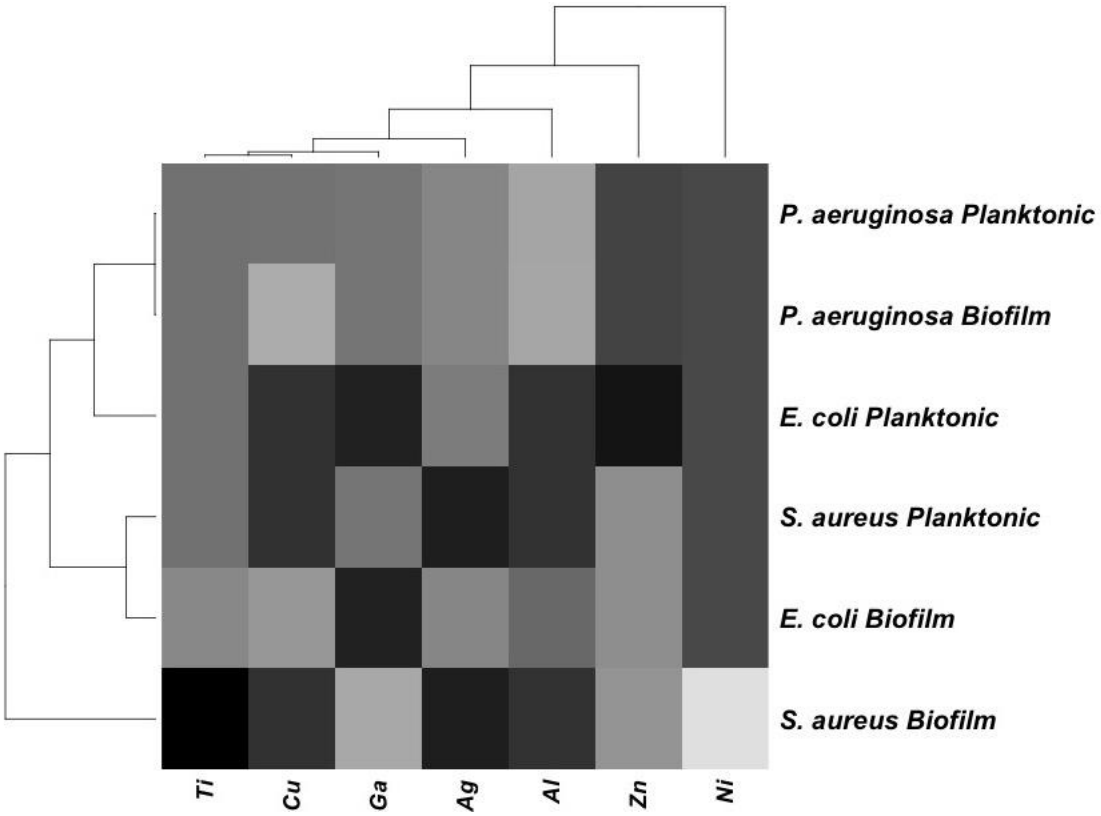

P. aeruginosa
Concentration (mM)
1024
512
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
Cu
Ag
Ti
Ga
Al
Challenge


S. aureus
Concentration (mM)
1024
512
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
Cu
Ag
Ti
Ga
Ni
Al
Zn
Challenge

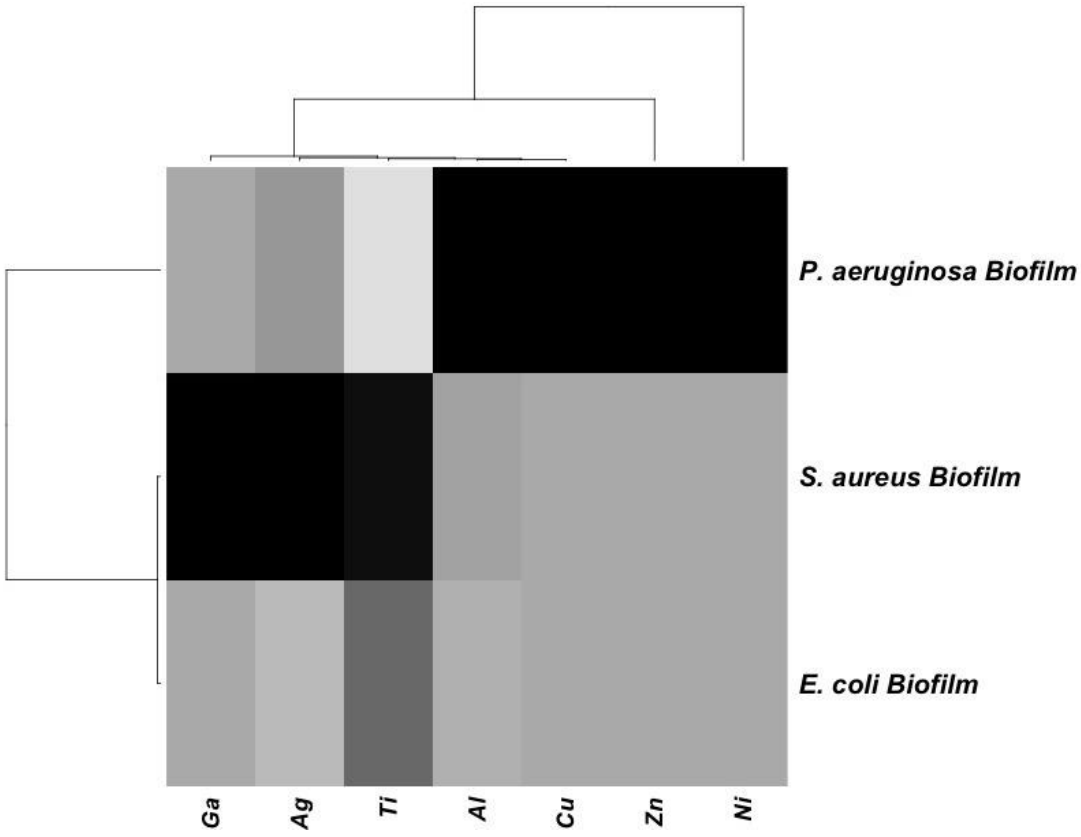

E. coli
Concentration (mM)
1024
512
256
128
64
32
16
8
4
2
1
0.5
0.25
0.125
0.0625
0.03125
Cu
Ag
Ti
Ga
Ni
Al
Zn
Challenge


P. aeruginosa
LOG(CFU/mL)
8
6
4
2
0
1
10
100
1000
Concentration (mM)

A)


P. aeruginosa Planktonic
P. aeruginosa Biofilm
E. coli Planktonic
S. aureus Planktonic
E. coli Biofilm
S. aureus Biofilm
Ti
Cu
Ga
Ag
Al
Zn
Ni

B)


P. aeruginosa Biofilm
S. aureus Biofilm
E. coli Biofilm
Ga
Ag
Ti
Al
Cu
Zn
Ni