Published in final edited form as:
Appl Microbiol Biotechnol. 2011 March ; 89(6): 1963–1970. doi:10.1007/s00253-010-2980-x.

Metallic copper corrosion rates, moisture content, and growth medium influence survival of copper-ion resistant bacteria

Jutta Elguindi[1], **Stuart Moffitt**[2], **Henrik Hasman**[3], **Cassandra Andrade**[1], **Srini Raghavan**[2], and **Christopher Rensing**[1]

[1]Department of Soil, Water, and Environmental Science, University of Arizona, Tucson, AZ 85721

[2]Department of Materials Science and Engineering, University of Arizona, Tucson, AZ 85721

[3]Danish Institute for Food and Veterinary Research, 1790 Copenhagen V, Denmark

Abstract

The rapid killing of various bacteria in contact with metallic copper is thought to be influenced by influx of copper ions into the cells but the exact mechanism is not fully understood. This study showed that the kinetics of contact-killing of copper surfaces depended greatly on the amount of moisture present, copper content of alloys, type of medium used, and type of bacteria. We examined antibiotic- and copper-ion resistant strains of *Escherichia coli* and *Enterococcus faecium* isolated from pig farms following the use of copper sulfate as feed supplement. The results showed rapid killing of both copper-ion resistant *E. coli* and *E. faecium* strains when samples in rich medium were spread in a thin, moist layer on copper alloys with 85% or greater copper content. *E. coli* strains were rapidly killed under dry conditions while *E. faecium* strains were less affected. Electroplated copper surface corrosion rates were determined from electro-chemical polarization tests using the Stern-Geary method and revealed decreased corrosion rates with benzotriazole and thermal oxide coating. Copper-ion resistant *E. coli* and *E. faecium* cells suspended in 0.8% NaCl showed prolonged survival rates on electroplated copper surfaces with benzotriazole coating and thermal oxide coating compared to surfaces without anti-corrosion treatment. Control of surface corrosion affected the level of copper ion influx into bacterial cells which contributed directly to bacterial killing.

Keywords

copper-ion resistant bacteria; metallic copper surface corrosion; corrosion inhibitors; survival assay

Introduction

Copper alloys and their antimicrobial properties have been shown to be effective in killing pathogenic and antibiotic-resistant bacteria (Wilks et al 2005, Noyce et al 2006). Studies demonstrated that pathogens are easily spread throughout the hospital environment on the

*To whom correspondence should be addressed: Christopher Rensing, Department of Soil, Water, and Environmental Science, University of Arizona, Shantz Bldg #38 Rm 429, Tucson, AZ 85721, rensingc@ag.arizona.edu, Telephone (520)626-8482, Fax # (520) 621-1647.

gloves of healthcare workers and that thorough disinfection of surfaces decreases the likelihood for transmission or acquisition of infection (Boyce et al 2007). Current hospital trials have shown that copper alloys may improve the ability to effectively disinfect surfaces (Casey et al 2010, Mikolay et al 2010). However, the exact mechanism through which copper is toxic to the cellular activity of bacteria remains under investigation, in particular the mechanism of killing on metallic copper surfaces. Copper in the ionic form, while toxic in high concentrations, is usually effectively managed in *E. coli* cells by copper homeostasis systems, such as the P-Type ATPase CopA, the CusCFBA system, and the multi-copper oxidase CueO (Rensing and Grass 2003, Baker-Austin et al 2006). *E. coli* strains may also contain plasmid-encoded determinants for additional copper resistance that further enhance their ability to withstand toxic copper concentrations (Lee et al 2002). *Enterococcus faecium* also maintains copper homeostasis through membrane transporter proteins of the P-type ATPase family, which are encoded by the chromosomal operon *cop*YZAB which is similar to *cop*YZAB described in *Enterococcus hirae* (Solioz and Vulpe 1996, Wunderli-Ye and Solioz 1999). More recently transferable copper resistance genes (*tcr*B genes) have been identified as being part of the plasmid-borne *tcr*YAZB operon in *E. faecium* which is similar to the *cop*YZAB operon and possibly mediates co-selection for resistance to macrolides and glycopeptides (Hasman and Aarestrup 2002, Hasman et al 2006). Isolates from animal farms containing the plasmid with *tcr*B showed resistance to copper and various antibiotics as well as resistance to compounds used for disinfection (Hasman and Aarestrup 2002, Aarestrup and Hasman 2004). Some of these genes involved in copper resistance and homeostasis also play a role in survival on copper alloy surfaces under specific conditions. It has been shown that disruption of the regulator in the *cin* operon in *Pseudomonas aeruginosa* results in faster killing rates on copper alloys when compared to the wild-type (Elguindi et al 2009). *Enterococcus hirae* Δ*cop*B and Δ*cop*AB deletion strains suspended in phosphate buffer were killed more rapidly on copper surfaces than was the wild-type, and observed differences in killing rates could be linked to copper ion concentrations in different media used for the incubation on copper alloys (Molteni et al 2010). Recent studies on copper alloys eliminating moisture on the surfaces revealed very rapid killing of *E. coli* cells which was inhibited by reactive oxygen species (ROS) quenchers but enhanced by inactivated superoxide-dismutase, suggesting an increase in ROS resulting from an increase in copper ion concentrations in the cell (Espirito Santo et al 2008).

In this study the survival rates of copper-resistant *E. coli* and *E. faecium* strains were tested on different copper alloys while altering moisture contents during inoculation following EPA recommended testing guidelines. The differences observed under different testing conditions may be related to moisture levels affecting copper surface corrosion and enhanced copper tolerance mechanisms of the strains. The measurement of corrosion rates presents a quantitative analysis of the level of copper ions released from the surface over time and determines how the flux of copper ions relates to bacterial survival. Surface treatments of copper alloys can influence corrosion rates and subsequently survival times. The commonly used organic corrosion inhibitor benzotriazole (BTA) is effective in binding copper ions in a single layer on the metal surface, thereby markedly reducing corrosion (Antonijevic and Petrovic 2008). A thermal oxide layer on a copper-electroplated surface provides a barrier to free copper ions as well and alters corrosion rates. In minimal medium

such as 0.8% NaCl, bacteria would respond directly to the concentration of bio-available copper ions since no sequestration of copper occurs due to other organic molecules in the medium. Copper-resistant *E. coli* and *E. faecium* cells inoculated in 0.8% NaCl on copper-electroplated surfaces showed significant differences in survival rates correlating with corrosion rates measured. The results presented indicate that copper resistance in bacteria does not play a significant role in survival on copper surfaces under the experimental conditions employed in this study. Some conditions only show a significant reduction of viable counts and not a complete die-off after 24 hours of exposure. This is significant regarding bacteria in the healthcare environment since they frequently acquire resistance to antibiotics and disinfectants, and total elimination of bacteria from fomites remains a difficult task in the food and healthcare industries.

Materials and Methods

Bacterial strains and growth conditions

The *E. coli* isolates (77-30009-6, 77-30013-2, 77-30253-2) and *E. faecium* isolates (75-30704-5, 75-30733-4, 75-30518-6) were collected as part of the DANMAP surveillance program (DANMAP 2004, DANMAP 2006) from healthy animals at or just prior to slaughter in 2003 (*E. faecium*) and 2005 (*E. coli*). These strains can be obtained through DTU, the National Food Institute, or by contacting Henrik Hasman (hhas@food.dtu.dk). All isolates had been tested previously for susceptibility to the following antimicrobial agents using a commercial prepared dehydrated panel (SensiTitre): avilamycin, bacitracin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin, quinupristin/dalfopristin, streptomycin, tetracycline, vancomycin, and virginiamycin according to CLSI standard and evaluated using CLSI breakpoints (Clinical and Laboratory Standards Institute 2008).

Cultures of *E. coli* copper-ion resistant strains were prepared in Luria Bertani broth at 37 °C while *E. faecium* copper resistant strains were grown in Lee's liquid medium (10 g tryptone, 10 g yeast extract, 5 g lactose, 5 g sucrose, 0.5 g dipotassium phosphate in 1^{-1} L). Following overnight incubation 100 μl of the cultures were placed in 3 ml of the respective liquid media and grown to mid log phase at OD_{600} 0.5 – 0.7. A 25 μl inoculum of these cultures was pipetted on each coupon to be tested and spread with a sterile glass rod. The samples were spread over the entire surfaces of the copper alloys and kept moist in a closed container (Wilks et al 2005). In order to simulate drop-size contamination the inoculum was placed as a droplet on the coupons and kept moist in a closed container. Additional experiments involved the methods described in EPA recommended protocols obtained from the Copper Development Association requiring the initial drying of the inocula for 30 minutes on copper alloys in open air followed by a timed survival assay (Nada S 2005) and a 24-hour repeat inoculation with intermittent sampling. For 24-hour repeat inoculation experiments a 10 μl inoculum was spread over copper alloy coupons every 3 hours and left drying in open air between applications (Nada S 2005). In order to compare survival rates to measured corrosion rates the bacteria were grown to mid-log phase in culture medium, pelleted at rcf 18,000×g for 2 minutes, and pellets re-suspended in 0.8% NaCl. After defined incubation times at room temperature (25 °C) specific for each of the experiments, the coupons were transferred into 50 ml centrifugation tubes which contained 10 ml sterilized PBS and 20 to

25 sterilized 2 mm diameter glass beads. After thorough mixing on a vortex mixer for 30 to 60 seconds serial dilutions were plated out on LB agar or Lee's agar plates and incubated at 37°C for 24 hours. The experiments were performed in triplicates and the mean and standard deviation calculated. For experimental controls *E. coli* and *E. faecium* copper-ion resistant strains were placed on stainless steel coupons.

MIC determination of copper sulfate by the agar dilution method

Copper-ion resistance was evaluated on Brain Heart Infusion agar plates containing 0, 1, 2, 4, 8, 12, 16, 20, 24 and, 28 mM copper sulfate ($CuSO_4 \cdot 5H_2O$) adjusted to pH = 7. Isolates were inoculated with a single drop (approximately $5x10^5$ cells) of cells picked from a blood agar plate and suspended in a 0.9% NaCl solution adjusted to McFarland = 0.5 according to CLSI standards. The plates were then incubated at 37°C for 48 hours and the growth assessed after 20 and 48 hours. The *tcr*-positive *E. faecium* A17sv1 (Hasman et al 2006) and the pRJ1004-carrying and *pco*-positive *E. coli* ED8739 (Courtesy of Dr. Jon Hobman, School of Biosciences, The University of Nottingham, Loughborough, UK) were used as positive controls. The plasmid-free and copper susceptible *E. faecium* BM4105 ($CuS0_4$ MIC=4 mM) and *E. coli* MT102 ($CuSO_4$ MIC=12 mM) were used as negative controls. In cases where the growth was difficult to evaluate, PCR was used to examine the presence of the *tcr* genes (*E. faecium*) and *pco* genes (*E. coli*) as described previously (Hasman et al 2006). Copper resistant *E. faecium* isolates had a $CuSO_4$ MIC value of 24 mM and copper resistant *E. coli* isolates had a $CuSO_4$ MIC of 20 mM.

Preparation of test surfaces

The different copper alloy coupons employed in this study were a gift from the International Copper Association and their compositions are listed in Table 1. Prior to use the 1 square inch copper alloy and stainless steel coupons were immersed for 30 seconds in a 3% NaOH solution heated to 70°C, rinsed in distilled H_2O, and followed by a 3- 5 seconds immersion in 10 % H_2SO_4 and rinsed with DH_2O. To prevent re-oxidation of the surfaces the coupons were rinsed in 95% ethanol, air-dried, and kept in a sterile closed container until used (Espirito Santo et al 2008). Copper films of thickness ~ 600 nm electroplated on silicon wafers were provided by Freescale Semiconductors Inc.. CuO_X films, 60 nm in thickness, were prepared by thermally oxidizing electroplated copper films at 300°C in a laboratory tube furnace in a high purity air ambient for 5 minutes. X-ray photoelectron spectroscopy results showed that a thin layer of CuO was present at the surface and as the layer was sputtered away, the film almost entirely consisted of Cu_2O. Using an electrochemical reduction process, the oxide film was characterized to be roughly 95% Cu_2O and 5% CuO. Copper-BTA film test surfaces were created by pre-treating electroplated copper samples with an Isopropanol and subsequent distilled H_2O rinse, then immersed for 60 seconds in 0.1M HCl and followed by a distilled H_2O rinse before being air dried. The samples were immersed 15 minutes in a BTA (99% pure, Sigma-Aldrich) and distilled H_2O based solution in concentrations of 0.016M, 0.001M, 0.0004M and 0.0001M for experiments, rinsed with distilled H_2O and air dried. Before testing the copper-electroplated samples were rinsed in 95% ethanol and air-dried.

Surface dissolution of copper and corrosion inhibition

Approximately 280 copper alloys containing more than 60% Cu have been registered with EPA as antimicrobial copper materials (EPA 2008). When exposed to aqueous solutions, depending on the pH and redox potential of the system, copper may dissolve as cupric ions or form oxides, Cu_2O and/or CuO. A potential-pH diagram for Cu-water system at a dissolved copper concentration of 10^{-6} M was constructed using a commercially available software package, STABCAL (Huang, Montana Tech. 2009). This diagram, shown in Figure S1, shows that at acidic pH values and under oxidizing conditions, i.e. more positive potential values, the oxidation of Cu to Cu^{2+} ions is favored. In the near neutral pH regions the formation of cuprous and cupric oxide is thermodynamically favorable if the solution potential is above zero. As a reference, the solution potential of 1 M NaCl is roughly 0.25 V (with respect to the standard hydrogen electrode). Hence, in saline test solutions at near neutral pH, copper surface is likely to be oxidized to copper oxide. At high pH values, depending on copper ion activity, copper oxides may be dissolved to form $HCuO_2^-$ and CuO_2^2 (Online Resource 1, Fig. S1).

Benzotriazole (BTA, $C_6H_5N_3$) is a well known corrosion inhibitor for copper in various applications in a wide range of environments (Cotton and Scholes 1967). The interaction of BTA with copper has been studied extensively. It is generally accepted that BTA (as well as BTA^- ion) chemisorbs on the copper (as well as cuprous oxide) surface and forms an insoluble cuprous surface complex. Under certain conditions, the formation of a thick, multilayered coating has been confirmed. The mechanism for BTA's unique film formed on copper is suggested to be a planar structure of stoichiometry 1:1 Cu:BTA (Cotton and Scholes 1967, Online Resource 1, Figs. 2.1, 2.2). The potential-pH diagram for the Cu-BTA-water system, constructed using the STABCAL software, is shown in Figure S3 for a copper ion concentration of 10^{-6} M and a BTA concentration of 10^{-4} M (Online Resource 1, Fig. S3) It may be noted from this figure that the formation of CuBTA (s) is thermodynamically favorable in the pH range of 3 to 10. Oxidation of CuBTA to Cu^{++} and CuO (s) is thermodynamically favorable under oxidizing (higher solution potential) conditions but it is kinetically very slow.

Results

Moisture content influences survival rates of copper-resistant bacteria

With 25 µl of bacterial culture spread thinly over the surface, copper alloys were kept in a moist, closed compartment, and survival rates of *E. coli* isolates were assessed by countable CFUs remaining. For *E. coli* 77-30009-6 on copper alloys containing >88% copper no countable CFUs were seen after 15 to 30 minutes while on 70 % copper alloys countable CFUS dropped to zero in 60 minutes (Fig. 1A). This method was also most effective in killing *E. faecium* isolates. Countable CFUs for *E. faecium* 75-30704-5 dropped to zero after 30 to 60 minutes. The 70% copper and 30 % zinc alloy was the least effective with only a 1 fold log decrease in countable CFUs after 60 minutes (Fig. 1B). This method has been frequently used to evaluate the efficacy of metallic copper surfaces against a variety of bacteria, but would be difficult to attain in the environment since the inocula dry within 10 to 15 minutes without cover in de-humidified surroundings.

Droplets of a bacterial suspension on copper alloys in a moist environment resulted in markedly prolonged survival rates of *E. coli* and *E. faecium* isolates. *E. coli* 77-30009-6 cells were killed within 120 to 360 minutes dependent on copper content of the alloy (Fig. 1C). *E. faecium* isolate 75-30704-5 showed a decrease of countable CFUs to zero after 360 minutes on 94.8 % copper but survival was still prolonged on the remaining alloys (Fig. 1D). However, *E. faecium* isolates 75-30733-4 and 75-30518 only showed a maximum of 1 fold decrease in countable CFUs after 480 minutes (data not shown). When bacteria were inoculated with increased moisture levels and a smaller area of exposure on the metal the kinetics of copper ions in rich medium were altered and significant differences in survival rates between individual strains were seen, signifying the strains' distinctive abilities to withstand copper-ion dependent toxicity.

The method of spreading the inocula of *E. coli* 77-30009-6 over the copper surfaces and letting it air dry for 30 minutes prior to sampling times left zero countable CFUs on all copper alloys (Fig. 1E). Countable CFUs on stainless steel controls were decreased by 0.5 to 1 log fold after 60 minutes. With this method a prolonged drying period was achieved, and recovery of all *E. coli* copper-ion resistant strains from all copper alloys tested showed no remaining countable CFUs, except on stainless steel (data not shown). *E. faecium* survival rates utilizing this testing method revealed that countable CFUs for 75-30704-4 were decreased by 3 log fold after 60 minutes. Differences between the alloys were not well-defined under drying conditions. There was no decrease in CFU count on stainless steel which indicated that the drying process itself did not have a negative effect on *E. faecium* (Fig. 1F).

A suspension of *E. coli* spread over copper surfaces and air dried between 8 successive inoculations revealed no countable CFUs on all alloys tested over a 1-24 hour period. However, on stainless steel controls countable CFUs increased over the 24 hour period (Fig. 2A). *E. faecium* incubation on 4 different copper alloys resulted in an overall decrease of countable CFUs ranging from 2 to 3 log fold over a 24 hour period, while stainless steel surfaces showed an increase in countable CFUs (Fig. 2B).

Accelerated killing of *E. faecium* 75-30704-5 was observed when incubated in 0.8% NaCl on 99.9% copper alloys. No countable CFUs remained after 30 min exposure in 0.8% NaCl (Fig. 3B). Therefore, a quantitative measurement of surface corrosion of copper materials was conducted in order to provide an estimate of the rate by which dissolved copper is released from the surface and how bacterial survival is influenced by that rate.

Corrosion rates influence survival of copper-resistant bacteria on copper

Electro-chemical measurements and calculation of corrosion rates of electroplated copper samples revealed that the corrosion inhibitor BTA at a concentration of 16 mM decreased the corrosion rate of electroplated surfaces by ~65%. In contrast, the 60 nm thermal oxide layer on electroplated surfaces decreased the corrosion rate by only 30% (Table 2). Bacterial survival rates on these surfaces also showed marked differences. *E. coli* 77-30009-6 cells in 0.8% NaCl on electroplated copper surfaces were killed within 60 min of exposure, but showed no decrease in countable CFUs after 60 min of exposure on BTA coated electroplated copper (Fig. 3A). Thermal oxide coating also prolonged survival and showed a

3 log-fold decrease of countable CFUs after 60 minutes Similarly, *E. faecium* cells in 0.8% NaCl were killed within 45 minutes on electroplated copper surfaces with and without thermal oxide coating, and countable CFUs were decreased 3 log fold after 60 minutes on BTA coated surfaces (Fig. 3B). However, bulk 99.9% copper surfaces were most effective in killing both *E. coli* and *E. faecium* strains suspended in 0.8% NaCl (Figs. 3A and 3B). *E. coli* cells were inoculated on electroplated copper surfaces bearing BTA concentrations ranging from 0.1 mM to 16 mM. After 30 minutes of exposure no die-off was seen at concentrations of ≥1 mM (Fig. 5). Exposure of copper surface to 0.016 M BTA solution resulted in a decreased level of copper release and rate of bacterial kill. Survival rates of *E. coli* were dependent on the BTA concentration in the solution in which copper surfaces were treated. (Table 2, Online Resource 1, Fig. S4). Copper ion concentrations on dry surfaces were not determined.

Images of *E. coli* cells were obtained utilizing an Environmental Scanning Electron Microscope and revealed no grossly damaged cell membranes after placing the cells on dry electroplated copper surfaces from 30 to 60 minutes. The most notable difference was cell shape which appeared rounded with live cells on stainless steel vs. the characteristic oblong shape of dead cells on copper (Online Resource 1, Figs S5.1 and S5.2).

Discussion

The findings of this study support the mounting evidence that the release and influx of bio-available copper ions from copper surfaces into bacterial cells is the driving force in the killing mechanism on metallic copper. Corrosion inhibitors such as BTA altered the concentration of copper ions (Cu^{+}/Cu^{2+}) released from copper surfaces, which directly affected bacterial survival in 0.8% NaCl medium. However, the exact concentration of copper needed to kill a particular microorganism would depend on other factors, such as moisture content, copper content of alloy, medium present, copper homeostasis mechanisms, and the membrane structure of e.g. Gram-negative or Gram-positive organisms. Altered moisture levels on copper surfaces resulted in marked differences in survival rates related to the copper content of the alloys. The observed faster killing of *E. coli* copper-resistant strains in dry environments suggests different interactions of Gram-negative bacteria with copper ions released from the metallic surfaces than those of Gram-positive bacteria. It has been reported that Gram-positives survive longer on dry copper surfaces (Espirito Santo et. al 2010), shown also with *E. faecium* in this study (Figs. 1F and 2B). However, in 0.8% NaCl medium the cells died off quickly correlating with different corrosion rates (Fig. 3B). The higher copper tolerance in a dry environment may be due to copper ions bound to components of rich medium, and exchange with the thick peptidoglycan layer could form a buffer to copper ion influx. Neutral copper complexes such as $Tris_2$-Cu or Cl_2-Cu may not exchange copper ions with other molecules and, as suggested by Molteni et al (2010), may actually facilitate cross-membrane transport of copper ions. Gram-negative *E. coli* may have an advantage when exposed to slow corrosion rates due to their periplasmic copper export systems which Gram-positives are lacking. At low corrosion rates through BTA coating, i.e. 4.9 μM in 1 minute, *E. coli* survival was not reduced after 60 minutes of exposure suggesting that existing copper homeostasis mechanisms were adequate at this rate. A 100% increase in corrosion rates, i.e. 10.2 μM in 1 minute through thermal oxide coating, reduced

survival rates 3 log fold after 60 minutes. However, these mechanisms offered no protection under dry conditions and most likely allowed a very rapid and massive influx of copper ions into the cells resulting in almost immediate cell death. Copper ion concentrations on a dry surface could not be determined with the method used in this study. It is presumed that dry copper also ionizes at the surface, and when making contact, cells can rapidly take up copper ions but cannot effectively transport them out of the cell possibly due to the lack of extracellular fluid. The rapid accumulation of copper in the cytoplasm was recently demonstrated with *E. coli* during 1 minute contact on dry copper surfaces suggesting cell membrane compromise as the underlying mechanism (Espirito Santo et al, personal communication). Bacterial contaminations in the food and healthcare environment can vary greatly in concentrations, exposure times, media, and moisture levels. The bacterial strains in this study were rapidly killed on moist copper surfaces regardless of their protective copper homeostasis mechanisms. Therefore, it is imperative for copper surfaces in the food and healthcare environments to maintain higher corrosion rates without application of corrosion inhibitors in order to achieve maximum bactericidal effects. Moreover, oxidized copper surfaces maintain sufficient corrosion rates and are effective for contact killing but may appear unclean when compared to stainless steel surfaces. It has also been shown that Tris-Cl medium may contribute to increased copper corrosion and consequently to more effective killing of *E. hirae* on copper surfaces (Molteni et al 2010). Therefore, copper alloys which have more durability and anti-corrosion properties may not perform well as bactericidal surfaces, as suggested previously (Wilks et al 2005). More studies will be needed to estimate corrosion rates in order to identify which copper alloys could provide the highest corrosion rates under most environmental conditions ensuring the maximum effectiveness of the anti-microbial copper materials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This research was supported by NIH grant GM079192 and a grant from the International Copper Association (ICA). Cassandra Andrade was supported by a MARC undergraduate fellowship from NIH grant T34GM008718. Copper alloy and stainless steel coupons were supplied by the International Copper Association.

References

Aarestrup FM, Hasman H. Susceptibility of different bacterial species isolated from food animals to copper sulphate, zinc chloride, and antimicrobial substances used for disinfection. Vet. Microbiol. 2004; 100(1–2):83–89. [PubMed: 15135516]

Antonijevic MM, Petrovic MB. Copper corrosion inhibitors: A review. Int J Electrochem. Sci. 2008; 3:1–28.

Baker-Austin C, Wright MS, Stepanauskas R, McArthur JV. Co-selection of antibiotic and metal resistance. Trends in Microbiology. 2006; 14(4):176–182. [PubMed: 16537105]

Boyce JM. Environmental contamination makes an important contribution to hospital infection. J.Hosp. Infect. 2007; 65:50–54. [PubMed: 17540242]

Casey A, Adams D, Karpanen TJ, Lambert PA, Cookson BD, Nightingale P, Miruszenko L, Shillam R, Christian P, Elliot TSJ. Role of copper in reducing hospital environment contamination. J. Hosp. Inf. 2010; 74:72–77.

Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing; 8th informational supplement. Wayne, Pa.: Clinical Laboratory Institute; 2008.

Cotton J, Scholes I. Benzotriazole and related compounds as corrosion inhibitors for copper. Brit. Corr. J. 1967; 2:5.

DANMAP. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. 2004 2005.

DANMAP. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. 2006 2007.

Elguindi J, Wagner J, Rensing C. Genes involved in copper resistance influence survival of *Pseudomonas aeruginosa* on copper surfaces. J. Appl. Microbiol. 2009; 106:1448–1455. [PubMed: 19239551]

EPA. [cited 2010 September 19] EPA registers copper-containing alloy products. 2008 May. 2008 Available from: http://www.epa.gov/pesticides/factsheets/copper-alloy-products.htm.

Espirito Santo CE, Taudte N, Nies DH, Grass G. Contribution of copper ion resistance to survival of Escherichia coli on metallic copper surfaces. Appl. Environ. Microbiol. 2008; 74(4):977–986. [PubMed: 18156321]

Espirito Santo CE, Morais PV, Grass G. Isolation and characterization of bacteria resistant to metallic copper surfaces. Appl. Environ. Microbiol. 2010; 76:1341–1348. [PubMed: 20048058]

Hasman H, Aarestrup FM. *Tcr*B, a gene conferring transferable copper resistance in *Enterococcus faecium:* occurrence, transferability, and linkage to macrolide and glycopeptide resistance. Antimicrob. Agents Chemother. 2002; 46(5):1410–1416. [PubMed: 11959576]

Hasman H, Kempf I, Chidaine B, Cariolet R, Ersboll AK, Houe H, Bruun Hansen HC, Aarestrup FM. Copper resistance in *Enterococcus faecium*, mediated by the *tcr*B gene, is selected by supplementation of pig feed with copper sulfate. Appl. Environ. Microbiol. 2006; 72(9):5784–5789. [PubMed: 16957194]

Lee SM, Grass G, Rensing C, Barrett SR, Yates CJ, Stoyanov JV, Brown NL. The *pco* proteins are involved in periplasmic copper handling in *Escherichia coli*. Biochem. Biophys. Res. Commun. 2002; 295(3):616–620. [PubMed: 12099683]

Mikolay A, Huggett S, Tikana L, Grass G, Braun J, Nies DH. Survival of bacteria on metallic copper surfaces in a hospital trial. Appl. Microbiol. Biotechnol. 2010

Molteni C, Abicht HK, Solioz M. Killing of bacteria by copper surfaces involves dissolved copper. Appl. Environ. Microbiol. 2010; 76(12):4099–4101. [PubMed: 20418419]

Nada S. Test method for efficacy of copper alloy surfaces as a sanitizer. ATS Labs, Protocol # CSC02032905.CUST.1. 2005

Nada S. Test method for the continuous reduction of bacterial contamination on copper alloy surfaces. ATS Labs, Protocol #CSC02032905.CUST.3. 2005

Noyce JO, Michels H, Keevil CW. Potential use of copper surfaces to reduce survival of epidemic methicillin-resistant Staphylococcus aureus in the healthcare environment. Hosp. Infect. Society. 2006; 63:289–297.

Rensing C, Grass G. *Escherichia coli* mechanisms of copper homeostasis in a changing environment. FEMS Microbiol. Rev. 2003; 27:197–213. [PubMed: 12829268]

Solioz M, Vulpe C. CPx-type ATPases: a class of P-type ATPases that pump heavy metals. Trends Biochem. Science. 1996; 21:237–241.

Wilks SA, Michels H, Keevil CW. The survival of *Escherichia coli* O157 on a range of metal surfaces. IntJFood Microbiol. 2005; 105:445–454.

Wunderli-Ye H, Solioz M. Copper homeostasis in *Enterococcus hirae*. Adv. Exp. Med. Biol. 1999; 448:255–264. [PubMed: 10079833]

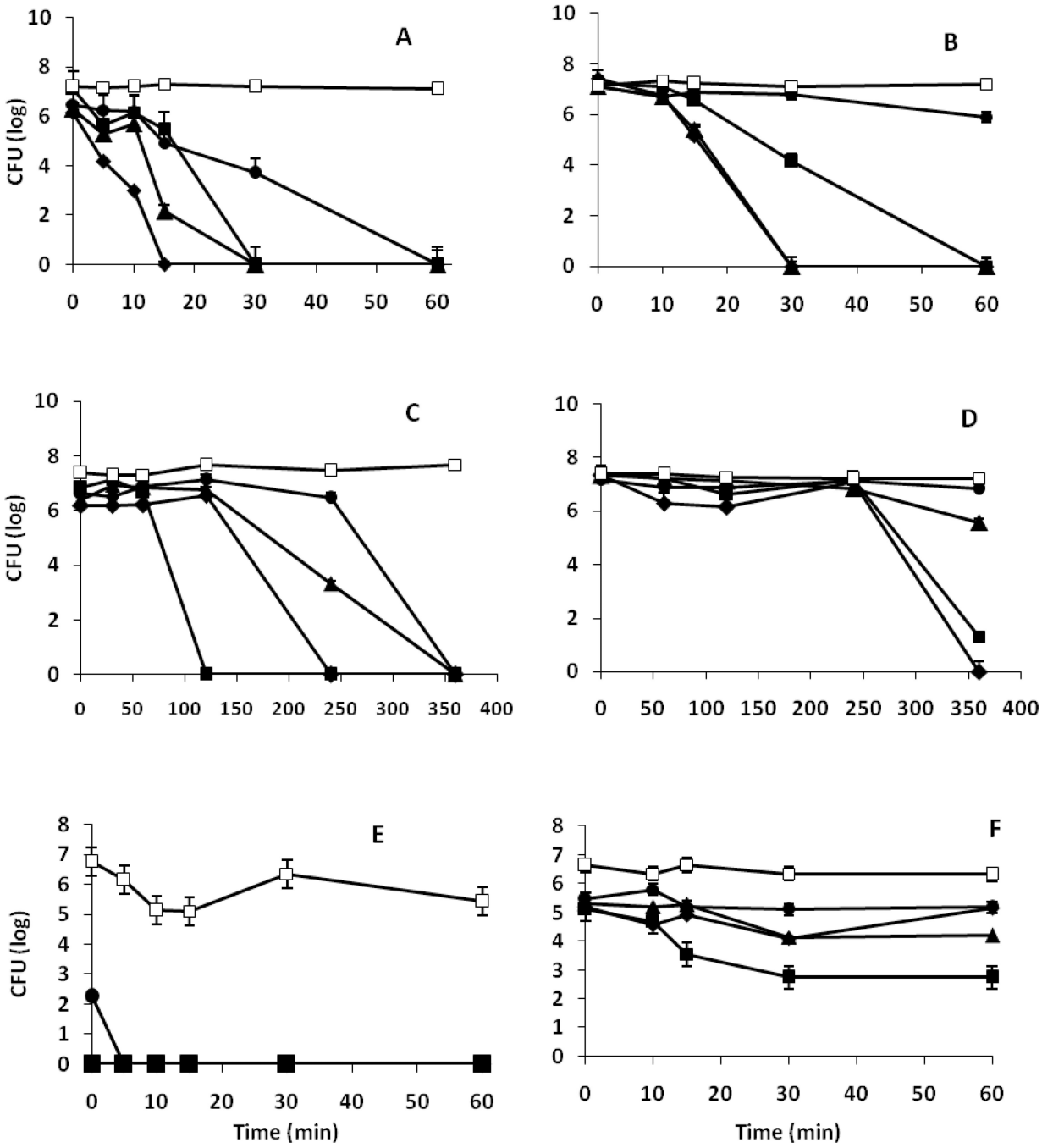


Figure 1. Survival rates of copper-ion resistant bacteria on copper alloys

Bacterial suspension of *E. coli* (A) and *E. faecium* (B) spread over copper alloys and kept in moist environment; *E. coli* (C) and *E. faecium* (D) applied as droplets on copper alloys and kept in moist environment; *E. coli* (E) and *E. faecium* (F) spread over copper alloys and dried 30 minutes before timing in dry environment. 99.9% copper (■), 94.8% copper (◆), 88.6% copper (▲), 70% copper (●), stainless steel control (□).

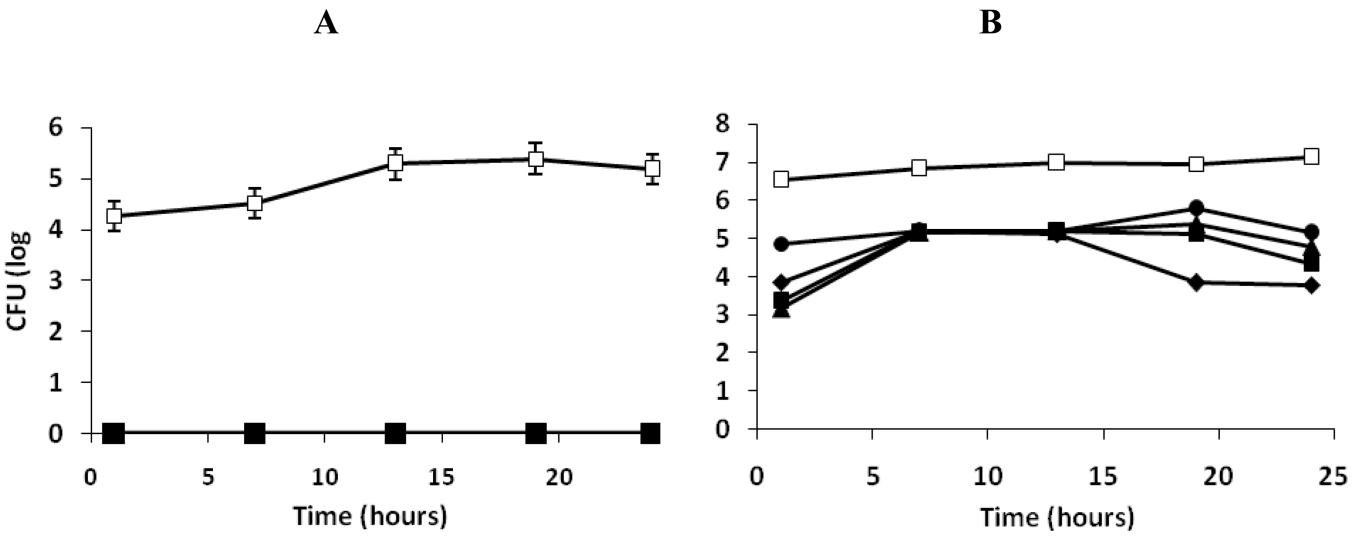
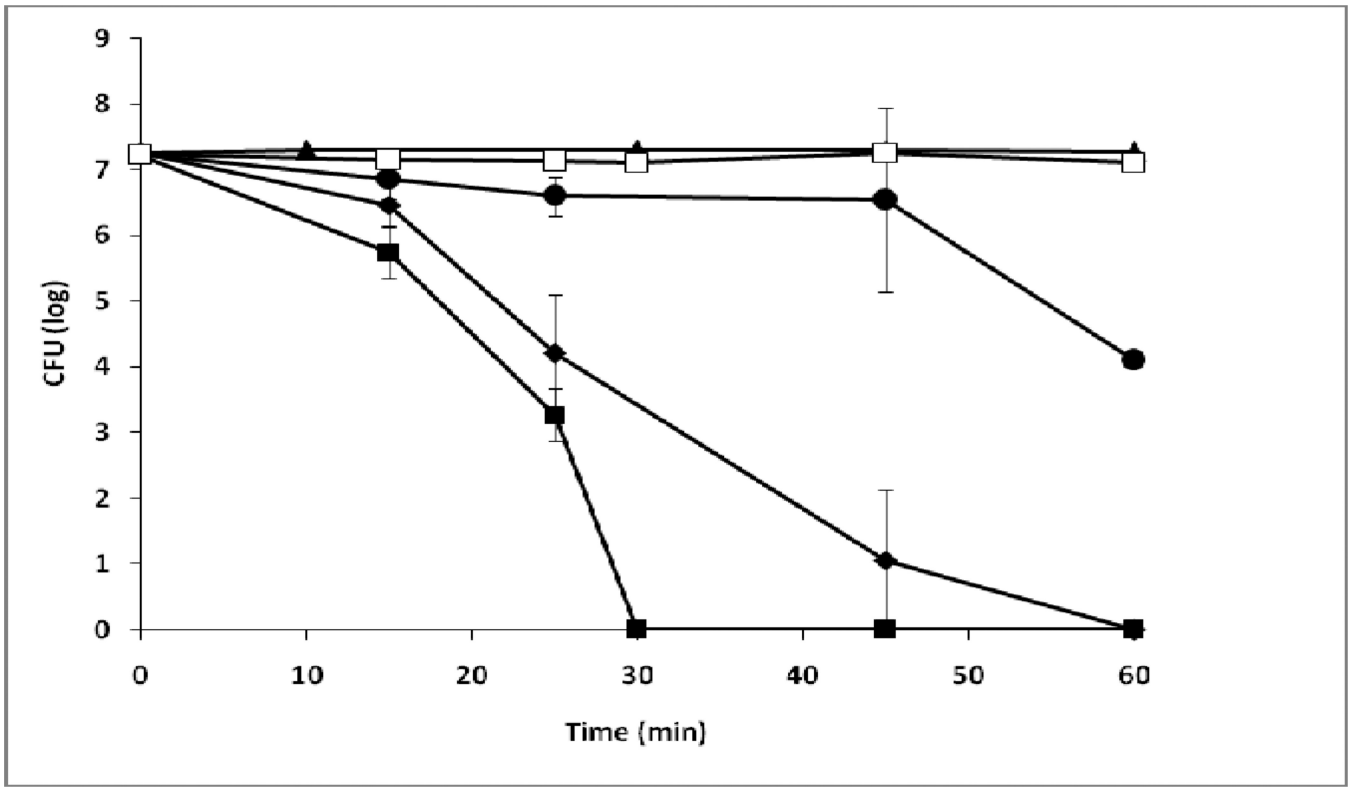


Figure 2. Survival of copper-ion resistant bacteria on copper alloys with multiple inoculation

E. coli (A) and *E. faecium* (B) suspensions were re-applied on copper alloys in 3 hour intervals without removal for a 24 hour period. Each 10 μl sample was spread over the surface and air-dried between applications. 99.9% copper (■), 94.8% copper (◆), 88.6% copper (▲), 70% copper (●), stainless steel control (□).



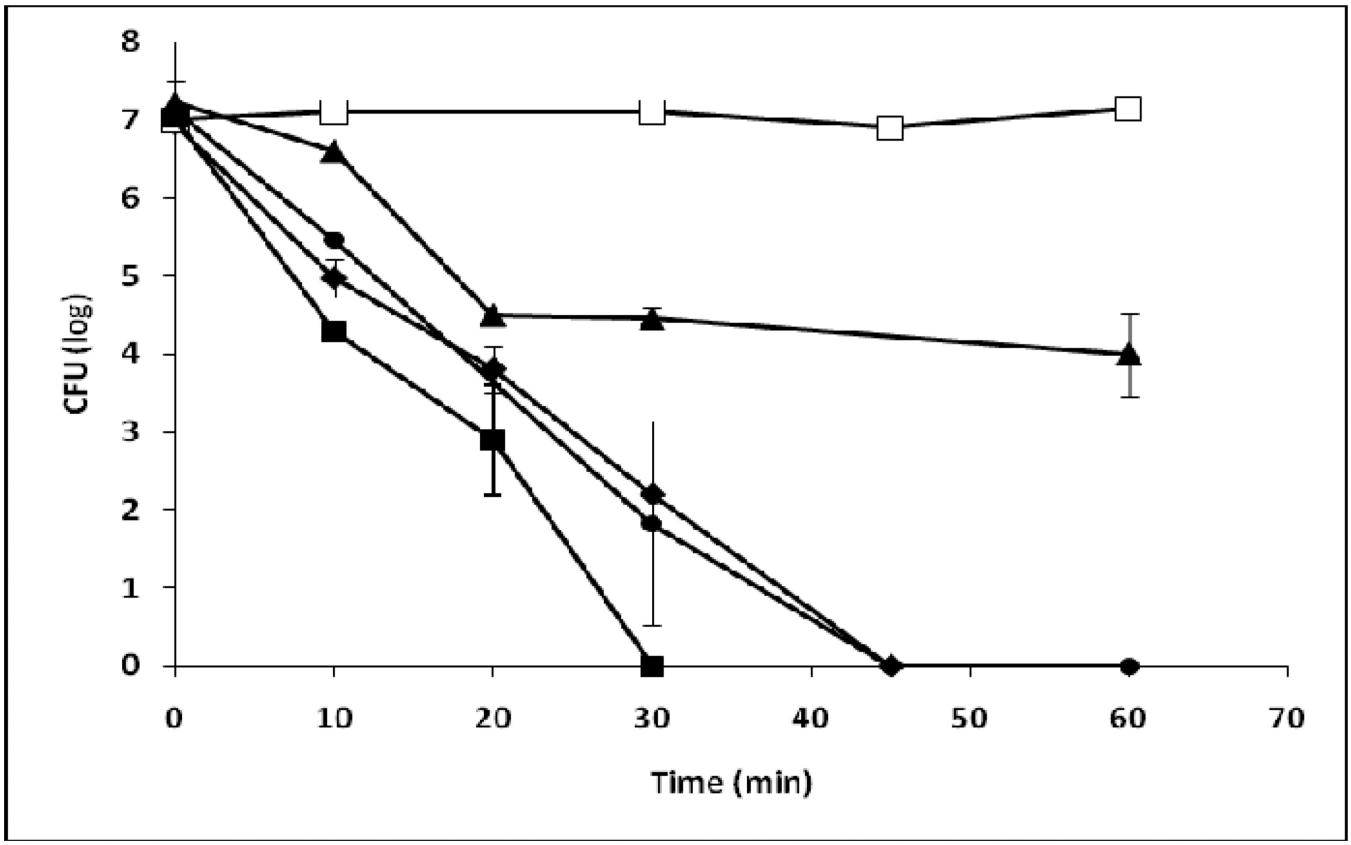


Figure 3.

Figure 3A. Survival of copper-ion resistant *E. coli* 77-30009-6 influenced by corrosion of copper surfaces. Solid metallic copper (■), electroplated copper (◆), electroplated copper with thermal oxide coating (●), electroplated copper with BTA 0.016 M (▲), stainless steel control (□).

Figure 4B. Survival of copper-ion resistant *E. faecium* 75-30704-5 influenced by corrosion of copper surfaces. Solid metallic copper (■), electroplated copper (◆), electroplated copper with thermal oxide coating (●), electroplated copper with BTA 0.016 M (▲), stainless steel control (□).

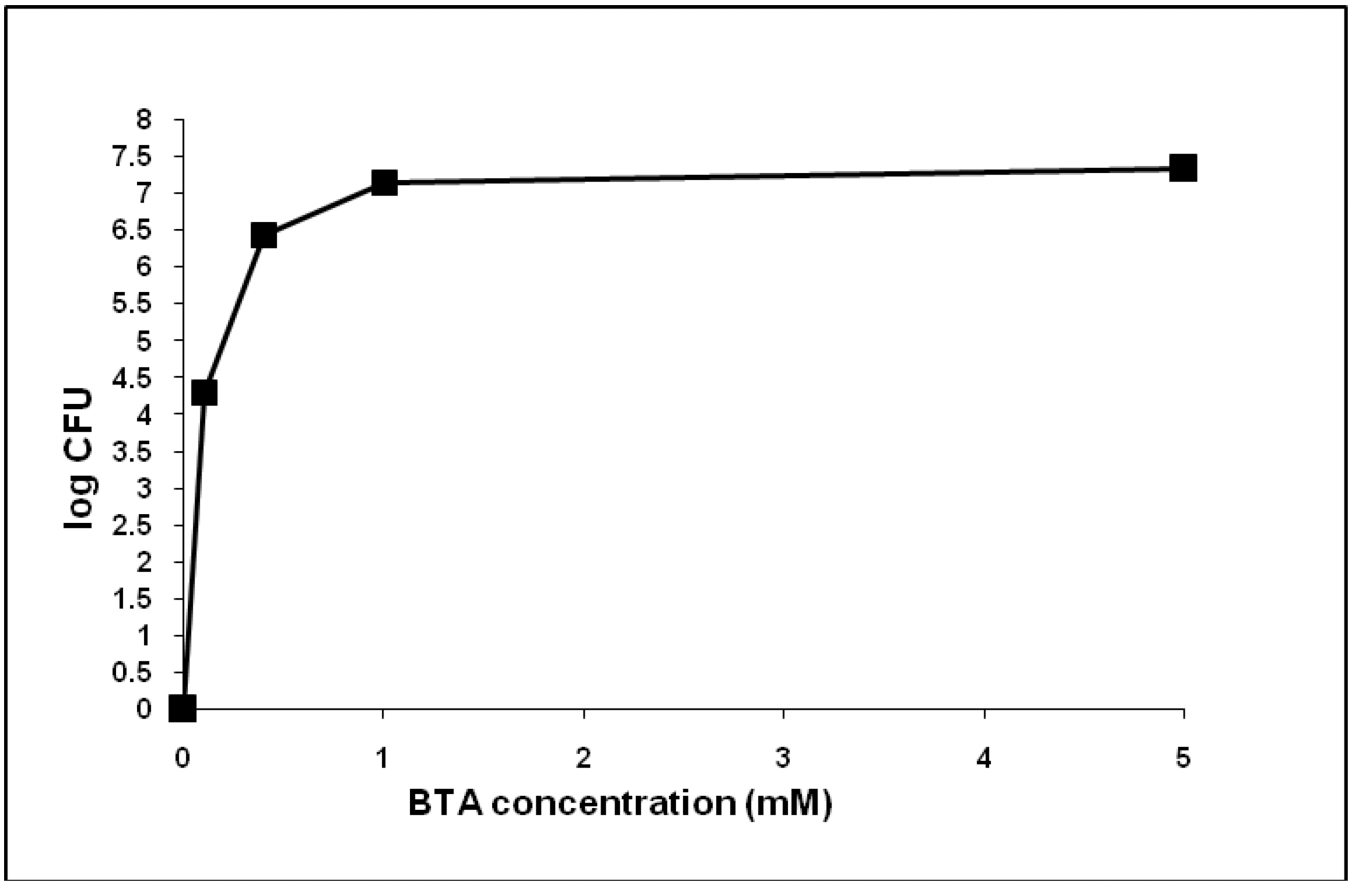


Figure 5. Survival of *E. coli* 77-30009-6 at 30 minutes on BTA coated electroplated copper surfaces

Table 1

Copper alloys and their constituent compositions in percent values

ALLOY	Cu	Zn	Sn	Ni	Fe	P
Cu 11000	99.9					
Cu 51000 (Bronze)	94.8		5.0			0.2
Cu-Ni 70600	88.6			10.0	1.4	
Cu-Zn 26000	70	30				
Stainless Steel	0			8	74	18

Table 2

Electrochemically determined copper ion release in one minute from untreated and treated electroplated copper surfaces in 0.8% NaCl electrolyte

Copper Surface	Cu+ ion concentration (μM)	CR - nm/min
Electroplated - No Oxide	14.4 ± 1.4	0.41 ± 0.04
Electroplated - BTA - 0.016M	4.9 ± 1.7	0.14 ± 0.05
Electroplated - BTA - 0.001M	3.1 ± 0.4	0.09 ± 0.01
Electroplated - BTA - 0.0004M	3.9 ± 0.6	0.11 ± 0.02
Electroplated - BTA - 0.0001M	9.3 ± 0.2	0.26 ± 0.003
Electroplated - Thermal Oxide	10.2 ± 2.1	0.29 ± 0.06