Death and genome destruction of methicillin-resistant and methicillin-sensitive strains of *Staphylococcus aureus* on wet or dry copper alloy surfaces does not involve Fenton chemistry

S. L. Warnes [#a *], C. W. Keevil [a]

[a] Centre for Biological Sciences, University of Southampton, Highfield, Southampton SO17 1BJ, United Kingdom.

Running title: Death of *Staphylococcus aureus* on copper surfaces

address correspondence to Sarah L. Warnes, s.l.warnes@soton.ac.uk

Abstract

The pandemic of hospital acquired infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) has declined but the evolution of strains with enhanced virulence, toxins and the increase of community-associated infections is still a threat. In previous studies, simulated droplet contamination of MRSA was killed on copper and brass surfaces within 90 minutes. However, contamination of surfaces is often via finger tips which dries rapidly and may be overlooked by cleaning regimes unlike visible droplets. In this new study a 5-log reduction of a hardy epidemic strain of MRSA (EMRSA-16) was observed following 10 minutes contact with copper and 4-log reduction observed on copper nickel and cartridge brass alloys in 15 minutes. A methicillin-sensitive strain (MSSA), from an osteomyelitis patient, was killed on copper surfaces in 15 minutes and a 4-log and 3-log reduction occurred within 20 minutes contact with copper nickel and cartridge brass, respectively. Bacterial respiration was compromised on copper surfaces and superoxide generated as part of the killing mechanism. In addition, destruction of genomic DNA occurs on copper and brass surfaces allaying concerns about horizontal gene transfer and copper resistance. Incorporation of copper alloy biocidal surfaces could help to reduce the spread of this dangerous pathogen.

Keywords: *Staphylococcus aureus*, methicillin, resistance, sensitivity, copper, brass, surface, contact killing

Introduction

Intrinsic penicillin resistance and acquisition of resistance to methicillin in the 1980s by *Staphylococcus aureus* led to a pandemic of infections worldwide. Initially the majority of infections were contracted in healthcare environments but incorporation of measures to control the spread including pre-admission screening, decolonisation, improved disinfection and antibiotic treatment have stemmed the tide [1]. The increased use of antibiotics required for the epidemic of infections caused by Gram-positive pathogens has allowed the evolution of multidrug resistant Gram-negative pathogens, effectively transforming some commensal gut bacteria into potential killers. However new strains of *S. aureus* that have acquired further virulence factors and toxins or have adapted to a specific environment, for example an increased ability to cause bacteraemia [2], are still a considerable threat. There is now widespread community associated methicillin-resistant *S. aureus* (CA-MRSA) and infections can spread within households, daycare centres and schools [3]. In addition, Guiffre observed an increasing incidence of MRSA in neonates [4]. The ability of some strains of MRSA to revert to methicillin susceptible isolates particularly in skin and soft tissue infections has been observed [5].

Colonisation with MRSA increases the risk of MRSA infection particularly following illness, surgical procedures and treatment with immunosuppressive drugs. Colonisation and or contracting infection may also occur from touching contaminated surfaces. In the community a recent study observed 58% and 82% surfaces in 19 Fire stations in Washington, USA were positive for MRSA and MSSA, respectively [6], and 37% fire service professionals had MRSA requiring medical attention. Otter et al. (2009) observed 8% sites tested in London public transport system had MSSA contamination but no MRSA was detected [7]. The use of biocidal surfaces may have a role in preventing infection transmission from contaminated surfaces when combined with stringent cleaning regimes. Laboratory studies have suggested copper surfaces may be effective against a range of bacteria, fungi and viruses [8–11] and the

Applied and Environmental Microbiology

irreversible pathogen nucleic acid destruction observed may allay fears of biocide resistance [12]. A previous study observed strains of MRSA were killed on copper surfaces in wet droplet simulated contamination[13]. However often surface contamination is by fingertip touch which dries rapidly. In this study we investigated the ability of several copper alloys to kill MRSA and MSSA, and the mechanism of bacterial death in simulated droplet and fingertip touch contamination of surfaces.

Material and Methods

Bacterial strains

Methicillin-resistant *Staphylococcus aureus* NCTC 13143 (EMRSA-16) was supplied by Public Health England, UK. Methicillin-sensitive *Staphylococcus aureus* ATCC 49230 (CDC 587) (MSSA) originally isolated from a patient with chronic osteomyelitis, was supplied by American Type Culture Collection, USA.

Metal surfaces

Metal samples were provided by the Copper Development Association (Table 1), cut into coupons (10 x 10 x 0.5 mm) which were cleaned and sterilised as described previously [22].

Bacterial survival on surfaces assessed by culture and *in situ* detection of respiring cells

For the simulated fingertip touch contamination aliquots of exponentially growing cultures of both species in Tryptone Soy Broth (TSB) were centrifuged to pellet the cells and resuspended in fresh growth medium to give approximately 10^7 colony forming units (cfu) per µL. One µL was spread over the surface of the coupons to allow rapid drying within seconds and incubated for various times. This size of inoculum represents a heavy bioburden and permits measuring an endpoint for inactivation. Cells were removed by vortexing in PBS with glass beads as described previously (22, no significant difference if chelators present to neutralise copper ions) and dilutions spread onto Tryptone Soy Agar (TSA) plates which were incubated at 37^{o}C for up to 48 hours and cfu per coupon calculated. Replicate coupons

were done for each time point. Simulated droplet contamination was done by inoculating the same number of cells in 20 µL per coupon. Cells were also inoculated in the presence of chelators ethylenediaminetetraacetic acid (EDTA; 20mM) and bathocuproine disulfonic acid (BCS, 20mM) to chelate Cu(II) and Cu(I) ions, respectively. Reactive oxygen species quenchers D-mannitol (20mM), 4, 5- dihydroxy-1,3-benzene disulfonic acid (Tiron) and superoxide dismutase (SOD, 500U/mL) were used to remove hydroxyl and superoxide radicals, respectively; 500U/ mL catalase and 10% sucrose (wt / vol) were also used to decompose hydrogen peroxide and investigate osmotic stress, respectively.

Respiring bacterial cells produce electrons which reduce the redox dye, CTC (5-cyano-2, 3-ditolyl tetrazolium chloride), to insoluble formazan which can be visualised as a red fluorescent stain using epifluorescence microscopy. SYTO-9 is a membrane permeable dye which binds to intact double stranded DNA and emits green fluorescence. The stain will not bind to degraded nucleic acid. Metal coupons were inoculated as described for culture assessment and stained *in situ* as described previously (22). Briefly, cells were dual stained with 5 mM CTC and 5 µM SYTO 9 for the final 90 and 30 minutes of the test 2 hour contact time, respectively. Bacterial cells were observed *in situ* for actively respiring cells (red) and all cells with intact DNA, live or dead (green). Cells were also inoculated in the presence of chelators or ROS quenchers as described for culture. Stainless steel was used as a control surface throughout.

In situ DNA destruction of MRSA (simulated fingertip touch contamination)

Bacterial cells in 1 mL of overnight culture were pelleted and washed in PBS. The cells were stained with 10 µM SYTO-9 for 30 minutes at room temperature. The stained cells were washed to remove excess stain and resuspended in 50 µL fresh culture medium: 1 µL was applied to metal coupons and fluorescence microscopy images recorded every minute for 20 minutes.

DNA fragmentation

The protocol was originally described by Fernandez et al [23] and has been adapted in our laboratory (22). Briefly, approximately 10^7 bacterial cells that had been exposed to copper, stainless steel, cartridge brass or Muntz metal for 2 hours were treated with 40U lysostaphin for 15 minutes at 37^{o}C. The cells were then encased in low melting point agarose on a slide previously coated with standard agarose. Following membrane permeabilisation the slides were dried and stained with SYBR Gold (Invitrogen, UK), which detects single and double stranded DNA, for 5 minutes at room temperature in the dark. Cells were observed with epifluorescence microscopy for the presence of DNA loops representing intact genome or dispersed fragments.

Statistical analysis

Data are expressed as mean ± standard errors of the mean (SEM) and are from multiple independent experiments. Statistical analyses and graphical representations were performed using GraphPad Prism Version 6.

Results and Discussion

Staphylococcus aureus can persist on surfaces for many months and is easily transferred to hands contacting surfaces (reviewed by Kramer [14]). This represents a significant infection risk especially in the healthcare environment, often via the hands of healthcare workers. In addition, a recent small study in Egypt observed 100% mobile phones belonging to patients and healthcare workers were contaminated with bacterial pathogens and MRSA was detected in >50% which may result in transferring pathogens out of the healthcare environment into the community [15].

A previous study (Noyce et al., 2006) suggested strains of MRSA applied as a wet droplet (10^7 bacteria in 20µL / cm^2) were killed on copper surfaces in 45 to 90 minutes contact, with EMRSA-16 being the most resilient. In this new study we have shown the same inoculum

size of EMRSA-16 applied in a low volume which dries in seconds, to simulate fingertip contamination, was killed in 20 minutes and almost a 5 log-reduction occurred within the first 10 minutes (Figure 1A). Fingertip touch will also contaminate the surface with biomolecules transferred from the skin surface so the cells were inoculated in bacteriological medium which contains a complex mixture of proteins, salts and sugars. There was a 2-log reduction in viable cells on cartridge brass (70% copper) and complete kill on copper nickel 10 (90% copper), which has been shown previously to have excellent antiviral activity, following 30 minutes contact. The results were similar for MSSA except complete kill on copper occurred within 15 minutes (Figure 1B). A previous study has observed exposure of MRSA to copper inhibits respiration and compromises DNA integrity [16]. Analysis of the bacterial DNA *in situ* on copper surfaces in the new study suggested rapid destruction occurs over the first 5 minutes contact which does not occur on stainless steel (Figure 2) and is equivalent to approximately 2-log reduction in viable cells observed in the culture results (Figure1). This suggests the development of copper resistance is unlikely and the problems that have arisen with using some biocides and concomitant antibiotic resistance [17] should not occur with the use of antimicrobial copper surfaces

Further investigations into the mechanism of copper kill of MRSA were done for both types of surface contamination. Chelators of Cu(I) and Cu(II) offered significant protection of EMRSA-16 on copper surfaces (Figure 3A, 3C) suggesting these moieties are directly or indirectly responsible for bacterial death. Generation of free radicals has been suggested to be involved in the copper kill mechanism and quenching potential superoxide generation with membrane permeable Tiron gave significant protection on copper surfaces for droplet contamination (Figure 3B) although the addition of superoxide dismutase (SOD) to dismute superoxide was not as effective. This may be because the enzyme activity is reduced at the neutral pH used in the test procedure or the large protein was unable to cross the cell

Applied and Environmental Microbiology

membrane to dismute intracellular superoxide. Tiron was protective for simulated dry touch contamination but only after the initial 10 minutes contact; thereafter there were still log-3 survivors on copper after 20 minutes (Figure 3D). D-mannitol displayed minimal protective effect suggesting hydroxyl radicals were not the primary instigators of bacterial death on copper (Figure 3B, 3D). It has been shown that generation of highly toxic hydroxyl radicals via Fenton reaction between Cu(I) and hydrogen peroxide combined with direct copper ion action led to the rapid death of Gram-negative bacteria on copper surfaces [18]. The results from this new study suggest reactive oxygen species (ROS) are involved in the bacterial kill on copper but not via Fenton chemistry. This is further supported when removal of copper ionic species (by chelators) and superoxide (by quenchers) allowed the bacterial cells to continue respiring and DNA to remain intact (Figure 4) but addition of D-mannitol to quench hydroxyl radicals and catalase to decompose hydrogen peroxide was not protective. Further evidence for the DNA damage can be observed using the genomic DNA fragmentation assay. Bacteria exposed to stainless steel display intact loops of genomic DNA emanating from and surrounding each lysed bacterial cell but in cells prior exposed to copper and copper alloys the DNA has degraded to small fragments that are too small to be visualised (Figure 5). The loss of genomic DNA was commensurate with cell death.

These results are comparable to the mechanism of copper toxicity observed by our laboratory for other Gram-positive bacteria i.e. pathogenic enterococci [19]. This supports our previous conclusions that the mechanism of bacterial death on copper surfaces is multifaceted, involves combination of direct copper ion attack of bacterial structural and metabolic biomolecules and suicidal generation of ROS. Although Gram-positive and Gram-negative bacteria die on copper surfaces the targets vary and rapid breakdown of nucleic acid is observed in the former.

Applied and Environmental Microbiology

The tide of MRSA infections observed in the 1980s has been reduced by implementation of many interventions including screening regimes, isolation, decolonisation, antibiotic stewardship and disinfection and has been superseded by multi-drug resistant *Enterobacteriaceae*. We must guard against complacency especially with the increase in *Staphylococcus aureus* community infections and the evolution of strains with increased virulence. This study is the first to show very rapid kill of fingertip contamination of MRSA and MSSA on copper alloy and the authors propose that incorporation of copper alloy surfaces may help to reduce the transmission of MRSA and MSSA from contaminated surfaces. The real life bacterial bioburden is considerably less than tested here suggesting kill times may be even faster and a hospital trial has already shown incorporation of just 6 copper surfaces in intensive care units significantly reduced MRSA colonisation and healthcare acquired infection over a 12 month period [20,21]. Further trials are now urgently needed to determine if the wealth of data from laboratory studies showing high efficacy of copper alloys to kill or inactivate a large range of microbial pathogens can be extrapolated to real life environments to reduce the number of infections contracted from touching contaminated surfaces.

Acknowledgements

This research was supported by the Copper Development Association (CDA), New York, and the International Copper Association, New York. CDA personnel were not involved in the development, design and execution of the experiments in this study.

Reference List

1. **Otto M** (2012) MRSA virulence and spread. Cell Microbiol **14**: 1513-1521. 10.1111/j.1462-5822.2012.01832.x [doi].

2. **Edgeworth JD, Yadegarfar G, Pathak S, Batra R, Cockfield JD, Wyncoll D, Beale R, Lindsay JA** (2007) An outbreak in an intensive care unit of a strain of methicillin-resistant *Staphylococcus aureus* sequence type 239 associated with an increased rate of vascular access device-related bacteremia. Clin Infect Dis **44**: 493-501. CID40723 [pii];10.1086/511034 [doi].

3. **Knox J, Uhlemann AC, Lowy FD** (2015) *Staphylococcus aureus* infections: transmission within households and the community. Trends Microbiol **23**: 437-444. S0966-842X(15)00070-0 [pii];10.1016/j.tim.2015.03.007 [doi].

4. **Giuffre M, Amodio E, Bonura C, Geraci DM, Saporito L, Ortolano R, Corsello G, Mammina C** (2015) Methicillin-resistant *Staphylococcus aureus* nasal colonization in a level III neonatal intensive care unit: Incidence and risk factors. Am J Infect Control **43**: 476-481. S0196-6553(15)00007-3 [pii];10.1016/j.ajic.2014.12.027 [doi].

5. **Patel AB, Hill E, Simpson EL, Hanifin JM** (2013) Reversion of methicillin-resistant *Staphylococcus aureus* skin infections to methicillin-susceptible isolates. JAMA Dermatol 149: 1167-1171. 1729129 [pii];10.1001/jamadermatol.2013.4909 [doi].

6. **Roberts MC, No DB** (2014) Environment surface sampling in 33 Washington State fire stations for methicillin-resistant and methicillin-susceptible *Staphylococcus aureus*. Am J Infect Control **42**: 591-596. S0196-6553(14)00135-7 [pii];10.1016/j.ajic.2014.02.019 [doi].

7. **Otter JA, French GL** (2009) Bacterial contamination on touch surfaces in the public transport system and in public areas of a hospital in London. Lett Appl

Microbiol **49**: 803-805. LAM2728 [pii];10.1111/j.1472-765X.2009.02728.x [doi].

8. **Wilks SA, Michels H, Keevil CW** (2005) The survival of *Escherichia coli* O157 on a range of metal surfaces. Int J Food Microbiol **105**: 445-454. S0168-1605(05)00346-6 [pii];10.1016/j.ijfoodmicro.2005.04.021 [doi].

9. **Weaver L, Michels HT, Keevil CW** (2008) Survival of *Clostridium difficile* on copper and steel: futuristic options for hospital hygiene. J Hosp Infect **68**: 145-151. S0195-6701(07)00417-3 [pii];10.1016/j.jhin.2007.11.011 [doi].

10. **Noyce JO, Michels H, Keevil CW** (2007) Inactivation of influenza A virus on copper versus stainless steel surfaces. Appl Environ Microbiol **73**: 2748-2750. AEM.01139-06 [pii];10.1128/AEM.01139-06 [doi].

11. **Warnes SL, Keevil CW** (2013) Inactivation of norovirus on dry copper alloy surfaces. PLoS One **8**: e75017. 10.1371/journal.pone.0075017 [doi];PONE-D-13-24101 [pii].

12. **Warnes SL, Highmore CJ, Keevil CW** (2012) Horizontal transfer of antibiotic resistance genes on abiotic touch surfaces: implications for public health. MBio **3**. mBio.00489-12 [pii];10.1128/mBio.00489-12 [doi].

13. **Noyce JO, Michels H, Keevil CW** (2006) Potential use of copper surfaces to reduce survival of epidemic meticillin-resistant *Staphylococcus aureus* in the healthcare environment. J Hosp Infect **63**: 289-297. S0195-6701(06)00037-5 [pii];10.1016/j.jhin.2005.12.008 [doi].

14. **Kramer A, Schwebke I, Kampf G** (2006) How long do nosocomial pathogens persist on inanimate surfaces? A systematic review. BMC Infect Dis **6**: 130. 1471-2334-6-130 [pii];10.1186/1471-2334-6-130 [doi].

15. **Selim HS, Abaza AF** (2015) Microbial contamination of mobile phones in a health care setting in Alexandria, Egypt. GMS Hyg Infect Control **10**: Doc03. 10.3205/dgkh000246 [doi];dgkh000246 [pii];Doc03 [pii].

16. **Weaver L, Noyce JO, Michels HT, Keevil CW** (2010) Potential action of copper surfaces on meticillin-resistant *Staphylococcus aureus*. J Appl Microbiol **109**: 2200-2205. 10.1111/j.1365-2672.2010.04852.x [doi].

17. **Webber MA, Whitehead RN, Mount M, Loman NJ, Pallen MJ, Piddock LJ** (2015) Parallel evolutionary pathways to antibiotic resistance selected by biocide exposure. J Antimicrob Chemother **70**: 2241-2248. dkv109 [pii];10.1093/jac/dkv109 [doi].

18. **Warnes SL, Caves V, Keevil CW** (2012) Mechanism of copper surface toxicity in *Escherichia coli* O157:H7 and *Salmonella* involves immediate membrane depolarization followed by slower rate of DNA destruction which differs from that observed for Gram-positive bacteria. Environ Microbiol **14**: 1730-1743. 10.1111/j.1462-2920.2011.02677.x [doi].

19. **Warnes SL, Keevil CW** (2011) Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact. Appl Environ Microbiol **77**: 6049-6059. AEM.00597-11 [pii];10.1128/AEM.00597-11 [doi].

20. **Michels HT, Keevil CW, Salgado CD, Schmidt MG** (2015) From Laboratory Research to a Clinical Trial: Copper Alloy Surfaces Kill Bacteria and Reduce

Hospital-Acquired Infections. HERD **9**: 64-79. 1937586715592650 [pii];10.1177/1937586715592650 [doi].

21. **Salgado CD, Sepkowitz KA, John JF, Cantey JR, Attaway HH, Freeman KD, Sharpe PA, Michels HT, Schmidt MG** (2013) Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit. Infect Control Hosp Epidemiol **34**: 479-486. 10.1086/670207 [doi].

22. **Warnes SL, Green SM, Michels HT, Keevil CW** (2010) Biocidal efficacy of copper alloys against pathogenic enterococci involves degradation of genomic and plasmid DNAs. Appl Environ Microbiol **76**: 5390-5401. AEM.03050-09 [pii];10.1128/AEM.03050-09 [doi].

23. **Fernandez JL, Cartelle M, Muriel L, Santiso R, Tamayo M, Goyanes V, Gosalvez J, Bou G** (2008) DNA fragmentation in microorganisms assessed *in situ*. Appl Environ Microbiol **74**: 5925-5933. AEM.00318-08 [pii];10.1128/AEM.00318-08 [doi].

Figure legends

Figure 1 Rapid death of an epidemic strain of methicillin-resistant *Staphylococcus aureus* (EMRSA) (A) and methicillin-susceptible *Staphylococcus aureus* (MSSA) (B) fingertip contamination on copper

Approximately 10^7 cfu in 1 µL were applied to 1 cm^2 test surfaces (C11000 (●), C26000 (■), S30400 (▲), C70600 (▼)) and immediately spread over the surface. Bacteria were removed after various contact time at 21°C as described in the text and assessed for viability by growth on agar. Results are expressed as cfu per coupon (± SEM of the mean) and are the result of multiple experiments.

Figure 2 Destruction of the DNA of an epidemic strain of methicillin-resistant *Staphylococcus aureus* (EMRSA) on copper surfaces

Approximately 10^7 bacterial cells were pre-stained SYTO9, which intercalates into intact DNA, and applied to copper surface. Cells with intact DNA appear green using epifluorescence microscopy. Images were recorded every minute (different fields of view to eliminate bleaching effect of excitation light source). A rapid reduction in cells with intact DNA occurred on copper surface following the first 5 minutes contact. In contrast, there was no reduction in staining of cells exposed to stainless steel for the experiment duration.

Figure 3 Determination of the role of copper ions and reactive oxygen species (ROS) in the rapid death of an epidemic strain of methicillin-resistant *Staphylococcus aureus* (EMRSA) on copper surfaces

Approximately 10^7 cells were applied to copper surface in 20µL to simulate wet droplet (A, B) or dry touch (C, D) contamination as described in the text. If cells were inoculated in the presence of chelators BCS and EDTA to chelate Cu(I) and Cu(II) respectively a protective effect was seen in both droplet and dry touch scenario compared to inoculation in PBS (A, C). Inoculation in the presence of Tiron to quench superoxide generation also had a significant protective effect especially in the droplet contamination (B) although superoxide dismutase (SOD) protection was much lower. D-mannitol, which quenches hydroxyl radicals, had a small protective effect in simulated droplet contamination (B).

Figure 4 Protection of bacterial DNA and respiration with chelators and reactive oxygen species quenchers in epidemic strain of methicillin-resistant *Staphylococcus aureus* (EMRSA) exposed to copper surfaces (simulated droplet contamination)

Approximately 10^7 cells were applied to copper surface in 20µL PBS or PBS supplemented with chelators or ROS quenchers. During 2 hours contact time at 21°C the cells were dual stained *in situ* with CTC and SYTO 9 to detect actively respiring cells (fluoresce red) and total

cells with intact DNA (fluoresce green) respectively as described in the text. Cells inoculated in PBS did not stain with either stain suggesting cells are not respiring and DNA has disintegrated. However, if EDTA, BCS and Tiron are present the bacterial DNA was protected and cells were respiring. This suggests Cu(II), Cu(I) and superoxide generation respectively are required for the killing mechanism on copper. No protective effect was observed on copper surfaces with D-mannitol, catalase or sucrose (not shown). Cells exposed to stainless steel surfaces in PBS or with any supplements displayed intact DNA and active respiration (not shown).

Figure 5 Exposure to copper and brass surfaces affects the DNA of epidemic strain of methicillin-resistant *Staphylococcus aureus* (EMRSA) (simulated droplet contamination) The DNA fragmentation assay allows the DNA integrity of individual bacterial cells to be observed. Bacteria were exposed to copper, brass and stainless steel surfaces for 2 hours at 21^{o}C (± 1), immersed in agarose, permeabilised and stained as described in the text. On steel individual cells with intact DNA loops protruding though the permeabilised membrane can be seen. There is virtually no stained DNA in cells exposed to copper and brasses, suggesting extensive disintegration of the DNA has occurred.

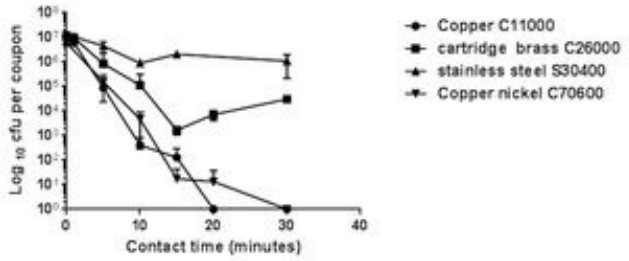
Table 1 Composition of metals used for the study

Metal type	UNS[a] no.	% composition					
		Cu	Zn	Sn	Ni	Fe	Cr
copper	C11000	100					
copper nickel 10	C70600	89-90			10	<1	
cartridge brass	C26000	70	30				
Muntz metal	C28000	60	40				
stainless steel '18/8'	S30400				8	74	18

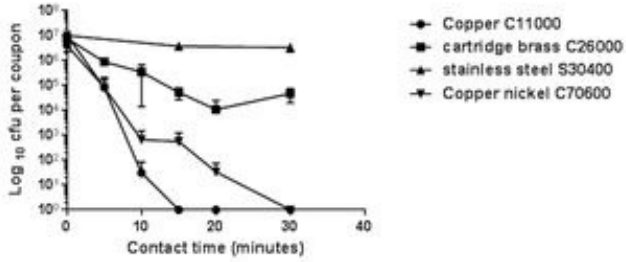
[a] Unified Numbering System

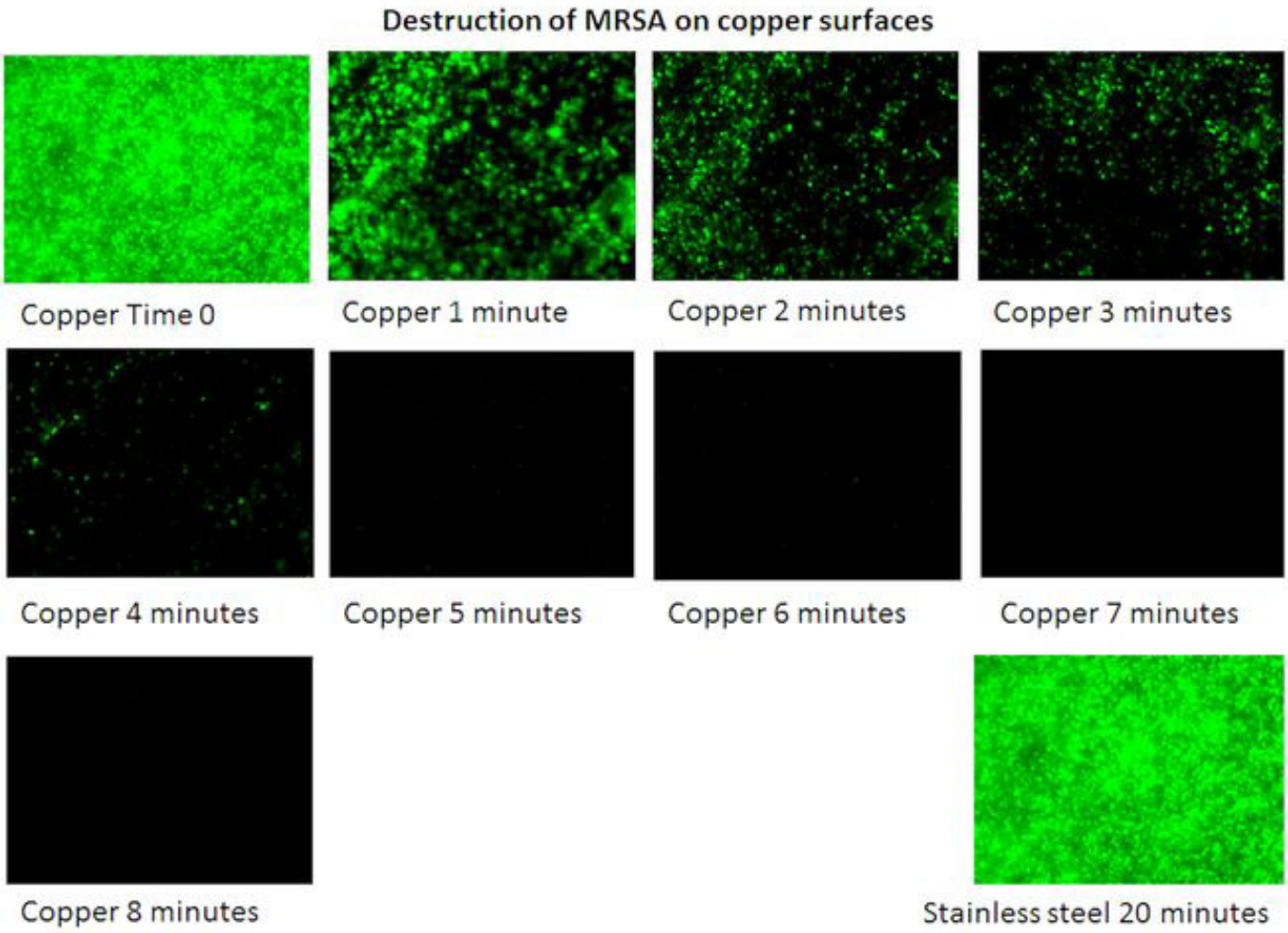
357

A

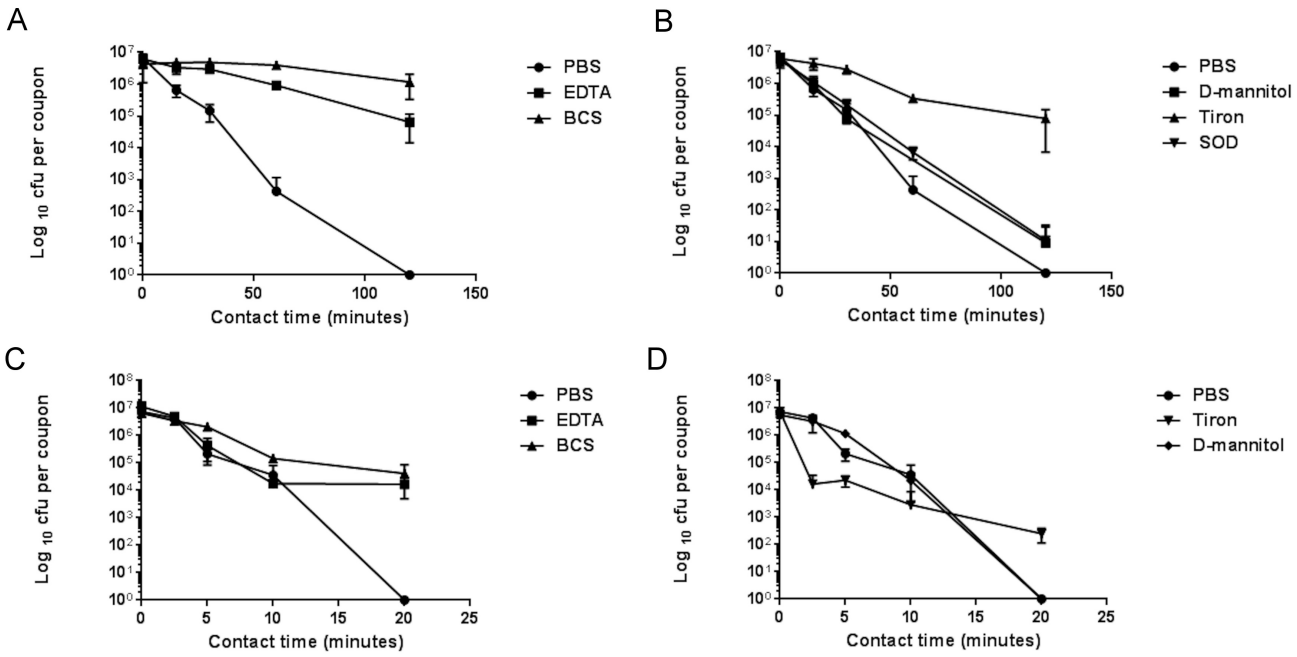


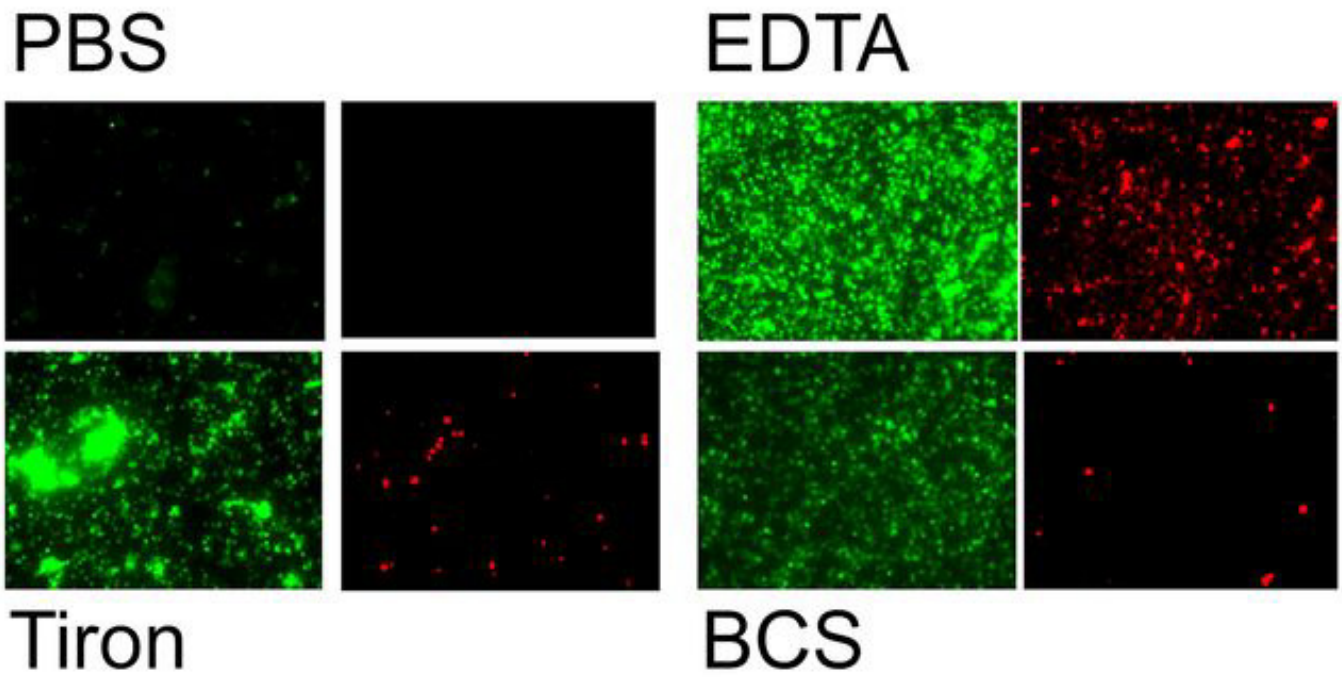
B

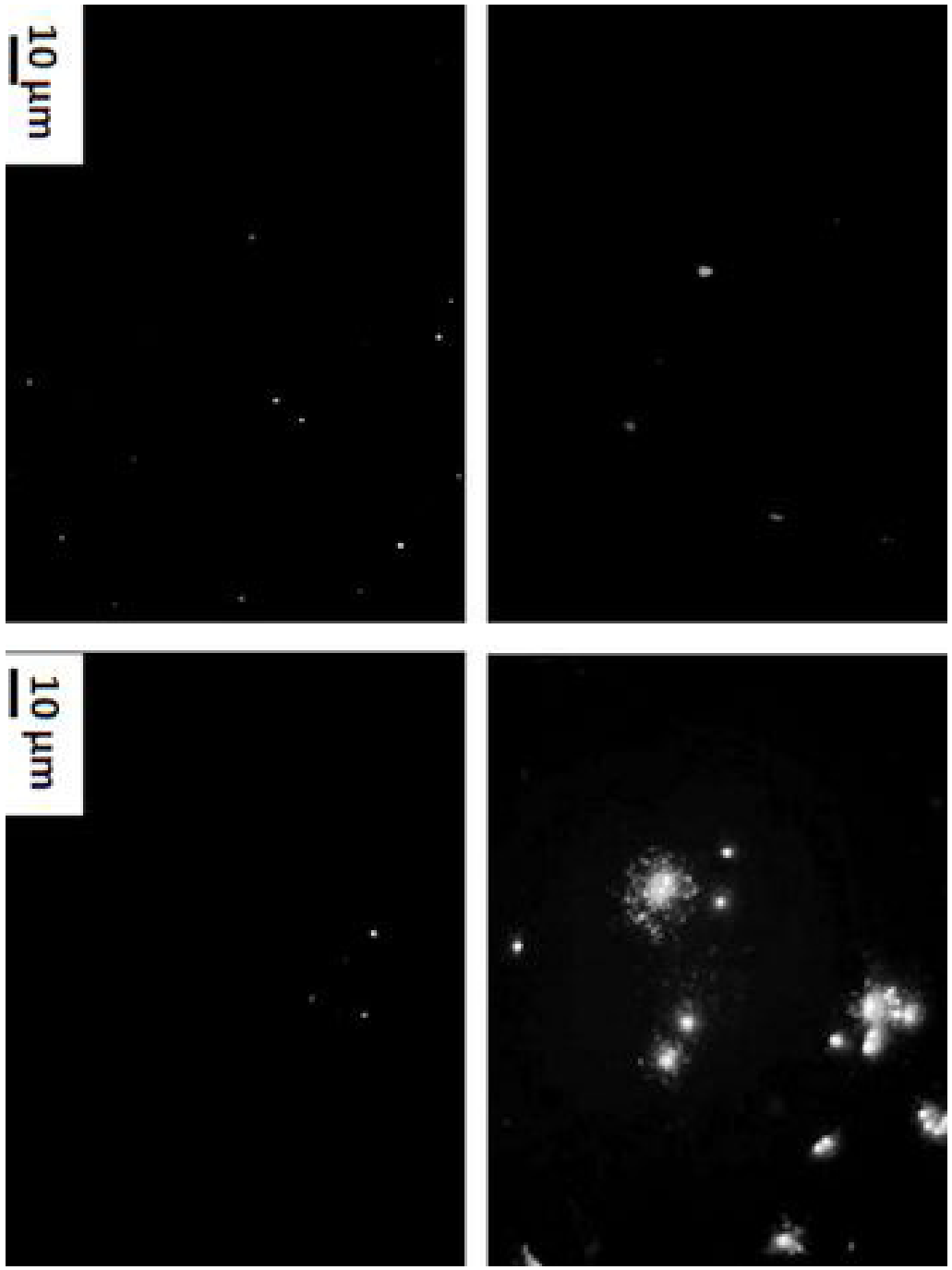




Applied and Environmental Microbiology






10 µm
10 µm