Contact killing of *Escherichia coli* K12 and *Staphylococcus cohnii* on copper containing and alloyed materials

DISSERTATION
ZUR ERLANGUNG DES DOKTORGRADES DER
NATURWISSENSCHAFTEN (DR. RER. NAT.)
DER NATURWISSENSCHAFTLICHEN FAKULTÄT III
- BIOLOGIE UND VORKLINISCHE MEDIZIN -
DER UNIVERSITÄT REGENSBURG

vorgelegt von
Claudia Hahn
aus Köln
im Juni 2016

Promotionsgesuch eingereicht am: 29.06.2016

Die Arbeit wurde angeleitet von: **Prof. Dr. Reinhard Wirth** (Universität Regensburg) in Zusammenarbeit mit **Dr. Petra Rettberg** (Deutsches Zentrum für Luft- und Raumfahrt e.V.)

Prüfungsausschuss:

Vorsitzender:	**Prof. Dr. Christoph Oberprieler**
1. Gutachter:	**Prof. Dr. Reinhard Wirth**
2. Gutachter:	**PD Dr. Christine Elisabeth Hellweg**
3. Prüfer:	**Prof. Dr. Reinhard Rachel**

Everyone is trying to accomplish something big, not realizing that life is made up of little things.

Frank Clark (1860 - 1936)

Table of contents

Abbreviations

Chemical elements and compounds were named after the International Union of Pure and Applied Chemistry (IUPAC).

AC	alternating current
Al	aluminium
Al-Ag	aluminium-silver
Al-Ag-Cu	aluminium-silver-copper
Al-Cu	aluminium-copper
ATP	adenosine triphosphate
BCC	body centered cubic
Bp	base pairs
CFU	colony forming unit
Cu	pure copper
CUB	cubic
DC	direct current
ddH_2O	double distilled water
DLR	German Aerospace Center
DNA	deoxyribonucleic acid
ds	double-stranded
DSB	double-strand breaks
DSMZ	German collection of microorganisms and cell cultures GmbH
E_{cal}	calculated eutectic point
EDTA	ethylenediaminetetraacetic acid
E_{exp}	experimental eutectic composition
EHT	extra high tension
EPA	United States Environmental Protection Agency
FCC	face centered cubic
HCP	hexagonal close packed
HS	heat-shock

KDPG	2-keto-3-deoxy-6-phosphogluconate
LB	Luria Bertani
MOPS	3-(N-morpholino)propanesulfonic acid
NB	nutrient broth
NCBI	National Center for Biotechnology Information
ND	not determined
Nt	nucleotides
PBS	phosphate-buffered saline
pH	potency of hydrogen
ppt	parts per trillion
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
rpm	rounds per minute
SD	standard deviation
SEM	scanning electron microscopy
SIM	stress-induced mutagenesis
sp	species (singular)
spp	species (plural)
ss	single-stranded
St	stainless steel
TAE	tris-acetate-EDTA
TCA	tricarboxylic acid cycle
TE	tris-EDTA
TGY	tryptone glucose yeast extract agar
Tris	trishydroxymethylaminomethane
TSB	tryptic soy broth
TSY	trypticase soy yeast
w/w	weight/weight

Abstract

Maintaining human health during long-term space missions is of major concern at any time. It is well known that space missions influence the immune system of the explorers and that they become more susceptible to bacterial infections. At the same time, bacteria exposed to the space environment have been shown to get more aggressive and more resistant to antibiotics. This particular combination can lead to severe infections and may jeopardise the mission; therefore a strong and uncompromising strategy inhibiting the propagation of bacteria is urgently needed. To achieve this, antimicrobial surfaces may just offer the answer. They provide a promising alternative to conventional cleaning procedures with e.g. disinfectants and contain organic/inorganic substituents or antimicrobial metals such as copper or silver. Copper is an essential metal but toxic at higher concentrations. On the contrary, silver is not essential and therefore toxic even at low concentrations. Due to their antimicrobial properties, both metals were used for medical issues and the storage of drinking water since thousands of years. While the toxicity is well known, the exact mechanisms of the toxicity are still unclear. In this study the antimicrobial effect of pure copper and new, potentially antimicrobial eutectic alloys composed of aluminium, silver and/or copper was investigated under wet contact killing conditions.

First, the survival of Gram-positive and Gram-negative cells after contact with antimicrobial materials was examined. Additionally, intracellular reactive oxygen species (ROS) production and damages of the membrane, DNA and RNA were determined for copper exposed samples. ROS production and membrane damages increased rapidly within one hour while the effect on cell survival was negligible even after two hours of exposure. Only longer exposure of up to four hours led to a rapid decrease in cell survival depending on the concentration of exposed cells/cm².

Further investigations were conducted with selected strains of the Keio collection. This collection is composed of *E. coli* strains which exhibit knockouts in one gene whereby each deleted gene was exchanged by a kanamycin cassette. To determine metabolic influences of pure copper and eutectic alloy exposed samples, 21 strains which had either a direct link to copper and/or silver transport mechanisms or a general function in the microorganism were selected. After pre-testing, different survivability's of *E. coli ΔcopA, ΔrecA, ΔcutA, ΔcueR* and *ΔcueO* were obtained. The survival was decreased in absence of CueO compared to *ΔcopA* strains when exposed to pure copper surfaces. In contrast to this, the survivability was vice versa when exposed to silver containing alloys whereas CueO had no function in silver oxidization.

Finally, the release of metal ions and the cell associated metal concentration was determined by taking the example of *Staphylococcus cohnii*, *Escherichia coli* K12 and *ΔcopA* to identify possible connections between cell associated ions and the survival of cells. These measurements indicated that the higher release of ions after exposure of cells in buffer is caused by an indirect dissolution. Despite a relatively high cell associated ion concentration, the experiments in the current study (with *ex vivo* and *in vivo* DNA/RNA and the above mentioned Keio strains) pointed out that these ions were possibly primarily present in the periplasm. Additionally, investigations on ternary, eutectic alloys were conducted. They showed that special casting protocols need to be in progress to obtain antimicrobial effects. Due to the antimicrobial effect and light weight, these materials could be a great opportunity to be applied in hospitals or in spacecraft facilities.

Zusammenfassung

Während Langzeitmissionen im All ist die Aufrechterhaltung der menschlichen Gesundheit von großer Bedeutung. Es ist bekannt, dass Weltraummissionen das Immunsystem von Astronauten beeinflussen und diese anfälliger für bakterielle Infektionen werden. Gleichzeitig verändern sich Bakterien, die den Umweltbedingungen des Weltalls ausgesetzt werden; sie werden aggressiver und resistenter gegen Antibiotika. Diese Kombination kann zu schwerwiegenden Infektionen führen und die Mission gefährden. Aus diesem Grund werden Strategien zur Inhibierung und Verbreitung von Bakterien benötigt, wobei der Einsatz von antimikrobiellen Oberflächen eine gute Alternative zu konventionellen Reinigungsverfahren mit Desinfektionsmitteln bietet. Sie können entweder aus organischen/anorganischen Substituenten oder antimikrobiellen Metallen wie Kupfer oder Silber bestehen. Kupfer ist ein essentielles Metall, das allerdings bei höheren Konzentrationen toxisch wirken kann. Im Gegensatz dazu ist Silber nicht essentiell und wirkt schon bei geringen Konzentrationen toxisch. Beide Metalle besitzen antimikrobielle Eigenschaften und wurden schon vor tausenden von Jahren in der Medizin und zur Lagerung von Trinkwasser eingesetzt. Während die Toxizität der Metalle bekannt ist, sind die genauen Wirkmechanismen noch unklar. In dieser Studie wurde daher neben der antimikrobiellen Wirkung von reinem Kupfer die potentielle antimikrobielle Wirkung von neuen Verbundmaterialien, die aus Aluminium, Kupfer und/oder Silber bestehen, in feuchten Kontakt-Tötungsversuchen untersucht.

Zunächst wurde das Überleben von Gram-positiven und Gram-negativen Stämmen auf antimikrobiellen Materialien ermittelt. Im Anschluss daran wurden die intrazelluläre reaktive Sauerstoffspezies (ROS) Produktion und Schäden an der Membran sowie der DNA und RNA nach der Exposition von *Escherichia coli* K12 auf Kupferoberflächen bestimmt. Bereits nach einer Stunde konnte eine erhöhte ROS Produktion sowie Schäden an der Membran festgestellt werden. Im Gegensatz dazu konnten, auch nach zweistündiger Exposition, keine Auswirkungen auf das Überleben der Zellen gemessen werden. Erst eine längere Exposition von vier Stunden führte zu einer raschen Abnahme des Überlebens in Abhängigkeit von der eingesetzten Zellkonzentration/cm^2.

Weitere Untersuchungen wurden mit ausgewählten Stämmen der Keio Kollektion durchgeführt. Diese Kollektion umfasst *E. coli* Stämme, die einen Knockout in einzelnen Genen aufweisen, wobei jedes Gen durch eine Kanamycin Kassette ausgetauscht wurde. Für diese Studie wurden 21 Stämme ausgewählt, die entweder einen direkten Einfluss auf den Kupfer- und/oder Silbertransport haben oder eine generelle Funktion im Mikroorganismus, um die Rolle dieser Gene beim Überleben von *E. coli* auf reinen

Kupferoberflächen und eutektischen Gemischen zu untersuchen. Detaillierte Experimente mit den Stämmen *E. coli ΔcopA, ΔrecA, ΔcutA, ΔcueR* und *ΔcueO* zeigten unterschiedliche Überlebensfähigkeiten. Nach der Exposition auf reinem Kupfer war das Überleben in Abwesenheit von CueO reduziert, wobei Stämme ohne CopA sehr resistent waren. Nach der Exposition auf silberhaltigen Legierungen überlebte der *ΔcueO* Stamm am besten, da er Silber nicht oxidieren kann.

Im Anschluss daran wurde die Freisetzung und zellassoziierte Kupferionen-Konzentration am Beispiel von *Staphylococcus cohnii*, *E. coli* K12 und *ΔcopA* bestimmt, um mögliche Verbindungen zwischen zellassoziierten Ionen und dem Überleben der Zellen zu ermitteln. Die Ergebnisse zeigten, dass die Freisetzung bei einer Exposition von Zellen sehr viel größer im Vergleich zu reinem Puffer war, was auf eine indirekte Freisetzung von Ionen hinwies. Neben der relativ hohen Konzentration an zellassoziierten Ionen, konnte durch Versuche mit *ex vivo* und *in vivo* DNA/RNA und den ausgewählten Stämmen der Keio Sammlung gezeigt werden, dass die Ionen möglicherweise eher im Periplasma, statt im Zytoplasma, vorliegen. Zusätzlich wiesen die Untersuchungen auf ternären, eutektischen Gemischen darauf hin, dass spezielle Gießverfahren eingehalten werden müssen, um einen antimikrobiellen Effekt hervorzurufen. Diese Materialien könnten, auf Grund des antimikrobiellen Effektes und ihres Gewichtes in Krankenhäusern oder der Raumfahrttechnik Anwendung finden.

1. Introduction

Since ancient times humans are fascinated by space. Astronomic research improved daily life e.g. by calculating the time of the Nile flooding; or in general the time for a day and night cycle or the duration of one year [1, 2]. From thereon, mankind and their technology developed but the natural curiosity to investigate new areas of life or discover new things is still there. One interesting and relatively new research field is named astrobiology. It addresses questions of the history of life on Earth back to its roots and the deciphering of planetary environments in our solar system including their satellites. These questions could give hints for the understanding of the origin, evolution, and distribution of life and its interaction with the environment, here on Earth and in the universe [3]. To pursue the aspects of astrobiology different spaceflight programs at NASA, ESA and Roscosmos were initiated [4]. Today, we are able to visit space and conduct long-term experiments in low Earth orbit on-board the International Space Station (ISS) to address these questions. Since 2000 an alternating crew ranging from three to ten members is working there. Due to the great distance to Earth and the external space (vacuum) conditions, working is subjected to certain restrictions. The ISS is a closed habitat and has limitations related to fresh air, water and food supply. These limitations and the fact that each crew member (~70 kg) consists of around 10^{13} human cells and 10^{14} microorganisms led to the development of a unique microbial diversity in the man-made environment of the ISS [5]. This diversity is mainly originated from the crew but did also include airborne germs [6]. To minimize a spread and contamination of microorganisms in air and on surfaces and to maintain astronauts' health, guidelines like the ISS Medical Operations Requirements Document (ISS MORD 2009) were drafted. They allow a maximal bacterial concentration in air of 1000 CFU/m³ and on internal surfaces of 10000 CFU/m³ [7]. These limits were observed by applying e.g. disinfectant wipes and air filtration systems [6, 8, 9].

The characteristics of human associated microorganisms differ depending on their occurrence. *Escherichia coli* for example is a Gram-negative, facultative anaerobic and rod-shaped microorganism (Figure 1). Most of them are harmless and live in the gut of endotherms (humans) where they support the digestion of food by producing e.g. vitamin K which cannot be synthesised by humans [10-12]. *Staphylococcus cohnii* is a Gram-positive, facultative anaerobic coccus (Figure 1) which forms grape-like clusters and lives on human skin in its protective acid mantle [13]. Both microorganisms prevent colonization of harmful fungi and pathogenic bacteria [14-16].

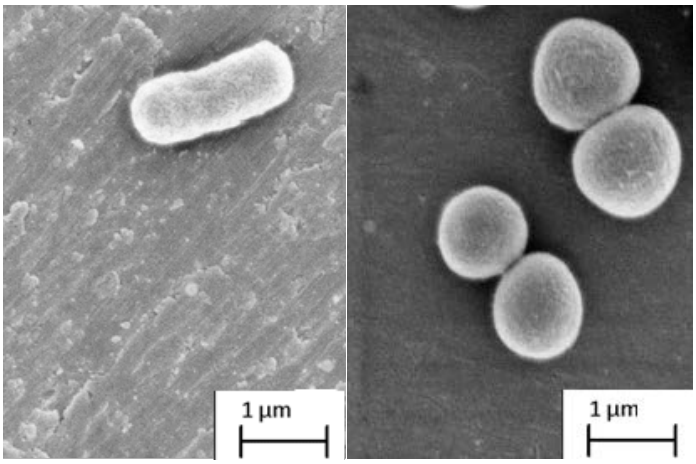


Figure 1: Representative Scanning Electron Microscopic (SEM) image of *E. coli* K12 (left panel) and *S. cohnii* (right panel).

Living in such a relationship can alter when microorganisms occupy other regions of the human body. *E. coli* and the common skin and mucous membrane associated microorganism, *Staphylococcus aureus* for example live in a commensal relationship with humans but once spread to wounds, they can cause nosocomial bloodstream infections [17, 18]. This problem is intensified when immune compromised persons are exposed to infections and diseases. Many patients are treated with antibiotics which decrease their natural bacterial flora. New and potentially harmful bacteria transferred to these patients do not have to compete with existing bacteria and can therefore increase their number on the new habitat.

The propagation of potentially pathogenic microorganisms occurs due to several ways. One way is evoked by contaminated transplants. As mentioned before, this leads to nosocomial bloodstream infections caused by e.g. staphylococci or enterococci [18]. Another way of propagation emerges through droplet infection or a skin to skin contact between the patient and other people. Thereby, people transferring harmful bacteria are not necessarily the host of these bacteria. They probably touched a light switch or door handle which was previously contaminated. Even if healthy people are not influenced by these microorganisms, a transfer of bacteria to an immune compromised person can lead to severe healthcare-associated infections. In Europe approximately 4,100,000 patients acquire such an infection every year, whereas 147,000 of them die [19]. To counteract these infections, patients are treated with antibiotics.

Antibiotics are produced by bacteria or fungi to develop benefits towards other microorganisms. They were discovered in 1928 by Alexander Fleming and from thereon used to fight bacteria [20]. However, the long exposure of antibiotics led to an unforeseen problem which is presented in Figure 2. Until 2010 many bacteria e.g. *E. coli* and

S. aureus developed resistances against specific antibiotics like third generation cephalosporins and meticillin which led to the need of new antibiotics or other ways to prevent microbial contaminations.

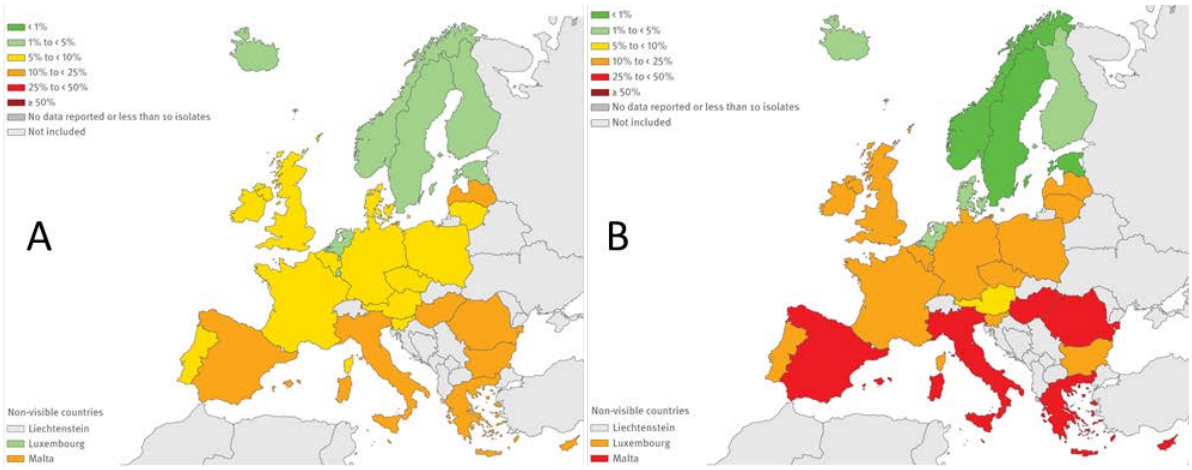


Figure 2: Percentage of invasive (blood and cerebrospinal fluid) isolates resistant to: A) third generation cephalosporins (*E. coli*) and B) meticillin (*S. aureus*). The colours indicate the percentage of reported isolates in 2010 (green: < 1 % - < 5 %; yellow: 5 % - < 10 %; orange: 10% - < 25 %; red: 25 % - < 50 %; grey: not included, no data reported or less than 10 isolates). Picture adapted from [21].

1.1 Antimicrobial materials

A promising alternative to minimize the accumulation and propagation of microorganisms on surfaces offer antimicrobial substances. Thereby, the term antimicrobial comprises all modes of action which inhibits growth and colonization of microorganisms. Antimicrobial materials contain organic/inorganic substituents or antimicrobial metals such as silver or copper ions. Historically, both metals have been used for medical treatment and the storage of drinking water [22-24]. Between 1890 and 1930, over 130 research projects and patents approached the toxic impact of metallic cations to cells, most of them dissecting the mode of function of copper and silver salts [25]. Von Nägeli discovered in 1893 the oligodynamic effect which describes a toxic effect of metal ions on various organisms such as viruses, eukaryotic and prokaryotic microorganisms [25, 26]. In 2008, Kawakami *et al*. examined the effect of metallic elements and detected that copper and silver were the most effective metals against bacteria (Figure 3) [27]. Today, they are utilized in many hygiene relevant areas of life, e.g. surgical textiles or special coatings in refrigerators [28].

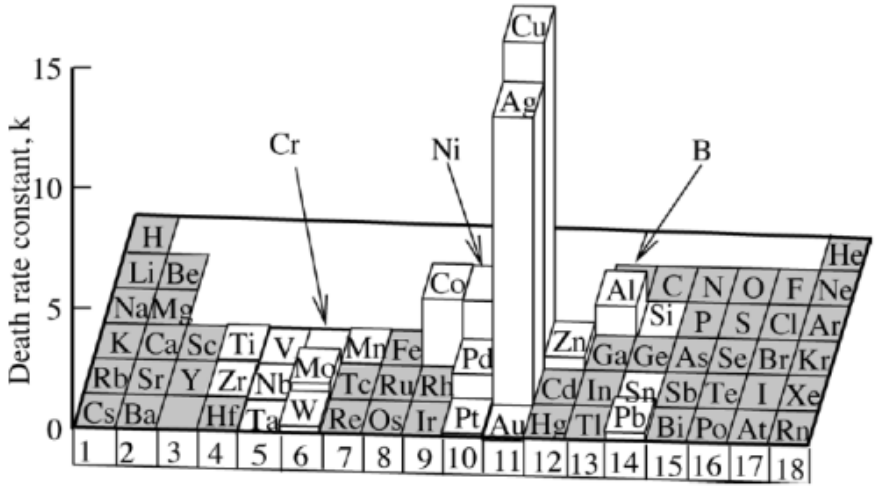


Figure 3: Death rate constants for *E. coli*. Hashed elements were not tested [27].

Based on the historical application, relatively low material costs and high antimicrobial properties, copper was selected as the major metal of interest for this study. One benefit of copper is the corrosive feature of its surface. Copper ions are released through oxidization and are taken up by cells which adhere to the surface. Once inside, copper ions damage cellular constituents and are ultimately responsible for cell death. However, the corrosive effect of copper surfaces leads to the formation of a patina. This “covering” is acquired through aging and exposure to the environment. Although the corrosive nature of copper is important for antimicrobial effects on cells, this process also leads to structural destabilisation over time. Further corrosive effects will detach more copper ions and therefore lead to the depletion of the surface. To optimize the features of surfaces, meaning a minimization of corrosion by a maximum antimicrobial effect, new materials composed of aluminium, copper and/or silver were conducted. These materials were eutectic alloys (paragraph 1.1.3), which means copper and silver ions exist as compounds with aluminium and were not available as single metals. This could lead to a diminished corrosive effect and longer lifetime of the corresponding materials.

1.1.1 Copper

Copper (from Latin: *cuprum*) is a soft and ductile metal displaying a reddish-orange colour. Nowadays, copper can be found in the Earth’s crust (with a concentration of 50 ppm) where it occurs as native copper or in minerals [29]. At the beginning of life on Earth, 3.5 billion years ago the atmosphere was anaerobic, containing H_2O, H_2S, NH_3 and CH_4. During that time copper was present in the water-insoluble Cu(I) state and therefore not available for biological processes until the Great Oxygen Event [30]. Cyanobacteria developed and produced oxygen via photosynthesis. Higher oxygen concentrations led to

an increased oxidization of copper to soluble Cu(II) [31]. Following this event, new copper-binding proteins, using copper as a cofactor, evolved. They are involved in processes of e.g. respiration and oxidative stress protection [32-34]. Today, copper is a trace element which is, in low concentrations, essential for many cellular processes.

Due to the reversible transition of Cu(I) and Cu(II) and the formation of radicals in a Fenton-like reaction, concentrations of copper ions need to be guarded by cells. The adjustment of the optimum intracellular copper concentration is called homeostasis and is achieved through specific or unspecific transporters. The unspecific transport is fast and driven by the chemiosmotic gradient across the cytoplasmic membrane while the specific transport of copper is very expensive for cells in terms of energy costs (ATP) and time. Therefore, specific transporters are only synthesized when they are needed whereas unspecific transporters are always available in the cell membrane. Based on this, a transport of heavy-metals into cells is always present [35, 36].

Elevated copper concentrations are cytotoxic and should be held at extremely low levels [37]. Increasing copper availability induces the necessary adaptation of cells in developing resistance and regulation of intracellular copper concentration via copper homeostasis [30, 37-39]. Mechanisms to balance the intracellular copper concentration are compartmentalization, sequestration and efflux [40].

Copper alloys

The well-established killing of bacteria by copper surfaces is also called contact killing [41]. As mentioned in paragraph 1.1, pure copper can act as an effective antimicrobial agent; however, due to its corrosive nature and therefore loss of stability over time, studies with copper alloys were performed. First antimicrobial efficiency tests with copper alloys were conducted by Phyllis Kuhn in 1982. Kuhn demonstrated that doorknobs made of brass exhibited a reduced microbial diversity in comparison to stainless steel doorknobs [42]. Thenceforward many copper alloy surfaces were produced and tested in laboratory studies conducted by the United States Environmental Protection Agency (EPA). Requirements of these alloys are that the killing of Methicillin-resistant *S. aureus* (MRSA), Vancomycin-resistant *Enterococcus faecalis* (VRE), *S. aureus*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, and *E. coli* O157:H7 should be higher than 99.9 % within two hours [43]. Nowadays more than 500 antibacterial copper alloys are registered, that are applied in various industrial branches.

Beside the application in the field of medicine [44] antimicrobial copper alloys are used in the field of e.g. water conditioning [45-47], food proceeding [48-50], textiles [28, 51, 52] and maritime industry [53, 54]. In food industry, the best known application of copper vessels is for brewery processes. Thereby copper is mainly used for its good heat conductor properties [55]. In hospital trials all over the world commonly touched surfaces were exchanged by copper alloys to determine whether and to what extend a reduction of microbial contamination occurred (Figure 4). These field studies were undertaken in South Africa (2010) [56], Germany (2010) [57], Finland (2010) [58], England (2010, 2012) [44, 59] and the USA (2012, 2013) [60-62]. Due to varying parameters, the obtained data from each study fluctuate greatly which made a comparison of the results difficult [63].

Figure 4: In hospital trials, typical touch surfaces which consisted of plastic were exchanged by copper surfaces [60].

Another application of copper is in nanoparticles. It was postulated that nanoparticles penetrate cells and lead to an acceleration of the bactericidal effect in comparison to metallic coatings [64]. Under aerobic and moist conditions copper surfaces oxidize and lead to the formation of CuO and Cu_2O whereby both exhibit antimicrobial effects [28, 51, 65-69]. Differences in the bactericidal effect of CuO and Cu_2O nanoparticles were analysed by Vargas-Reus *et al*. (2012) [67]. They determined that the antimicrobial effect of Cu_2O nanoparticles is more pronounced compared to CuO nanoparticles [70].

Summarizing, it is assumed that the antimicrobial effect of copper alloys is inferior compared to pure copper surfaces. Additionally, the antimicrobial effect of Cu^+ is determined to be higher compared to Cu^{2+}. To examine the most effective surface, related

to corrosion and stability, under frequent environmental conditions, ensuring the maximum effectiveness of the antimicrobial copper material, more studies need to be performed [70].

Cellular toxicity of copper

Copper ions have a toxic effect on viruses, fungi and bacteria even at very low concentrations (< 1 mg/l) [71-73]. The toxicity of copper occurs due to several modes of actions whereas the most important one is the production of free radicals (Figure 5). Free Cu^+ catalyses a Fenton-like reaction which synthesizes reactive oxygen species (ROS) [74-76].

$$Cu^+ + H_2O_2 \rightarrow Cu^{2+} + OH\cdot + OH^-$$

These free radicals in turn induce oxidative degradation of the lipid double layer (lipid peroxidation) [77], DNA, RNA and proteins [78, 79]. Additional damaging effects occur through a direct interaction of Cu^+ and Cu^{2+} with proteins, DNA, RNA and the membrane.

The negatively charged bacterial membrane is the first barrier for toxic agents [80]. Cations like Cu^+ and Cu^{2+} can be absorbed and therefore sequestered outside of cells. In contrast, higher concentrations of ions severely compromise the cytoplasmic membrane by interfering of the membrane potential [81, 82]. In dry contact killing experiments, Espirito Santo *et al*. (2011) determined membrane damages as the primary effect of the treatment [83]. However, Warnes *et al*. (2011, 2012) claimed that not all bacteria killed by copper necessarily exhibit membrane damages. This leads to the hypothesis, that further intracellular damages play a critical role during contact killing [84, 85]. Cu^+ has a high affinity to thiol and thioether groups (cysteine and methionine) [86, 87] and leads to an oxidation, mutation and/or cleavage of proteins [79, 88]. They also displace essential metals and therefore inactivate proteins and enzymes. Cu^{2+} has a high affinity to oxygen and imidazole nitrogen groups (aspartic and glutamic acid, or histidine) and degrades DNA and RNA [89]. In general, copper has two binding sites in the DNA double helix. One is present every four nucleotides; the other is an intercalating site which is present in every base pair [90]. DNA damage can be either the disordering of DNA helical structure, DNA strand breakage and crosslinking, or DNA mutation [33, 35, 72, 91, 92].

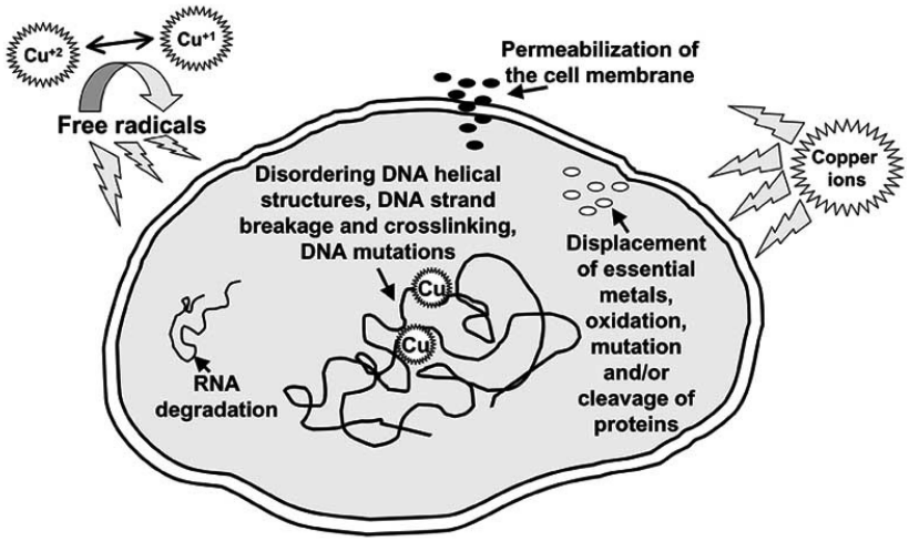


Figure 5: Toxicity mechanisms of copper on microorganisms [71].

Resistance strategies of bacteria against copper

To prevent damages caused by heavy metals, cells developed several mechanisms of resistance (Figure 6). They reduce the uptake of metals due to exclusion by the permeability barrier and the extra-cellular sequestration of copper ions by forming copper-complexes. Once copper ions are in cells, there are several other mechanisms of resistance. In *E. coli,* specialized proteins are synthesised for intracellular sequestration. Additionally, cells modify metals to a less toxic component or detoxify them via enzymatic reaction. Still, the most important mechanism of resistance is the efflux of metals via pumps and transporters [33, 71, 93].

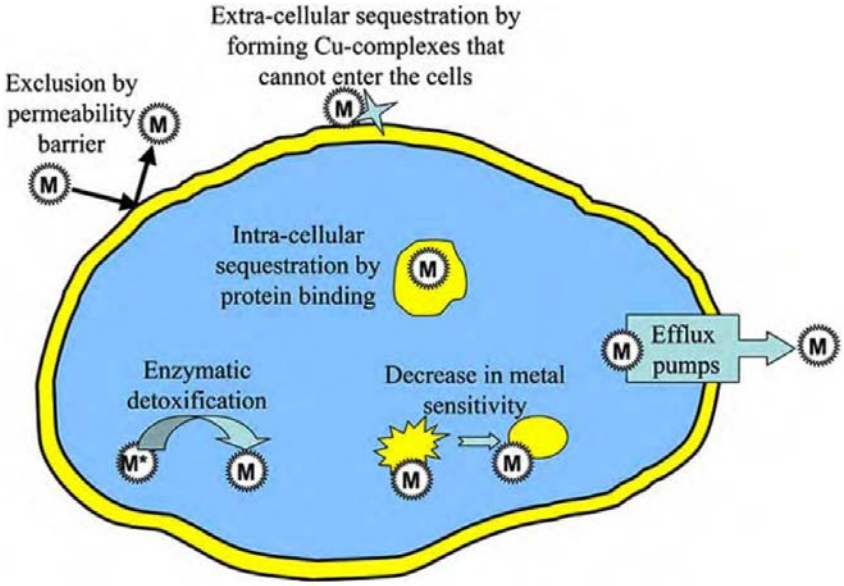


Figure 6: Resistance strategies of bacteria to heavy metals [71].

These transporters are responsible for copper homeostasis in *E. coli* and are comprised of the resistance nodulation cell division (RND)-efflux system (CusCFBA), the P-type ATPase (CopA) and the multi copper oxidase (CueO*)* proteins (Figure 7). CopA transports copper ions under energy loss from the cytoplasm to the periplasm. There, copper ions are bound by CusF which transports them to the CusCBA system and then outside the cell. CueO is present in the periplasm and oxidizes Cu^{+} to the less toxic Cu^{2+}. Additionally, the CueO oxidizes the siderophore enterobactin which binds free copper ions and therefore diminishes the copper concentration in the periplasm [33, 71, 94].

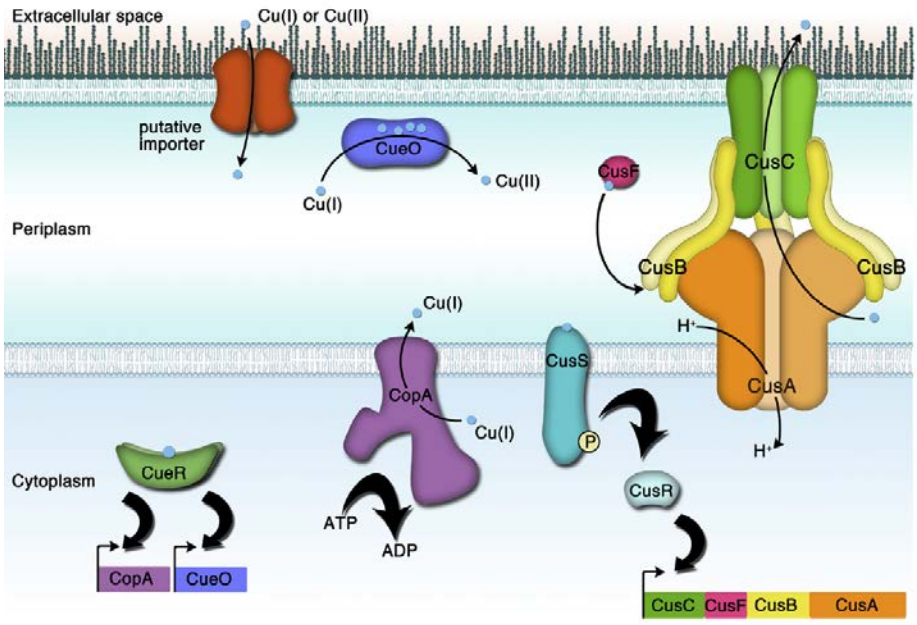


Figure 7: Copper homeostasis system in *E. coli* [95].

1.1.2 Silver

Silver (from Latin: *argentum*) is a soft metal with a characteristic silver sheen. It occurs naturally in the Earth's crust (with a concentration of 70 ppb) in a free form or embedded in minerals [29]. In contrast to copper, silver is not an essential metal and poisonous even at extremely low concentrations [36]. Beside the mentioned research and applications in paragraph 1.1, Gibbard showed in 1932 that the oxidization of silver is of high importance to obtain antimicrobial effects [96]. Already in 1932, silver was used for water treatment and as a preservative in the food industry. Today, it is applied for water purification (e.g. on-board the ISS) [97] and in clinical fields e.g. in implants or catheters [98, 99] to prevent the adhesion and proliferation of microorganisms [100-102].

Silver and copper possess similar chemical properties. Therefore it is assumed that they also exhibit related mechanisms of action [103-105] and damaging effects to the DNA, RNA, proteins and membrane [81, 82]. However, in contrast to copper ions which exist as Cu^{+} and Cu^{2+}, silver is only available as Ag^{+}. Therefore silver does not perform the Fenton-like reaction and as a consequence no ROS were produced by silver ions [105].

Both metals possess antimicrobial properties with similar mechanisms of action but the application of an equal concentration of single ions resulted in different response of bacteria [27, 73, 106]. This observation was the beginning of several studies which combined both ions to determine possible synergistic effects [46, 73, 107].

1.1.3 Eutectic alloys composed of aluminium, copper and/or silver

Synergistic and single effects were evaluated here with eutectic alloys which have never been tested before for their antimicrobial properties. The promising advantages of these alloys are relative low material costs, lightweight and lower corrosion compared to pure copper surfaces. Significant antimicrobial effects may lead to the evidence of an application for future space stations like the ISS, where the weight of materials is always of major importance and can be an exclusion criterion.

All prepared surfaces were implemented as eutectic compositions. A eutectic system consists of minimum two components and is plotted in a phase diagram with two degrees of freedom; the temperature (Y-axis) and concentration (X-axis) (Figure 8). On the left side of the X-axis 100 % of component A is available. From the left to the right the concentration of component A decreases and the concentration of component B increases until 100 % of component B is available on the right side of the X-axis. The straight line

displays the solidus line. At a temperature below this line both components are stable in the solid state. At a temperature above the liquidus line the components are liquid.

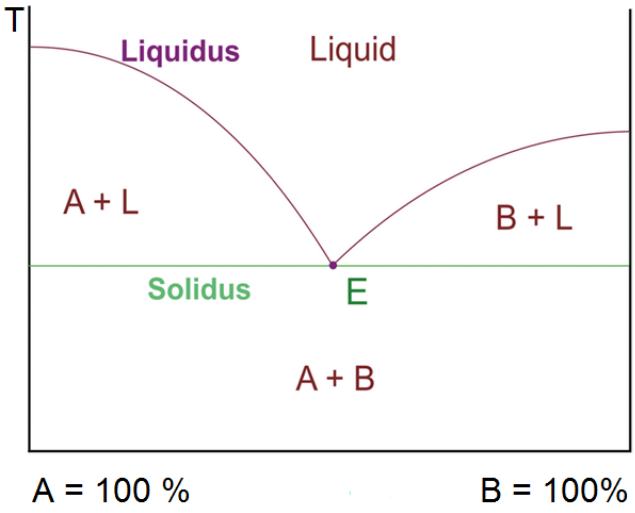


Figure 8: Phase diagram of a eutectic system. Picture modified after [108].

At high concentrations of component A e.g. 80 % and a temperature crossing the liquidus line, component A begins to crystallize. With this crystallization the concentration of component A decreases whereas the concentration of component B increases in the mixture. Due to the alteration of the concentration the solidification temperature also changes. As a consequence the crystallization of component A stops immediately. Further decrease of the temperature leads to a repetition of the described process until the concentration and temperature reaches the eutectic point (E). By reference to Figure 8, the concentration of component A and B at this point is 50 %. The liquidus and solidus line cross each other and the whole melting crystallizes.

A starting composition of 50 % of component A and B displays a eutectic composition. At that composition the temperature can be decreased until the eutectic point (E) is reached and both components crystallise immediately at the same time.

The eutectic system of the materials composed of Al-Cu, Al-Ag and Al-Ag-Cu are based on this principle but are a bit more complicated. The phase diagrams of Al-Cu and Al-Ag are attached in the supplements, while the eutectic system composed of three metals (aluminium, copper and silver) is presented in Figure 9. Similar to the phase diagram of two components, the eutectic point can be considered. This point is reached at a composition of 69.1 mol% aluminium, 18.1 mol% silver and 12.8 mol% copper (the red arrows in Figure 9 indicate how to determine the composition). At a temperature below the eutectic point Al, Al_2Cu and Ag_2Al crystallize.

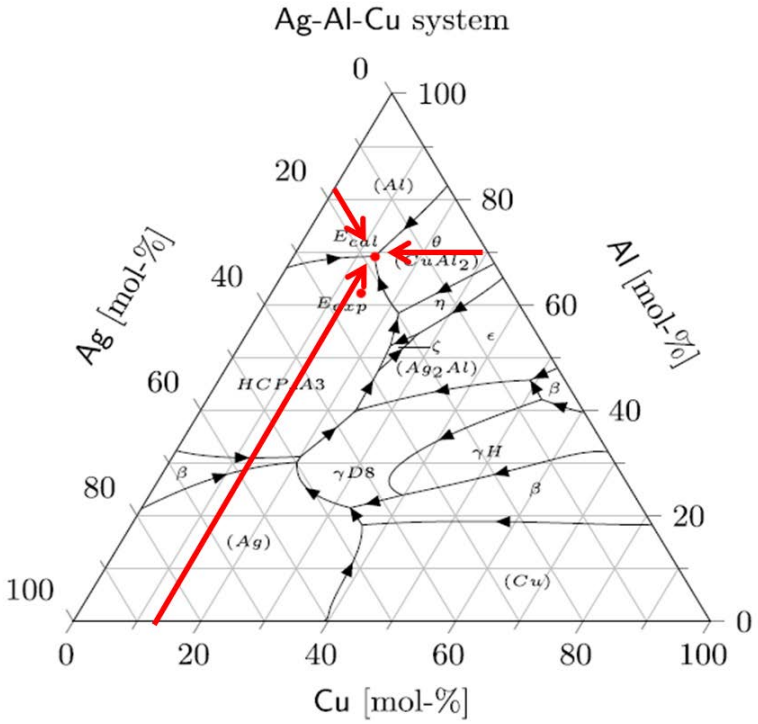


Figure 9: Phase diagram of a eutectic system composed of the three components aluminium, copper and silver. Picture modified after [109].

1.2 Aim of this work

Antimicrobial studies were implemented with copper and silver salts, since von Nägeli discovered the oligodynamic effect of metal ions on organisms in 1893 [26]. Nearly 100 years later, first experiments with antimicrobial surfaces were implemented by Kuhn in 1982 [42].

Based on the historical application, the relative low material costs and high antimicrobial properties, experiments on pure copper surfaces were conducted to analyse the toxic effects on DNA, RNA and the cell membrane under wet conditions. To ascertain new antimicrobial surfaces and detect their toxic effects on microorganisms, eutectic alloys composed of aluminium, silver and copper were examined. Thereby, single effects of copper and silver in correspondence with aluminium were determined, in addition to synergistic effects of alloys composed of all three metals.

Furthermore, analyses on selected strains of the Keio collections were implemented to clarify which cellular pathway or transport system is of highest importance when getting into contact to different potentially antimicrobial surfaces.

Previous studies pointed out that the corrosion and release of ions are very important for the antimicrobial effect of surfaces [83, 110]. To verify this effect and to determine differences in the ion release, ICP-MS measurements were conducted after the exposure to potentially antimicrobial surfaces. Thereby, the total release of metal ions and the cell associated concentration was determined.

These investigations should identify effective, new antimicrobial surfaces which could then be applied in spacecraft facilities or exchange plastic surfaces, e.g. doorknobs or light switches in hospitals to reduce microbial contaminations.

2. Material and Methods

2.1 Bacterial strains

The bacterial strains used in this work are human associated microorganisms or isolates from the environment and were either obtained from the DSMZ – German Collection of Microorganisms and Cell Cultures GmbH, Braunschweig, Germany or Prof. Dr. Lars Leichert, Bochum University, Germany (Table 1). More strains were investigated however not considered further. The selected strains from the Keio collection exhibit single knockouts whereby the deleted genes were exchanged by a kanamycin cassette. Therefore 30 µg/ml kanamycin was added to LB growth medium [111].

Strains obtained from the DSMZ were reactivated as followed by recommendations of the DSMZ. Except *Deinococcus radiodurans* R1 which was grown at 30 °C to the stationary phase in 40 hours, all other strains were grown at 37 °C overnight (16 hours) to stationary phase. An optimal oxygen concentration in liquid cultures was ensured through a shaken incubation at 200 rpm (HT Multitron, Infors GmbH, Sulzemoos, Germany).

Table 1: List of bacterial strains used in this work and their cultivation conditions. a = obtained from the DSMZ, Braunschweig, Germany; b = obtained from the Bochum University, Germany; c = 30 µg/ml Kanamycin was added to the medium.

Strains	**Genotype**	**Growing medium**
Deinococcus radiodurans R1[a] (DSM 20539)	Type strain	2xTGY
Escherichia coli K12[a] (DSM 498)		LB
E. coli BW25113[b]		
E. coli ΔcusA[b] (Keio:JW0564) [112]	Resistance-Nodulation-Cell Division (RND) Transporter system for the detoxification of copper and silver ions in *E. coli* as part of the *cusCFBA* copper/silver efflux system	LB[c]
E. coli ΔcusB[b] (Keio:JW0563) [112]		
E. coli ΔcusC[b] (Keio:JW0561) [112]		
E. coli ΔcusF[b] (Keio:JW0562) [112]		
E. coli ΔcusR[b] (Keio:JW0560) [113]	*CusRS* two-component system: Regulates expression of the c*usCFBA* operon	
E. coli ΔcusS[b] (Keio:JW5082) [113]		
E. coli ΔcueO[b] (Keio:JW0119) [34]	Multicopper oxidase: Role in copper homeostasis	
E. coli ΔcueR[b] (Keio:JW0476) [114]	Regulates the expression of *cueO* and	

	copA	
E. coli ΔcopA[b] (Keio:JW0473) [112]	Copper efflux P-type ATPase	
E. coli ΔcutA[b] (Keio:JW4097) [115]	Copper-binding protein	
E. coli ΔcutC[b] (Keio:JW1863) [116]	Copper transporter protein	
E. coli ΔleuC[b] (Keio:JW0071) [117, 118]	Activates isopropylmalate dehydratase with LeuD which catalyzes the second step in leucine biosynthesis	
E. coli ΔfumA[b] (Keio:JW1604) [119]	Fumarase isozymes participate in the TCA cycle	
E. coli Δedd[b] (Keio:JW1840) [120]	Encodes 6-phosphogluconate dehydratase, for the Entner-Doudoroff (KDPG) pathway	
E. coli ΔrecA[b] (Keio:JW2669) [121]	Repair of DSBs by homologous recombination	
E. coli ΔrpoS[b] (Keio:JW5437) [122]	Encodes the alternative sigma factor σ^S Master regulator of the general stress response	
E. coli ΔsodA[b] (Keio:JW3879) [123]	Superoxide dismutases	
E. coli ΔkatE[b] (Keio:JW1721) [124]	Scavenger at high H_2O_2 concentrations [125]	
E. coli ΔkatG[b] (Keio:JW3914) [124]	Scavenger at low H_2O_2 concentrations [125]	
E. coli ΔacrA[b] (Keio:JW0452) [126]	Multidrug efflux pump; Promoting stress-induced mutagenesis (SIM) response	
E. coli ΔacrB[b] (Keio:JW0451) [126]		
Staphylococcus cohnii[a] *(DSM 20260)*	Type strain	TSY

2.2 Media

All buffers, media and solutions were adjusted to the corresponding pH with either NaOH or HCl and autoclaved at 121 °C for 20 minutes.

The manufacturer's information for chemicals used in this work is listed below:

[a] AppliChem GmbH, Darmstadt, Germany

[b] Difco Laboratories, Sparks, USA

[c] Fluka, Sigma-Aldrich Chemie GmbH, Steinheim, Germany

[d] Sigma-Aldrich Chemie GmbH, Steinheim, Germany

[e] VWR International GmbH, Darmstadt, Germany

2x TGY medium

Bacto-Trypton [b]	10 g
Bacto Yeast-Extract [b]	6 g
D-Glucose-Monohydrat [e]	2 g
ddH_2O	ad 1000 ml

TSY medium

Trypticase Soy Broth [c]	30 g
Yeast extract [b]	3 g
ddH_2O	ad 1000 ml

LB medium

Luria Broth [d]	20 g
ddH_2O	ad 1000 ml

NB medium

Nutrient Broth [e]	8 g
ddH_2O	ad 1000 ml

Solid medium:

For the preparation of solid media 15 g/l agar [b] was added to the medium described above.

Buffer and solutions

1x MOPS buffer (3-(N-morpholino)propanesulfonic acid) pH 7

MOPS [e]	20.9 g
ddH_2O	ad 100 ml

6x Loading dye

Sucrose [e]	4 g
Bromophenol blue [d]	25 mg
ddH_2O	ad 10ml

1x Phosphate-buffered saline (PBS) pH 7

Na_2HPO_4 x 2 H_2O [e]	7 g
KH_2PO_4 [e]	3 g
NaCl [e]	4 g
ddH_2O	ad 1000 ml

50x Tris acetate EDTA (TAE) buffer pH 8.0

Tris [a]	224 g
Na_2-EDTA (0.5 M) [d]	100 ml
Glacial acetic acid [e]	57.1 ml
ddH_2O	ad 1000 ml

2.3 Surfaces and alloys

The following surfaces and alloys were tested for their antimicrobial potential.

V2A stainless steel (AISI 304: X5CrNi18-10), pure copper and oxidized (Cu_2O and CuO) pure copper surfaces were obtained from the Department of Functional Materials (Saarland University, Material Engineering Center Saarland, Germany).

In addition, alloys composed of aluminium (Hydro, Bonn, Germany), silver (ESG Edelmetall-Service GmbH & Co. KG, Rheinstetten, Germany) and/or copper (Alfa Aesar, Karlsruhe, Germany) were used to examine the effect of the following eutectic compositions: aluminium (Al), aluminium-copper (Al-Cu), aluminium-silver (Al-Ag) and aluminium-copper-silver (Al-Ag-Cu). They were produced at the Institute of Materials Physics in Space, DLR Cologne, Germany.

2.4 Surface and alloy preparation

To ensure consistent surface roughness, all surfaces were smoothed down by using sandpapers of different granulation. The first sandpaper had a roughness of P320 followed by a paper of P1000, P2500 and P4000. Afterwards, the MasterPrep solution (Buehler, Düsseldorf, Germany) was used for polishing, whereby the eutectic alloys were used without polishing.

V2A stainless steel (AISI 304: X5CrNi18-10), which consists of 71.5 % w/w iron, 0.5 % w/w carbon, 18 % w/w chrome and 10 % w/w nickel, and copper samples from 99.99 % rolled sheet copper were cut into 2.25 cm^2 coupons with a height of one mm, sanded and polished. Subsequently, they were disinfected with ethanol (VWR International GmbH, Darmstadt, Germany) and stored in a protective gas atmosphere (nitrogen).

For the production of Cu_2O layers on pure copper surfaces these were sanded, polished, washed thoroughly with ethanol and air dried at room temperature. After exposure at 200 °C for 20 hours to air a Cu_2O layer was formed on top of the pure copper surface. For the formation of a CuO layer, no sanding and polishing of pure copper surfaces was necessary. The surfaces were washed thoroughly with ethanol, air dried at room temperature and heated for 150 minutes at a temperature of 350 °C. In contrast to the obtained layer after the exposure to 200 °C, initially a Cu_2O layer was build and on top of this the CuO layer was formed [127]. Both surfaces were slowly cooled down and stored in a protective gas atmosphere.

The eutectic materials were composed of aluminium (Al) (100 mol%), aluminium-silver (Al-Ag) (65.7 mol%, 34.3 mol%), aluminium-copper (Al-Cu) (82.5 mol%, 17.5 mol%) and aluminium-silver-copper (Al-Ag-Cu) (69.1 mol%, 18.1 mol%, 12.8 mol%). Each material has an individual melting point. This point is reached at a temperature of ~660 °C for Al, Al-Ag melts at a temperature of ~567 °C, Al-Cu at ~548 °C and Al-Ag-Cu at a temperature of ~501 °C.

For the experiments two different batches were casted. The first batch comprises all eutectic alloys while in the second batch only Al-Ag-Cu was casted. For both batches, each composition was weighed and filled in a hot crucible (760 °C). Inside, the components were slowly molten and stirred. After one hour, each component was completely molten and intermixed well. In case of the first, “fast” batch, the melting was poured into a mould with a diameter of 15 mm and a height of 100 mm. For the second, “slow” batch the melting was casted in a quadratic form of 16 cm x16 cm and a height of one – two cm. Afterwards, smaller pieces of this cast were molten, mixed and casted in a

12 mm mould. After cooling down at room temperature, the materials were eroded by the Böhm company (Troisdorf-Spich, Germany).

Once eroded, all surfaces were scrubbed and provided many possible adhesion points for microorganisms. Therefore, surfaces were standardized due to sanding which smoothed the surface structure. Different surface appearance, before and after sanding, are displayed in Figure 10.

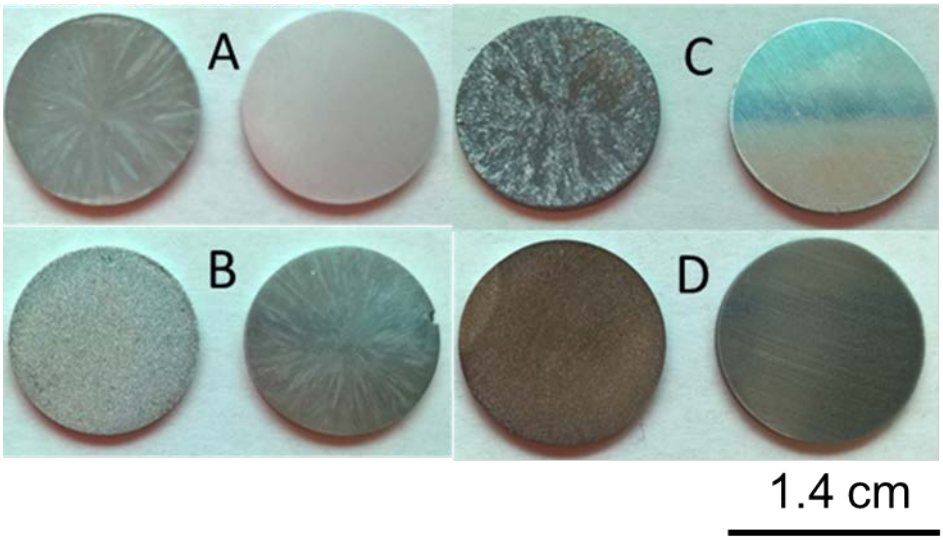


Figure 10: Eutectic alloys (A: Al; B: Al-Cu; C: Al-Ag; D: Al-Ag-Cu), the left surface displays samples before sanding and on the right after sanding.

2.5 Cultivation of bacteria

For the preparation of a working suspension one colony was picked and incubated in 10 ml of the respective medium in a test tube. Cells from the stationary phase were transferred in 15 ml Falcon-tubes, centrifuged for 15 minutes at 3000 x g, washed twice and resuspended in 10 ml buffer. Corresponding to the experiment requirements this buffer was either 0.1x PBS, 1x PBS or 0.1 mol/l MOPS.

2.6 Colony forming unit (CFU)

The determination of living cells, before treatment and after exposure on antimicrobial surfaces, was conducted by determining the number of colony forming units (CFUs). A 1:20 dilution of the working suspension or exposed cell suspension was prepared, followed by a 1:10 serial dilution. The last four dilution steps were then spread on

corresponding agar plates. The number of colonies could be determined by counting after one (e.g. *E. coli* K12) or three (e.g. *S. cohnii*) days of incubation. In case of cells exposed on antimicrobial surfaces, growth was delayed.

All CFU data were determined with the following calculation.

$$\frac{CFU}{ml} = \frac{(\sum_{i=1}^{\infty} x_i * 10^i) * a}{n}$$

x = counted colonies per dilution step

i = dilution step

n = counted dilution steps

a = dilution of the working suspension

For further analysis the relative survival of cells was determined by dividing the obtained CFU after the exposure to antimicrobial surfaces (N) by the CFU obtained after the exposure to stainless steel (N_0).

Data of the current study are mean values with standard deviations. Statistical significant differences were determined using Student's t-test whereby p-values < 0.05 were considered as significant.

2.7 Desiccation of cells

The ability of cells surviving desiccation was investigated with $5x10^6$ cells suspended in 50 µl 1x PBS in an 1.5 ml Eppendorf tube and air dried in the laminar flow (Hera Safe KS12, Thermo Scientific, Schwerte, Germany) for one day. The dried pellet was stored in the incubator (ICP 700, Memmert, Schwabach, Germany) at 30 °C. After one and seven days, the pellet was resuspended in one ml 1x PBS for 30 minutes at room temperature in a table-top shaker (TB1 Thermoblock, Biometra, Göttingen, Germany) and CFUs were determined.

2.8 Minimal inhibitory concentration (MIC)

To evaluate the effects of solubilized copper ions in solution on *E. coli* K12 and *S. cohnii*, the minimal inhibitory concentration (MIC) was determined. Therefore, 10 ml medium with different concentrations of $CuSO_4$ (VWR International GmbH, Darmstadt, Germany)

ranging from 0.1 mmol/l to 10 mmol/l were inoculated and incubated for up to 48 hours. On the basis of OD_{600} and CFU measurements the MIC was determined.

According to the MIC measurements of *E. coli* K12, the most important genes during $CuSO_4$ stress were determined for selected strains of the Keio collection. Overnight cultures were washed twice in 1x PBS and 1:100 dilutions were prepared in a 24 well plate containing 1.6 ml medium per well and $CuSO_4$ concentrations ranging from 1 mmol/l to 10 mmol/l. Growth was measured for 30 hours at 37 °C spectroscopically at OD_{600} in the TECAN Reader (TECAN infinite M200 pro, Tecan group, Männedorf, Switzerland). Before each measurement (every 30 minutes) the well plate was shaken in the TECAN Reader by the orbital shaker for 10 seconds.

2.9 Scanning Electron microscopy

Scanning Electron Microscopy (SEM) experiments were conducted to determine the distribution and structure of cells on antimicrobial surfaces. Dried samples were applied to the SEM and scanned with a focussed beam of electrons. Corresponding to the interaction of electrons in the beam with the sample, a loss of energy produces various signals that can be detected [128]. These signals contain information about the topography of the surfaces and the composition.

At the beginning of the experiment 10^4 to 10^8 washed cells/cm² of *E. coli* K12 and *S. cohnii* were air dried in a laminar flow on stainless steel surfaces. Single samples were placed in one well of an eight well plate and covered with 2 % glutaraldehyde (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in 0.1 mol/l sodium cacodylate buffer (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 20 minutes. This fixative solution was taken off and samples were covered two times with 0.1 mol/l sodium cacodylate buffer for five minutes (washing step). The third washing step lasted 10 minutes. Another fixation step with 1 % OsO_4 (Electron Microscopy Sciences, Hatfield, United Kingdom) in 0.1 mol/l sodium cacodylate buffer was conducted. As described before, the samples were washed three times with 0.1 mol/l sodium cacodylate buffer followed by three washing steps with ddH_2O. All washing steps lasted five minutes. Then, samples were dehydrated by covering them for five minutes with an increasing concentration of acetone (VWR International GmbH, Darmstadt, Germany) starting with 10 %, to 20 %, 40 %, 60 %, 80 % and finally 100 % of acetone. The last step in 100 % acetone was repeated three times, while the first step lasted five minutes; step two and three 10 minutes. After dehydration, samples were stored overnight at 4 °C in 100 %

acetone, followed by complete drying with the Critical Point Drying machine (CPD300, Leica Microsystems GmbH, Wetzlar, Germany) and finally sputtering with two nm Pt (Scancoat Six, Edwards, West Sussex, United Kingdom). All images were either conducted with the TM3000 (Hitachi, München, Germany) in Cologne, Germany or with the FE-SEM LEO 1530 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany) with Gemini optic in Regensburg, Germany. Images obtained with the TM3000 were conducted at an extra-high tension (EHT) of 15 kV. In case of FE-SEM LEO 1530 an EHT of 2 kV and the inlens detector was used. With these settings no precise determination of the surface structure but an accurate presentation of microorganisms was possible. All samples were analysed in high vacuum without an alteration of the pressure.

2.10 Contact killing experiments

To evaluate the antimicrobial efficiency of different surfaces, contact killing experiments were conducted as previously described by Molteni *et al*. (2010) [110]. Briefly, different organisms and cell concentrations were exposed in liquid on various surfaces. These surfaces were exposed at 30 °C in a water-filled desiccator to ensure the maintenance of a relative humidity of 83 %. The inactivation rate of the organisms was determined by CFU analysis after exposure (paragraph 2.6). Subsequent investigations about how damage occurs are described in the following paragraphs.

2.10.1 Cell survival after exposure to antimicrobial materials

Overnight cultures of *E. coli* K12 and *S. cohnii* were washed twice in 0.1x PBS and suspensions of different cell concentrations were prepared. The number of cells was measured by using the haemocytometer (Neubauer) followed by the determination of the optimal cell concentration to obtain a monolayer of cells on surfaces. Approximately, 10^8 to 10^4 cells/cm² of *E. coli* K12 and *S. cohnii* were exposed to stainless steel and pure copper surfaces for zero, two and four hours. According to the corresponding exposure times, CFUs were determined. Additionally, 10^6 cells/cm² of *E. coli* K12 and *S. cohnii* were exposed to copper oxide layers (Cu_2O and CuO) with a subsequent CFU analysis.

Beside the experiments conducted on stainless steel and pure copper surfaces, the putative antimicrobial effect of eutectic alloys (paragraph 2.4) was investigated. Overnight cultures of *E. coli* K12 and *S. cohnii* were washed twice with 0.1 mol/l MOPS and 10^6 cells/cm² were exposed. For a better comparison with cells exposed in 0.1x PBS, cell

suspensions in 0.1 mol/l MOPS were also exposed on pure copper and stainless steel surfaces.

Investigations with selected strains of the Keio collection were conducted with the wildtype, *E. coli ΔcopA*, *E. coli ΔcueO*, *E. coli ΔcueR*, *E. coli ΔcutA* and *E. coli ΔrecA*. Therefore, overnight cultures of 10^6 cells/cm² were resuspended: 1) in 0.1x PBS and exposed for up to four hours to stainless steel and pure copper surfaces and 2) in 0.1 mol/l MOPS and exposed for up to four hours on all materials including eutectic alloys.

2.10.2 Metabolic activity

To analyse the metabolic activity Alamar Blue staining (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was applied after the exposure of cells to different antimicrobial surfaces. In presence of reduced metabolites (e.g. FADH, NADH), Alamar Blue (resazurin) is reduced to pink resorufin. This shift was monitored spectrophotometrically at 570 nm and 600 nm as described previously [129, 130]. Overnight cultures of *E. coli* K12 and *S. cohnii* were washed twice in 0.1 mol/l MOPS and contact killing experiments were conducted for up to six hours. Thereby, approximately 10^6 and 10^5 cells/cm² were exposed on stainless steel, pure copper surfaces and eutectic alloys. To determine the metabolic activity, a 96 well plate was prepared with 200 µl medium, containing 39.8 mmol/l Alamar Blue. After the corresponding times, 40 µl of exposed cell suspension (containing 10^5 or 10^4 cells) was added. Afterwards, the well plate was incubated for 16 hours at 37 °C in the TECAN Reader. Every 30 minutes the well plate was shaken by the orbital shaker for 10 seconds and the OD_{570} and OD_{600} were measured to determine the reduction of the dye.

2.10.3 Membrane integrity

For the evaluation of membrane integrity the LIVE/DEAD staining assay (LIVE/DEAD® BacLight™ Bacterial Viability Kit (L13152) (Thermo Scientific, Schwerte, Germany)) was used [131]. The green dye Syto9 penetrates all membranes while the red dye propidium iodide only penetrates damaged membranes (loss of membrane potential). As a result the stained cells can be divided into two different conditions; green (Live) and red (Dead / Injured) cells. To visualize differences, the following two approaches were conducted:

Fluorescence microscopy

Overnight cultures of *E. coli* K12 and *S. cohnii* were washed twice in 0.1x PBS and 10^7 and 10^5 cells/cm² were exposed to stainless steel and pure copper surfaces for zero and four hours. After exposure, the whole suspension was taken off the surfaces, centrifuged for 10 minutes at 4000 x g (Z 216 MK, Hereaus, Thermo Scientific, Schwerte, Germany), washed twice in 200 µl sterile filtered ddH_2O (cellulose acetate filters, 0.2 µm, Whatman GmbH, Dassel, Germany) and resuspended in 50 µl sterile filtered ddH_2O. For staining, Syto9 and propidium iodide were added to the suspension with an end concentration of 6 µmol/l and 30 µmol/l, respectively. After incubation for 15 minutes in the dark at room temperature, fluorescence images were recorded with a fluorescence microscope (Axio Imager M2, Carl Zeiss MicroImaging GmbH, Göttingen, Germany) and evaluations were performed with the AxioVision 4.8.2 software and GIMP 2.6.12.

Flow cytometry

For additional quantification of the membrane integrity after exposure to stainless steel and pure copper surfaces, flow cytometry was conducted. Therefore, overnight cultures of *E. coli* K12 and *S. cohnii* were washed twice in 0.1x PBS and 10^7 and 10^5 cells/cm² were exposed for zero, one, two and four hours on the surfaces. After the corresponding times, cells were taken off the surfaces and centrifuged for 10 minutes at 4000 x g. The pellet was resuspended in 50 µl sterile filtered ddH_2O and stained with 6 µmol/l Syto9 and 30 µmol/l propidium iodide. Approximately, 900 µl sterile filtered 0.1x PBS was added and samples were measured with the flow cytometer FACScan (BD Biosciences). The FACScan contains an argon laser (488 nm) for excitation and three fluorescent emission detection channels (FL1 515-545 nm, FL2 564-606 nm, FL3 > 670 nm). These fluorescence channels can separate between red and green fluorescent cells. The voltage of the diode/photomultipliers were set as following: FSC: E01 (log), SSC: 375 V (log), FL1: 600 V (log), FL2: 150 V (log), FL3: 650 V (log), without compensation. All results were evaluated with the Flowing Software 2 (Freeware by Perttu Terho. Turku Center for Biotechnology. University of Turku, Finnland, http://flowingsoftware.com).

Before each experiment, five controls were measured to adjust the instrument settings. One sample was unstained and used to define the region of interest by reference to the cell size. This region was defined in a dot plot of FSC against SSC and used for all further dot plots. Another sample was stained green with Syto9 to optimize the voltage for FL1 (green fluorescence). FL3 (red fluorescence) was optimized with a propidium iodide stained control which was previously treated for one hour with 30 % H_2O_2. At last one

untreated and one stressed sample (one hour with 30 % H_2O_2) were stained with Syto9 and propidium iodide to determine the appearance of double stained cells. All obtained results are presented in dot plots, with the green fluorescence on the X-axis against the red fluorescence on the Y-axis (Figure 11).

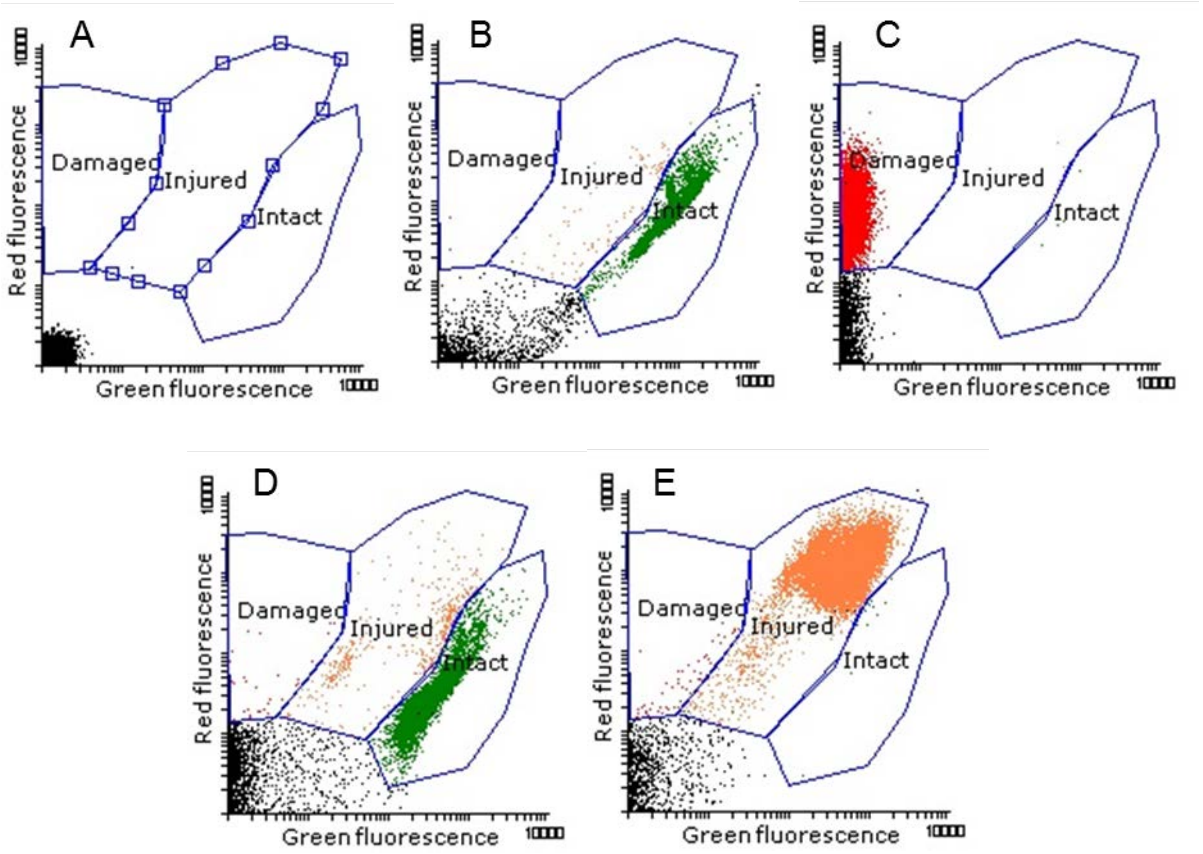


Figure 11: Dot plots for the control measurements of 10^5 *E. coli* K12 cells/cm² in 0.1x PBS. A: unstained control, B: green (Syto9) stained control, C: maximal (one hour treated with 30 % H_2O_2) stressed, red (propidium iodide) stained control, D: double stained, non-stressed control and E: double stained, maximal (one hour treated with 30 % H_2O_2) stressed control.

2.10.4 Detection of reactive oxygen species (ROS)

Exposure on antimicrobial surfaces could lead to production of reactive oxygen species which results in severe damaging effects. ROS were visualized by staining with CellROX Green (Thermo Scientific, Schwerte, Germany). The dye is non fluorescent in the reduced state and cell-permeable. After oxidization by ROS and binding to DNA, a green photo stable fluorescence is exhibited which has an excitation/emission maximum of ~ 485/520 nm [132]. Overnight cultures of *E. coli* K12 were washed twice in 0.1x PBS and 10^7 and 10^5 cells/cm² were exposed to stainless steel and pure copper surfaces for zero, one, two

and four hours. After the corresponding times, cells were taken off the surfaces and one µl CellROX Green was added. The samples were incubated for 30 minutes in dark at room temperature and directly measured with the FACScan. The instrument settings were: FSC: E01 (log), SSC: 375 V (log), FL1: 800 V (log), FL2: 150 V (log), FL3: 150 V (log), without compensation. All results were evaluated with the Flowing Software 2 (Freeware).

For each experiment, three controls were prepared to determine the instrument settings. Thereby, one sample was unstained to define the region of interest by reference to the cell size. This region was defined in a dot plot of FSC against SSC and used for all further dot plots. Another sample was treated for one hour with 30 % H_2O_2 and stained with CellROX green to optimize the voltage for FL1 (green fluorescence). Additionally, one untreated sample was stained to determine ROS production of non-stressed cells. Figure 12 displays the results for the control measurements. Counts were plotted on the Y-axis against the green fluorescence (relative ROS production) on the X-axis.

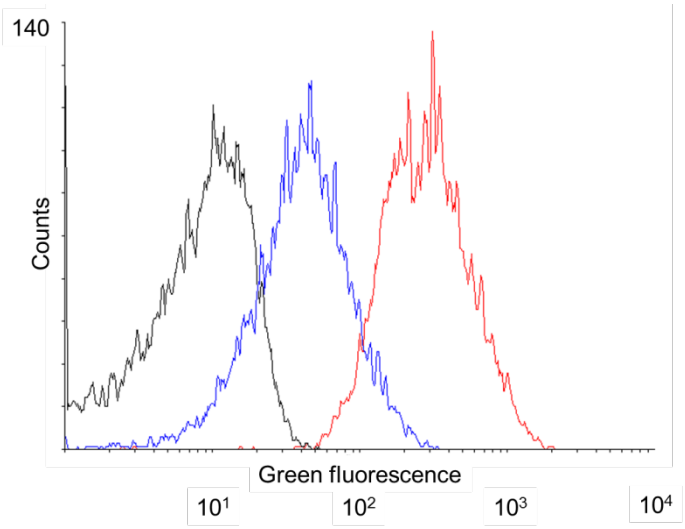


Figure 12: Control measurement to determine ROS production in *E. coli* K12 with CellROX green. Events of the unstained, non-treated control (black peak), non-treated stained control (blue peak) and maximal (one hour treated with 30 % H_2O_2) stressed and stained control (red peak) were plotted against the measured green fluorescence.

2.10.5 Nucleic acid integrity

To detect whether DNA or RNA damage occurs after the exposure to pure copper and stainless steel surfaces, experiments with DNA and RNA were conducted with 10^7 *E. coli* K12 cells/cm². For *ex vivo* experiments the isolation of DNA and RNA was achieved before the exposure and in case of *in vivo* experiments the isolation was performed after the exposure.

For *in vivo* analysis 10^7 cells/cm² were exposed on stainless steel and pure copper surfaces at a temperature of 30 °C and a relative humidity of 83 % (paragraph 2.10). After the corresponding times of zero, one, two and four hours 10^8 cells were used for RNA and DNA isolation.

Additionally, RNA and DNA were isolated from 10^8 cells for *ex vivo* experiments. For the measurement, 15 µl RNA and DNA solution was exposed on 0.12 cm² of the surfaces. The purity was measured in a spectrophotometer (NanoDrop™ 2000c Spectrophotometer, Thermo Scientific, Schwerte, Germany). Further analysis was conducted with gel electrophoresis (gel electrophoresis apparatus, Biostep, Burkhardtsdorf, Germany; power supply: major science mini - 300, Saratoga, USA) and Bioanalyzer (Bioanalyzer 2100r, Agilent Technologies, Karlsbrunn, Germany) as described in the following paragraphs.

DNA and RNA isolation

DNA was isolated with the peqGOLD Bacterial DNA Kit and RNA with the peqGOLD Bacterial RNA Kit. All buffers except Lysostaphin were available in the Kit. An overnight culture of *E. coli* K12 or *S. cohnii* was washed twice with 0.1x PBS. Approximately, 10^8 cells were lysed with either 10 µl Lysozyme (10 mg/ml) in case of *E. coli* K12 or 10 µl Lysostaphin (5 mg/ml; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for *S. cohnii* [133]. Followed by the instructions of the Kits, DNA and RNA were isolated. Before continuing, the quantity and purity was determined with Nanodrop. For experiments, DNA was either used directly or after storage in Elution buffer at -20 °C. In case of RNA experiments, RNA was either used directly or after the storage in RNAse free water at a temperature of -80 °C.

Absorption measurement

The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. Approximately, one µl DNA or RNA sample was applied to the spectrophotometer Nanodrop which then conducted a wavelength scan of absorbance. A ratio of pure DNA is around 1.8 and for RNA 2.0 [134]. The scan also provides first hints of possible molecule fragmentations. These fragmentations were visualized with gel electrophoresis. Additionally, fragmentation of RNA was visualized by capillary gel electrophoresis using the Bioanalyzer.

Gel electrophoresis

Gel electrophoresis was used to detect possible fragmentations of DNA and RNA. Genomic DNA was separated in a 0.8 % agarose gel (Serva Electrophoresis GmbH, Heidelberg, Germany) and RNA in a 2 % agarose gel. Exactly, 100 ml 1x TAE buffer containing 0.8 % or 2 % agarose was boiled until the agarose was completely dissolved. After cooling down to 40 °C, two µl ethidium bromide (10 mg/ml, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) was added and well mixed. The solution was then poured into the gel equipment with a well forming comb and completely cooled down until the gel was cured. After the removal of the comb the gel was placed in the electrophoresis unit and completely covered with 1x TAE buffer. Then 300 ng of each sample, mixed with two µl loading dye was loaded to the chambers. For DNA a High Range DNA Ladder (Fermentas, Thermo Scientific, Schwerte, Germany) and for RNA the RiboRuler High Range RNA Ladder (Thermo Scientific, Schwerte, Germany) was loaded on the gel. After 30 minutes of separation by applying an electric field of 7 V/cm the different bands were visualized by using the ImageQuant LAS4000 (GE Healthcare Life Sciences, Freiburg, Germany) and labelled in Microsoft PowerPoint 2010.

Capillary gel electrophoresis

For detailed analysis of RNA fragmentation, samples were measured by using the Bioanalyzer and the Agilent RNA 6000 Nano Kit. The principle of the Bioanalyzer is based on capillary electrophoresis which leads to the separation of fragments. At the beginning of this experiment the gel-dye mix was prepared according to the manufacturer's instructions and loaded on the RNA Nano chip. Then the Marker was added in each well followed by the application of the samples and the RNA ladder. Finally, the chip was transferred to the Bioanalyzer and measured with the program "Prokaryote total RNA Nano Series II.xsy" of the 2100 expert software. The obtained results were labelled in Microsoft PowerPoint 2010.

2.10.6 Measurement of ion release

The concentration of released ions was measured with the inductively coupled plasma mass spectrometry (ICP-MS). The nebulized sample was ionized in plasma, which was obtained by a high frequency field of alternating current of 10000 K argon gas [135]. The ionized particles were separated towards their mass and charge through a quadrupole-MS. The ion current proceeds through four parallel conductive bars. By suitable

superposition of opposite DC voltage and phase shifted, high-frequency AC voltages, ions are accelerated and forced on a spiral path. The frequency and amplitude of the applied high frequency voltage can be used as filters. These filters are adapted with the result that ions with a certain mass-to-charge ratio can pass the analyser exclusively with an approximate detection limit for Al, Ag and Cu of 10 ppt (personal communication with Dr. Christine Hein, Saarland University, Germany).

In three different experimental setups, the release rate of copper ions after contact killing was determined. First of all, *E. coli* K12, *E. coli ΔcopA* and *S. cohnii* were washed twice in 0.1x PBS and applied to stainless steel and pure copper surfaces. The second experiment was conducted with *E. coli* K12 washed twice in 0.1 mol/l MOPS and exposed to stainless steel, pure copper surfaces and eutectic alloys. Additionally to contact killing experiments, *E. coli* K12 was exposed in 0.1x PBS containing different concentrations of $CuSO_4$.

For both contact killing experiments, 10^6 cells/cm² were exposed for zero, one, two, three and four hours. To determine the difference between the cell associated copper concentration and the supernatant the whole suspension was centrifuged for 10 minutes at 6000 x g and the supernatant separated from the pellet. Cell associated copper comprises of copper ions inside the cell and that adhered to the cell membrane (Figure 13).

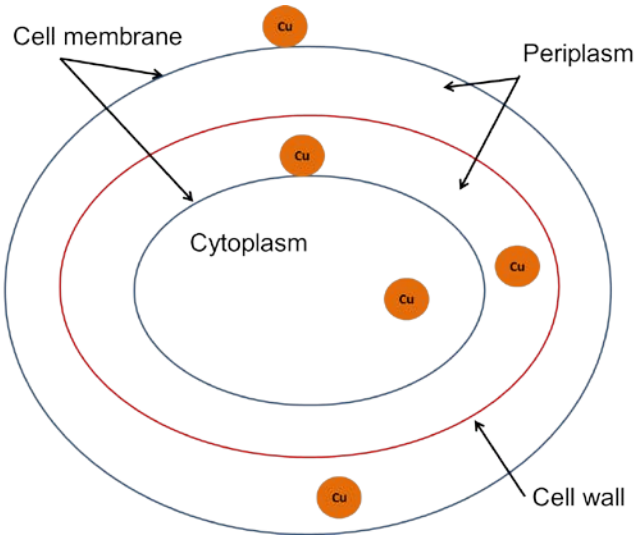


Figure 13: Schematic image of an *E. coli* cell and cell associated copper ions.

The pellet was resuspended in 350 µl 0.1 x PBS and cells were lysed by using 10 µl Lysozyme (10 mg/ml, Serva Electrophoresis GmbH, Heidelberg, Germany) and 10 µl DNAse (10 mg/ml, Serva Electrophoresis GmbH, Heidelberg, Germany) at a temperature of 30 °C for 30 minutes. In case of *S. cohnii* 10 µl Lysostaphin (5 mg/ml) was used instead of Lysozyme. Each sample (350 µl) was diluted 10-fold in ddH_2O, containing 150 µl

0.065 % nitric acid (VWR International GmbH, Darmstadt, Germany) and 3.5 µl of an internal standard (10 ppm Ho, 10 ppm Sc). The copper concentration was quantified by using the ICP-MS 7500 Series, Agilent Technologies (Inorganic solid state chemistry, Saarland University, Saarbrücken, Germany) which runs with the power of 1550 W in the Spectrum Multi Tune mode. The flow rate of the carrier gas was 1.05 l/min and of the plasma cooling gas 15 l/min. As internal standard ^{165}Ho and ^{45}Sc were applied to each sample and measured additionally to ^{63}Cu, ^{107}Ag and ^{27}Al. The sample was pumped with a velocity of 0.3 rps (rounds per seconds) until equilibrium was reached. Thereafter, during measurement of the sample the pumping velocity was 0.1 rps. Previously to each measurement a copper, silver and aluminium calibration curve was recorded. All data were accumulated in triplicates for 100 msec and listed as cps (counts per second). The results, given in ppb or ng/ml, were analysed by using the calibration curve.

Calculation of released aluminium, silver and/or copper ions and cell associated copper ions was conducted as followed. The obtained concentration (x) in ppb or ng/ml was multiplied by 3.5 ml to determine the total amount of copper in ng in the whole sample volume.

$$A = x * 3.5\,ml\ *\ 10^{-9}$$

A = total amount of copper [g]

After that, these values were corrected by subtracting the blank value of PBS or MOPS.

$$B = A - PBS_0\ or\ MOPS_0$$

B = corrected data [g]

PBS_0 or $MOPS_0$ = blank PBS or MOPS value [g]

Two different calculations were conducted afterwards. One calculation was related to the amount of released aluminium, silver and copper ions in solution. The other measurement determined the cell associated ion concentration in *E. coli* K12, *E. coli* Δ*copA* and *S. cohnii* after the exposure to antimicrobial surfaces.

The total amount of released ions after exposure was achieved through addition of the obtained data for the cell pellet and the supernatant. For the calculation it was assumed that 63.55 g copper (107.87 g silver and 26.98 g aluminium) in solution corresponds to a one molar solution. The following equations were conducted for copper ions.

$$C = \frac{B * 1000}{63{,}55}$$

C = copper ions [mmol]

To determine the cell associated ion concentration the following calculations were conducted. Approximately 3.5×10^{6} cells were exposed (y) and the wet weight (z) of *E. coli* K12 and *S. cohnii* was assumed as 9.5×10^{-13} g/cell [136].

$$D = \frac{B}{y}$$

y = number of exposed cells [cells]

D = amount of copper [g/cell]

$$E = \frac{D * 100\ \%}{z}$$

z = wet weight of one cells [g/cell]

E = cell associated copper concentration [%]

3. Results

Desiccation tolerance

Desiccation tolerance was examined for several environmental isolates and human associated strains, however not considered further (see paragraph 4). Figure 14 presents representative survival data of the Gram-positive *Staphylococcus cohnii* and Gram-negative *Escherichia coli* K12 in addition to the well-known model organism *Deinococcus radiodurans* R1after desiccation for one and seven days ($5x10^6$ cells per sample).

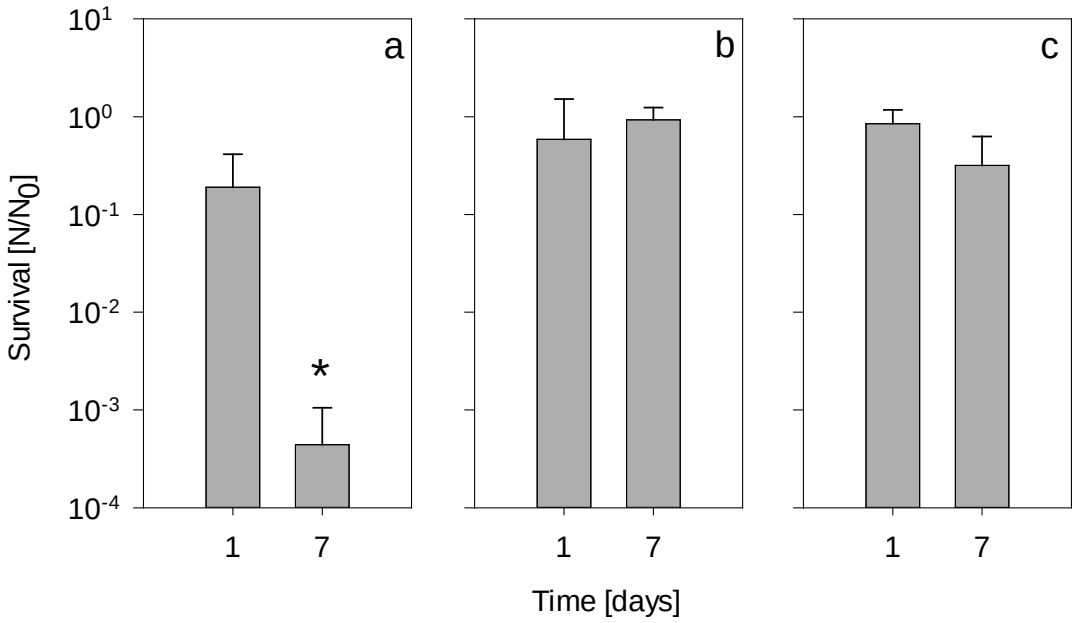


Figure 14: Survival of $5x10^6$ cells per sample after desiccation. The obtained CFUs after desiccation at 30 °C (N) were divided by the obtained CFU before desiccation (N_0) (n = 3). a: *E. coli* K12, b: *D. radiodurans* R1, c: *S. cohnii*, * significant decrease in cell survival (p-value < 0.05).

Concerning the survivability after seven days of desiccation, *D. radiodurans* R1 and *S. cohnii* were not influenced unlike *E. coli* K12 which was decreased in cell survival after seven days of desiccation by three orders of magnitude. Nevertheless, *D. radiodurans* R1 was not selected for further experiments.

Minimal inhibitory concentration (MIC) of $CuSO_4$

The following experiments were conducted with *E. coli* K12 and *S. cohnii*. Both strains exhibit similar growth conditions but differ in their cell wall and membrane structure. To identify the copper concentration which was needed to inhibit cell growth, the MIC of $CuSO_4$ in medium was determined for concentrations ranging between 0.1 mmol/l to 10 mmol/l. The obtained $CuSO_4$ MIC for *E. coli* K12 was 5 mmol/l and for *S. cohnii* 7 mmol/l.

Further experiments, so called contact killing experiments, were conducted on antimicrobial surfaces. To determine the antimicrobial effect on all cells, the prevention of shielding effects and a direct contact of cells with the surface were of major concern. This was achieved for cells lying in a monolayer. Therefore, investigations to obtain optimal cell concentrations for a monolayer of cells were conducted with SEM analysis.

SEM analysis of cell distribution

SEM images of different cell concentrations were evaluated and visualized in Figure 15. Therefore 10^8 to 10^5 cells/cm² were exposed to stainless steel surfaces.

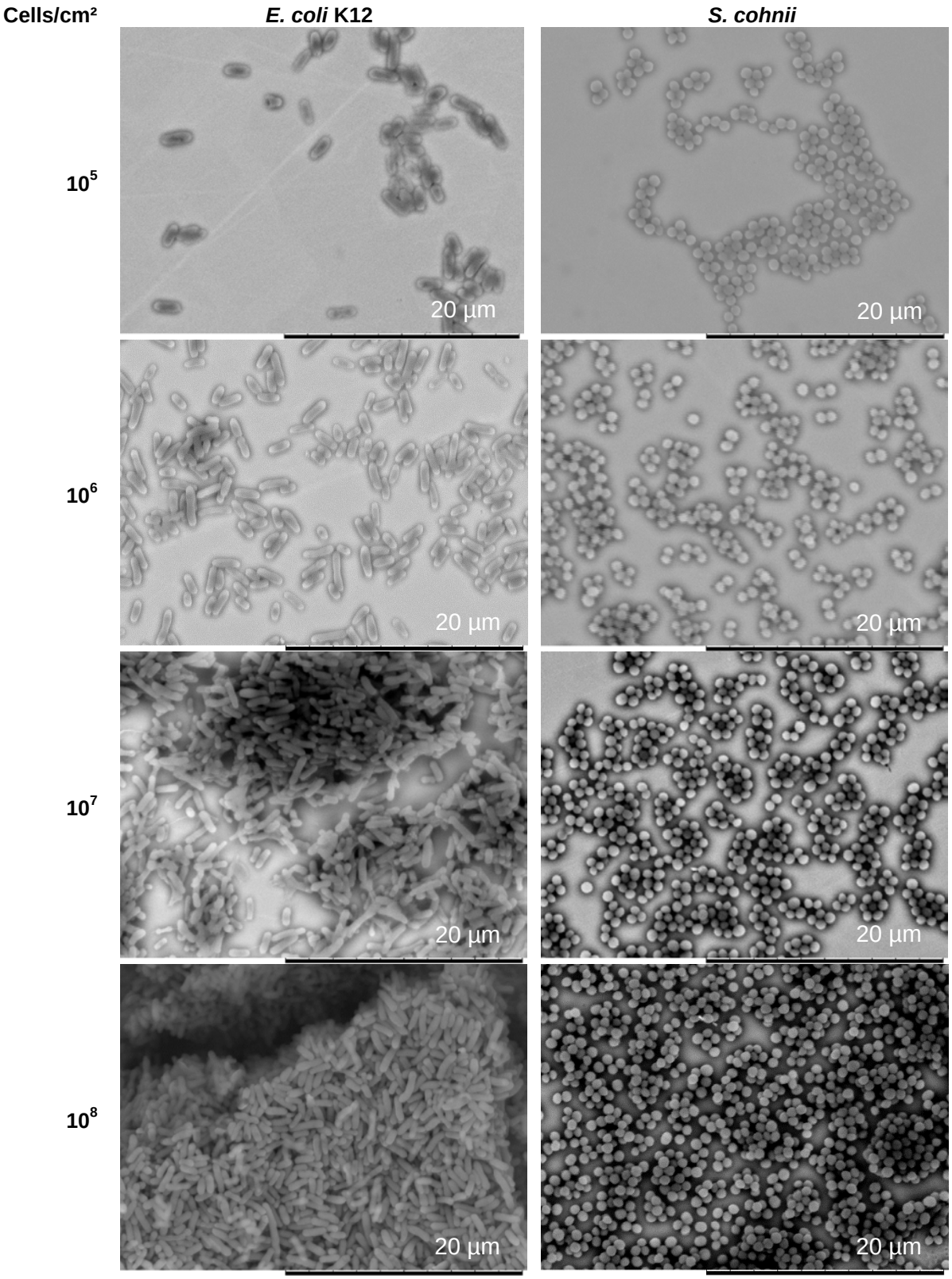


Figure 15: SEM images of 10^8 to 10^5 cells/cm² of *E. coli* K12 and S*. cohnii* applied in 0.1x PBS to stainless steel surfaces.

SEM images showed that an application of 10^8 and 10^7 cells/cm² led to the formation of multilayers on the surface. After the application of 10^6 cells/cm², most of the cells were in

direct contact with the surfaces. Only a couple of cells were detected to be on top of other cells. A complete monolayer of cells was determined after an application of 10^5 cells/cm².

3.1 Contact killing on pure copper surfaces

This chapter comprises the survival data of different cell concentrations exposed to pure copper surfaces and a comparison of cell survival after the exposure to Cu_2O and CuO layers. Additionally, the release of copper ions, ROS production and damages of the membrane, DNA and RNA after exposure to pure copper surfaces was conducted.

3.1.1 Optimal cell concentration for exposure

To confirm the results obtained by SEM analysis and to determine the antimicrobial effect of pure copper surfaces to a multi- and a monolayer of cells, contact killing experiments with cell concentrations from 10^8 to 10^4 cells/cm² were evaluated by CFU analysis. As expected, no significant decrease in cell survival was detected for stainless steel exposed samples. Therefore these results were used as controls (N_0) in the following experiments.

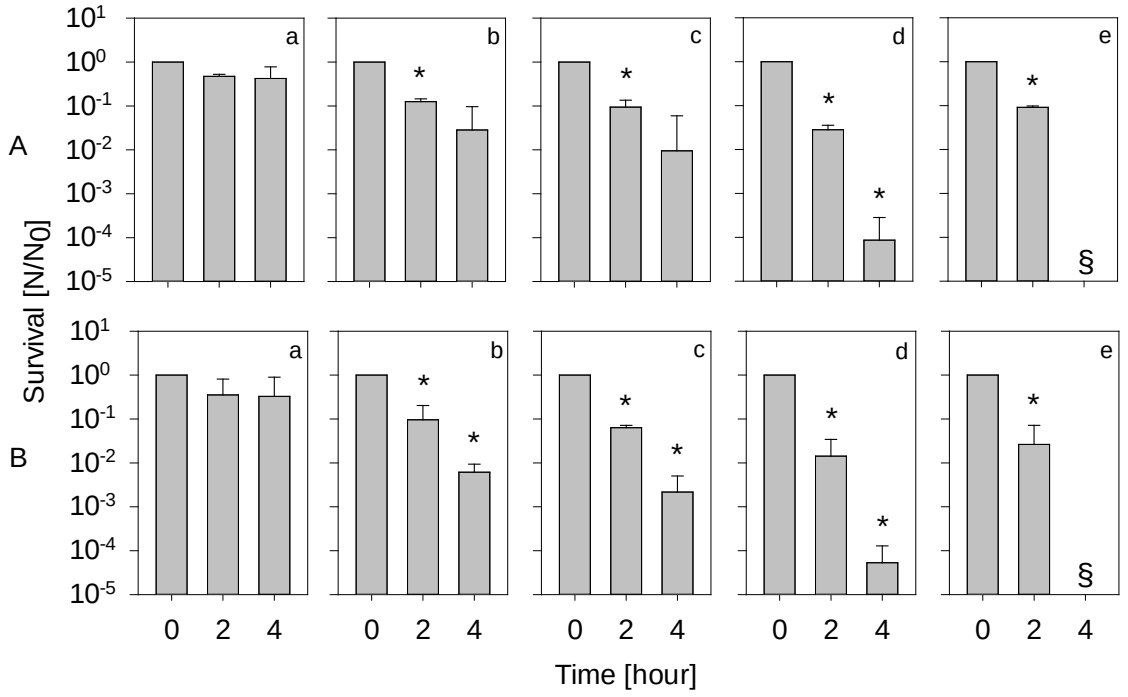


Figure 16: Survival of *E. coli* K12 (A) and *S. cohnii* (B) suspended in 0.1x PBS after the exposure to stainless steel (N_0) and pure copper surfaces (N) (n = 3). a: 10^8 cells/cm², b: 10^7 cells/cm², c: 10^6 cells/cm², d: 10^5 cells/cm² and e: 10^4 cells/cm²; * significant decrease in cell survival (p-value < 0.05), § below detection threshold.

After the exposure of 10^8 cells/cm² of *S. cohnii* and *E. coli* K12 for two and four hours to pure copper surfaces, no significant differences in cell survival were determined. Opposed to this, exposure of 10^7 to 10^4 cells/cm² for two hours led to equal reduction in cell survival of around one to two orders of magnitude. Exposure for four hours resulted in higher variances of cell survival. An exposure of 10^7 *E. coli* K12 cells/cm² led to a decrease in survival by 1.5 orders of magnitude, while the survival of *S. cohnii* cells decreased by two orders of magnitude. The survivability of 10^6 *E. coli* K12 cells/cm² decreased by two and in case of *S. cohnii* by 2.6 orders of magnitude. After the exposure of 10^5 cells/cm², both strains were reduced in cell survival of about 4.2 orders of magnitude, whereas no survival was detected for both strains after an exposure of 10^4 cells/cm² for four hours to pure copper surfaces.

These results pointed out that no significant differences in the survivability of *E. coli* K12 and *S. cohnii* existed. This in turn possibly indicates that differences in the cell membrane and cell wall have no impact on cell survival after contact killing on pure copper surfaces.

3.1.2 Influence of different copper ions

From pure copper surfaces Cu^{+} and Cu^{2+} can be dissolved whereby both ions possess different contact killing effects on cells. To determine which copper ion has the most toxic effect, 10^6 cells/cm² of *E. coli* K12 and *S. cohnii* were exposed to copper oxide layers (CuO and Cu_2O – see Figure 34) which were thermally prepared on pure copper surfaces.

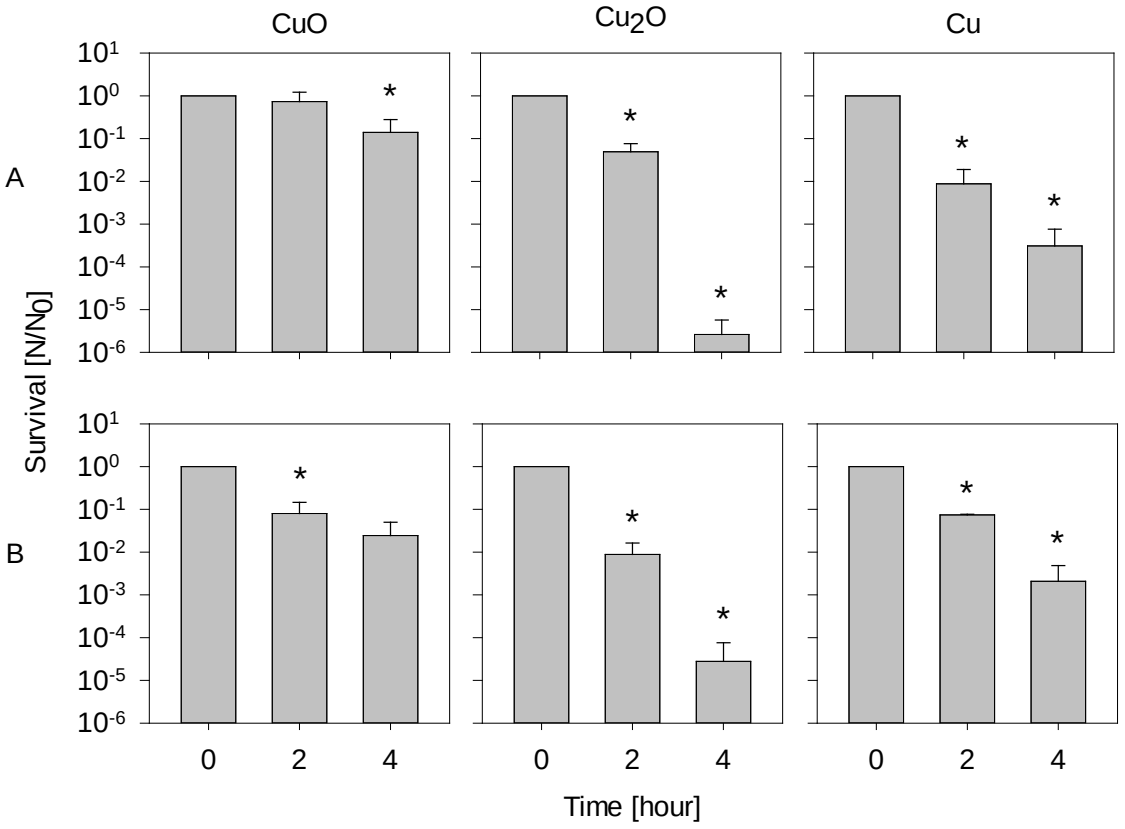


Figure 17: Survival of 10^6 cells/cm² of *E. coli* K12 (A) and *S. cohnii* (B) suspended in 0.1x PBS after the exposure to pure copper surfaces and copper oxide layers (N) and stainless steel (N_0) (n = 3). * significant decrease in cell survival (p-value < 0.05).

After four hours of exposure on CuO layers, cell survival decreased by one order of magnitude for *E. coli* K12 and 1.5 for *S. cohnii*. Contrary to this, cell survival decreased after four hours of exposure on Cu_2O layers by 4.5 orders of magnitude in case of *S. cohnii* and 5.5 for *E. coli* K12. This indicates that the most effective copper oxide layer was Cu_2O, from which Cu^{+} ions are mostly dissolved, followed by the pure copper surface (results were obtained from paragraph 3.1.1). The CuO layer was also effective in killing bacteria but the toxic effect was not as strong as for the Cu_2O layer or pure copper surfaces.

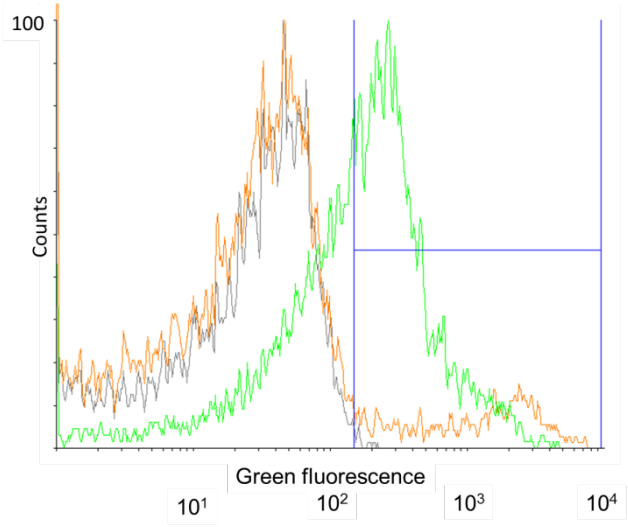
3.1.3 Damaging effects of copper ions on different cellular components

Several experiments related to ROS production, membrane integrity, DNA and RNA fragmentation were implemented to identify damaging effects caused by copper ions.

Production of reactive oxygen species (ROS)

Intracellular ROS production was monitored by using CellROX green followed by subsequent flow cytometry. An exposure of 10^7 *E. coli* K12 cells/cm² to stainless steel and pure copper surfaces and analysis of ROS production did not result in significant changes in ROS production. Therefore the following experiments were conducted with 10^5 *E. coli* K12 cells/cm² exposed to stainless steel and pure copper surfaces. Subsequently, cells were stained and intracellular ROS production measured.

Figure 18 presents the obtained results for an unstained control in comparison to four hours stainless steel and pure copper exposed samples. The grey peak presents the unstained control and by reference to this peak a region of interest was set. Every cell which was obtained in this region exhibited green fluorescence whereby less than 5 % of the unstained cells were determined in this region. The four hours on stainless steel exposed sample (orange peak) displays that a few cells were determined to exhibit green fluorescence. Thereby, a much higher detection of green fluorescence was obtained for four hours copper exposed sample (green peak).



	Exposure [hour]	ROS production [%]
Control	**0**	11
Steel	**1**	16
	4	15
Copper	**1**	59
	2	62
	4	58

Figure 18: ROS production in *E. coli* K12 was determined with CellROX green after the exposure of 10^5 cells/cm² for zero, one, two and four hours to stainless steel and pure copper surfaces. In the Histogram, events of the unstained, non-treated control (grey peak), stainless steel (orange peak) and pure copper (green peak) exposed samples were plotted against the measured green fluorescence. Only 5 % of unstained cells were present in the blue marked region. This region contains fluorescent cells which exhibit higher fluorescence and therefore higher ROS production. Percentages of CellROX green fluorescent cells are presented in the table.

Non-stressed cells possess a general ROS production which was determined to be 11 % (control). A slight increase in ROS production, which is in the range of the measurement accuracy, of about 4 % was obtained for stainless steel exposed samples. In contrast to this, exposure to pure copper surfaces led to a rapid increase in ROS production. The maximum was reached after one hour of exposure with 59 % and did not significantly change after longer exposure.

These results indicate that ROS production occurs quickly during contact killing in cells. To investigate further impact of copper ions, experiments were conducted to determine membrane, DNA and RNA damages.

Membrane integrity

Previous research has shown that copper ions have negative effects on the membrane [83]. To confirm these results, experiments on the membrane integrity were investigated. *E. coli* K12 and *S. cohnii* cells were exposed to stainless steel and pure copper surfaces, subsequently stained with the LIVE/DEAD kit and analysed via fluorescence microscopy and flow cytometry.

For the visualization of membrane damage, the LIVE/DEAD kit contains two different staining dyes. The green dye (Syto9) stains all cells whereas the red dye (propidium iodide) only penetrates membrane damaged cells. Figure 19 displays the obtained results after microscopic analysis. Approximately 10^7 and 10^5 cells/cm² were exposed to stainless steel and pure copper surfaces. Similar to contact killing experiments, no differences in membrane integrity were obtained for stainless steel exposed samples. For 10^7 cells/cm² exposed to pure copper surfaces only a couple of cells (around 10 %) were red stained. The majority of cells were green stained which indicates an intact membrane potential. After the exposure of 10^5 cells/cm² to pure copper surfaces the number of cells which were red stained increased for *E. coli* K12 and *S. cohnii* cells up to 75 %.

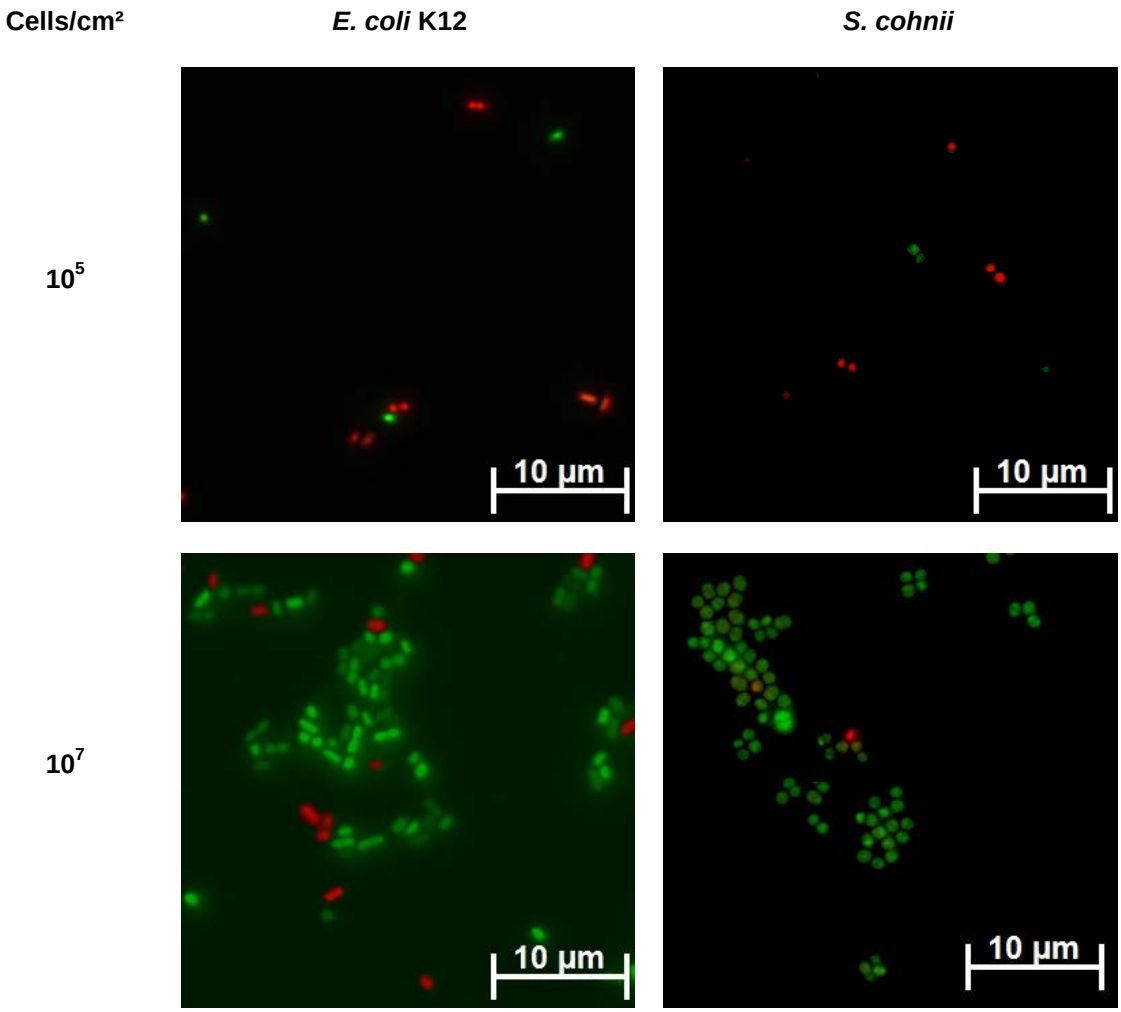


Figure 19: Fluorescence microscopy image of 10^7 and 10^5 cells/cm² of *E. coli* K12 and *S. cohnii* after the exposure of four hours in 0.1x PBS to pure copper surfaces. Cells were stained with Syto9 and propidium iodide and analysed with fluorescence microscopy.

To quantify these measurements, further experiments were conducted with flow cytometry.

Similar to the ROS experiments, an exposure and analysis of 10^7 *E. coli* K12 cells/cm² did not result in significant changes of the membrane integrity. Therefore investigations were performed with 10^5 cells/cm² exposed to stainless steel and pure copper surfaces. The samples were subsequently LIVE/DEAD stained and analysed with flow cytometry as previously described in paragraph 2.10.3.

After the classification of the regions of interest, the surface exposed samples were measured and the obtained results listed in Table 2. Here, only green and the sum of red and orange fluorescent cells are listed and named as injured. The reason for this is explained by the results obtained in Figure 11. After the control measurements, it became obvious that a double staining of maximally (one hour treated with 30 % H_2O_2) stressed cells led to orange fluorescent cells. This was attributed to the existence of both dyes in cells which possess membrane damage. The regions of interest were modified depending on each single experiment; therefore the results vary by a few percent.

Table 2: Percentage of alive (green) and injured (orange) fluorescent *E. coli* K12 cells after the exposure of 10^5 cells/cm² to stainless steel and pure copper surfaces.

	Control	Steel		Copper		
Exposure	**0 hour**	**1 hour**	**4 hours**	**1 hour**	**2 hours**	**4 hours**
Alive (Green)	77 %	66 %	74 %	39 %	28 %	19 %
Injured (Orange)	21 %	33 %	25 %	60 %	72 %	80 %

Approximately 21 % of the control sample was orange stained (injured) and 77 % green (alive). Similar results were obtained after the exposure of up to four hours on stainless steel. Here, 74 % of all cells were green stained. For samples exposed on pure copper surfaces, the viability decreased rapidly as indicated by the loss of green fluorescence. After one hour on pure copper only 39 % of all cells were detected with an intact membrane. Longer exposure on pure copper led to further decrease in the amount of alive cells. After four hours of exposure, only 19 % of all cells were detected as being alive.

Based on these intriguing results, further investigations into how copper is interacting with the DNA and RNA were conducted. To identify whether and to what extend DNA and RNA damages occur under copper stress conditions further analysis was conducted.

Nucleic acid fragmentation

Damages of the DNA and RNA refer to the binding of copper ions possibly followed by fragmentation of these molecules. *Ex vivo* and *in vivo* experiments were conducted to determine these damages. For *in vivo* experiments, 10^7 cells/cm² were exposed to

stainless steel and pure copper surfaces and subsequently genomic DNA and total RNA were isolated. The final amount of exposed, isolated DNA was 1800 ng and in case of RNA 5000 ng was exposed. For *ex vivo* analysis, the genomic DNA and total RNA were isolated and 15 µl DNA (540 ng) and RNA (2500 ng) solution was exposed to 0.12 cm².

The visualization of DNA fragmentation was confirmed by agarose gel electrophoresis (Figure 20) whereas RNA fragmentation was confirmed by experiments with a chip based technique, the Bioanalyzer (Figure 21).

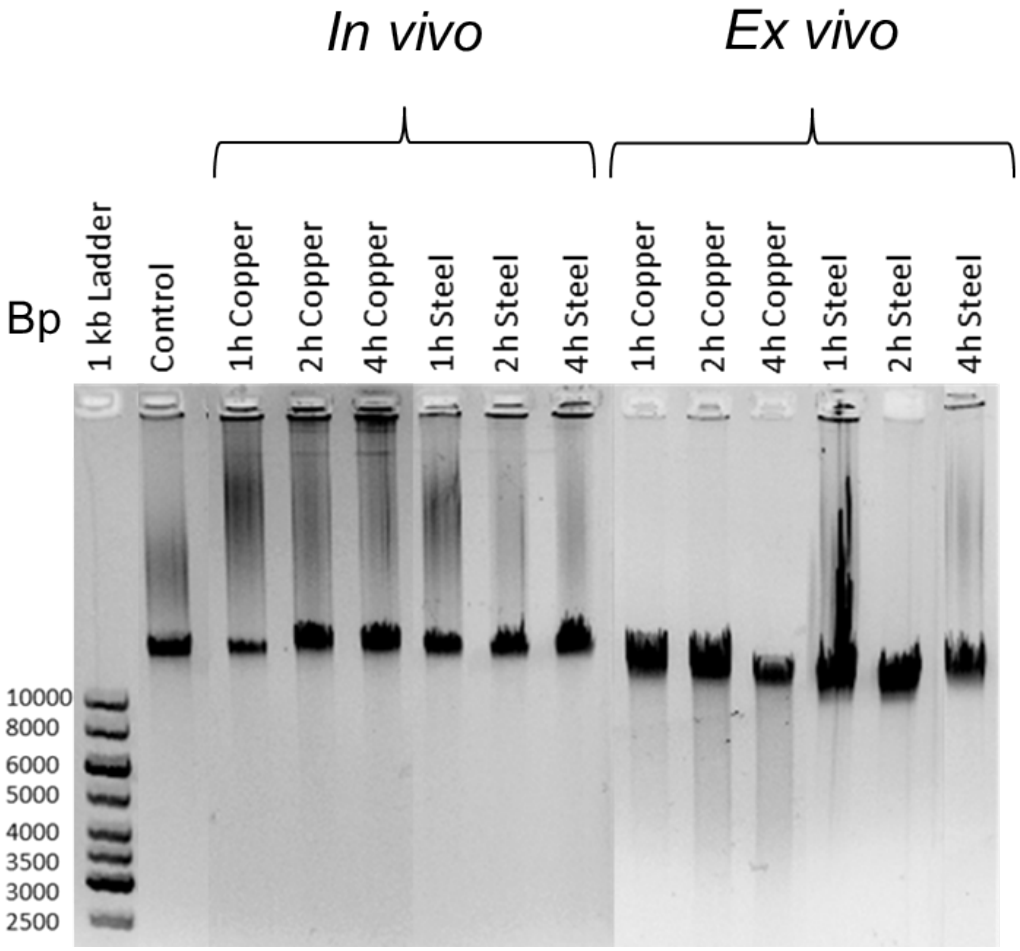


Figure 20: A typical gel electrophoresis image of *in vivo* and *ex vivo* exposed DNA separated in a 0.8 % agarose gel; Bp indicates base pairs. For *in vivo* analysis, 10^7 cells/cm² were exposed to stainless steel and pure copper surfaces. For *ex vivo* analysis, 15 µl DNA solution was exposed to 0.12 cm² stainless steel and pure copper surfaces. After the exposure and isolation 300 ng of each solution was separated on the agarose gel.

In case of *in vivo* stainless steel and pure copper exposed samples and *ex vivo* stainless steel exposed samples, apparently no fragmentation was detected. Fragmentation was only detectable for pure copper, *ex vivo* exposed samples. After one hour of exposure on pure copper surfaces slight fragmentation was determined, this increased with further

exposure times. Following these experiments, *in vivo* and *ex vivo* exposure of RNA on stainless steel and pure copper surfaces were conducted.

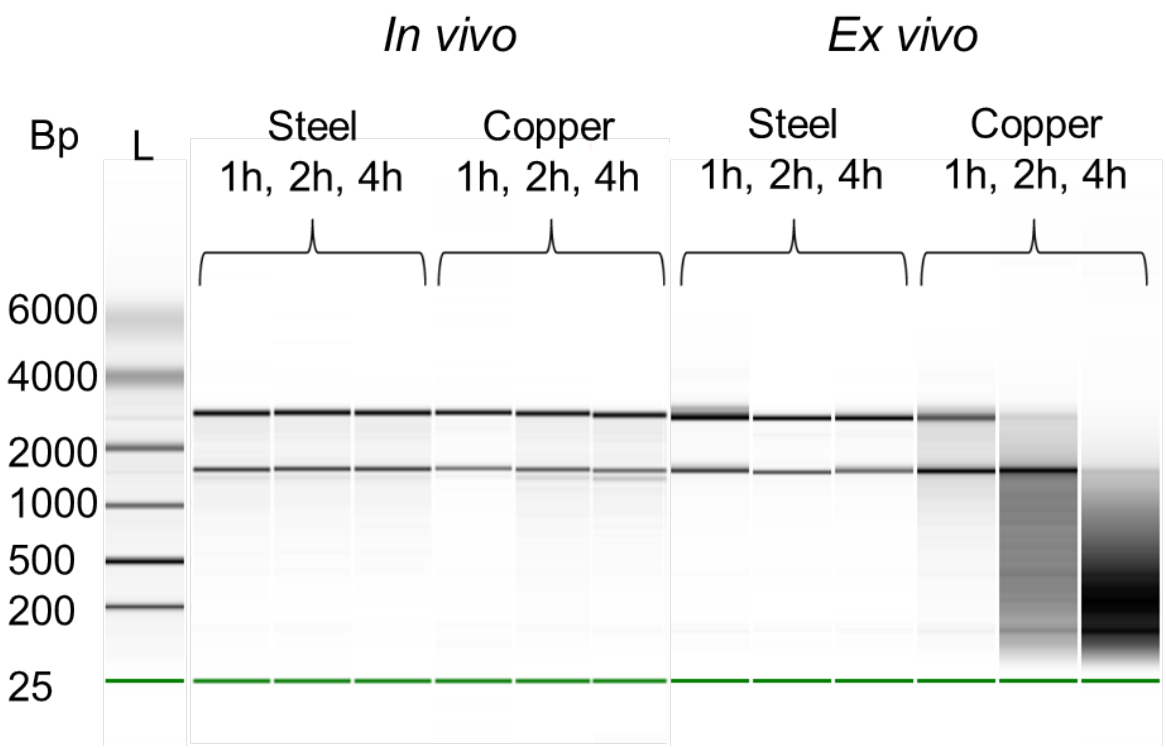


Figure 21: A typical image of *in vivo* and *ex vivo* chip based electrophoresis with 300 ng *E. coli* K12 RNA; Bp indicates base pairs and L is the abbreviation for the Ladder. For *in vivo* analysis, 10^7 cells/cm² were exposed to stainless steel and pure copper surfaces. For *ex vivo* analysis, 15 µl RNA solution was exposed to 0.12 cm² stainless steel and pure copper surfaces. After the exposure and isolation, the RNA was separated with the Bioanalyzer.

The obtained results in Figure 21 display fragmentation of different RNA samples. Intact RNA is represented by two distinct band patterns, referring to 16 S RNA and 23 S RNA. The 16 S RNA was detected at 1500 base pairs and the 23 S RNA at 2900 base pairs. Similar to DNA experiments, apparently no fragmentation was detected for *in vivo* samples and *ex vivo* stainless steel exposed samples. A fragmentation was only detected for *ex vivo* pure copper exposed samples. After one hour of exposure, a minor fragmentation was detected. Both RNA fragments and only some smaller fragments in comparison to the control and stainless steel exposed samples were detected. The fragmentation increased after two hours of *ex vivo* exposure. Most fragments of the 23 S RNA were degraded and the amount of smaller fragments increased. Further exposure of up to four hours led to a complete disappearance of 23S RNA fragments. Additionally, most of the 16 S RNA fragments were also degraded and a lot of smaller fragments were detected.

Beside the DNA and RNA separation images obtained after exposure to stainless steel and pure copper surfaces, similar results were obtained by spectrophotometric measurements of the DNA and RNA purity. Results obtained from the 260/280 ratio are listed in Table 3.

Table 3: Purity of nucleic acids was indicated by the absorbance ratio of 260/280. To determine the purity, 10^7 *E. coli* K12 cells/cm² (*in vivo*) were exposed and 15 µl isolated DNA/RNA (*ex vivo*) was exposed to 0.12 cm² stainless steel and pure copper surfaces (n = 4). The total amount of DNA for *in vivo* exposure was 1800 ng and 540 ng *ex vivo*. The total amount of RNA for *in vivo* exposure was 5000 ng and 2500 ng *ex vivo*. SD indicates standard deviation.

		DNA		RNA	
		In vivo	*Ex vivo*	*In vivo*	*Ex vivo*
Time [hour]	**Exposed to**	**260/280 ratio mean ± SD**	**260/280 ratio mean ± SD**	**260/280 ratio mean ± SD**	**260/280 ratio mean ± SD**
0		1.85 ± 0.06	1.82 ± 0.02	2.09 ± 0.05	2.05 ± 0.03
1	**Steel**	1.72 ± 0.03	1.82 ± 0.02	2.10 ± 0.01	2.00 ± 0.13
2	**Steel**	1.83 ± 0.01	1.84 ± 0.01	2.10 ± 0.02	2.07 ± 0.02
4	**Steel**	1.81 ± 0.01	1.85 ± 0.01	1.92 ± 0.06	2.05 ± 0.08
1	**Copper**	1.74 ± 0.08	1.44 ± 0.06	2.12 ± 0.02	1.48 ± 0.09
2	**Copper**	1.81 ± 0.01	1.33 ± 0.02	2.13 ± 0.02	1.37 ± 0.05
4	**Copper**	1.77 ± 0.03	1.28 ± 0.01	2.04 ± 0.05	1.33 ± 0.01

For DNA a ratio of ~ 1.8 indicated pure DNA and for pure RNA the ratio is ~ 2.0. In case of stainless steel and *in vivo* exposed RNA and DNA apparently no changing in the 260/280 ratio was detected. A difference in the ratio maxima was only detected for *ex vivo* exposed DNA and RNA on pure copper surfaces. *Ex vivo* exposed RNA on pure copper surfaces led to a decrease of the ratio from 2.05 to 1.48 even after one hour of exposure. Similar results were obtained for DNA measurements; the ratio decreased from 1.82 to 1.44 after one hour of exposure.

The results indicate that copper ions are able to degrade DNA and RNA into smaller fragments. However, no clear statement could be done if these damaging effects occur under normal conditions or if these molecules are main targets when cells were exposed to copper containing surfaces.

3.1.4 Release and uptake/binding of copper ions

Beside the experiments to determine different kinds of damages, the question aroused how many copper ions were released from pure copper surfaces and how many of these ions were associated with cells. Cell associated copper ions comprise all ions which were

attached to the cell membrane and wall or were present in the cytoplasm and periplasm. The results for control measurements on stainless steel did not result in significant differences in the release of copper ions. At the beginning of the measurement, 0.05 ± 0.00 nmol copper was determined in cell suspension. After four hours of exposure to stainless steel surfaces, the measured copper concentration was 0.08 ± 0.04 nmol and therefore not significantly higher compared to the beginning. With the chosen experimental setup, the total release of copper ions through the exposure of 0.1x PBS alone and 10^6 cells/cm² of *E. coli* K12 or *S. cohnii* suspended in 0.1x PBS for different time points was determined (Figure 22). This setup ascertained whether there was a difference in the release of copper ions after the exposure of buffer alone and cell suspensions. Additionally, possible differences in the release of copper ions after the exposure of Gram-positive or Gram-negative cells could be determined.

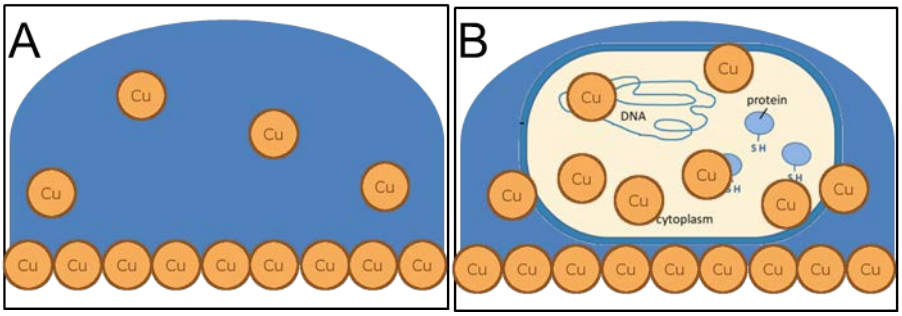


Figure 22: Total release of copper ions after the exposure of buffer alone (A) and cells in buffer (B).

Table 4 displays the total amount of released copper ions. The data for zero hour exposed cell suspension display the total amount of copper ions which was present in cells under non-stressed conditions.

Table 4: Total amount of copper ions in 350 µl suspension (10^6 cells/cm²) after the exposure to pure copper surfaces (n = 3). SD indicates standard deviation.

	PBS exposed	*E. coli* K12 in 0.1x PBS	*S. cohnii* in 0.1x PBS
Time [hour]	**Cu ions [nmol] mean ± SD**	**Cu ions [nmol] mean ± SD**	**Cu ions [nmol] mean ± SD**
0	0.00 ± 0.00	0.03 ± 0.01	0.01 ± 0.02
1	2.51 ± 0.08	9.46 ± 0.46	5.83 ± 1.30
2	2.72 ± 0.29	11.14 ± 0.36	7.87 ± 0.80
3	2.95 ± 0.15	14.53 ± 1.00	10.46 ± 1.22
4	2.79 ± 0.16	14.77 ± 0.47	13.09 ± 0.03

After one hour of exposure on pure copper surfaces, the amount of released copper ions in buffer solution increased quickly to 2.51 nmol. Further exposure for up to four hours led

to a slight, but not significant, increase in the amount of copper ions to 2.79 nmol. The application of cell suspensions in buffer on pure copper surfaces led to a higher increase of the release of copper ions. In case of *E. coli* K12, an amount of 14.53 nmol copper ions were measured after three hours of exposure. This amount did not change significantly after longer exposure times of up to four hours. For *S. cohnii*, the amount of copper ions increased until a maximum of 13.09 nmol was reached after four hours of exposure. Comparing these results, differences in the release of copper ions after the exposure of buffer alone and cell suspensions were determined.

The exposure of 0.1x PBS for four hours on copper surfaces led to a 280-fold increase of copper ion concentration in solution whereby exposure of *E. coli* K12 and *S. cohnii* in 0.1x PBS led to a 500-fold increase of copper ions. These results indicate that twice as much copper ions were released after the exposure of cell suspensions. Comparing the copper release of Gram-negative *E. coli* K12 and Gram-positive *S. cohnii*, the release increased faster for *E. coli* K12 but resulted in similar amounts of copper ions after four hours of exposure.

The amount of cell associated copper ions was determined in percent relating to the wet weight of cells and is displayed in Table 5. The intracellular copper concentration of controls was between 0.05 % and 0.07 % of the whole cell weight. Similar to the total release of copper ions described above, the cell associated copper ion concentration for *E. coli* K12 cells increased faster compared to *S. cohnii*. After one hour of exposure of *E. coli* K12, the cell associated copper ion concentration reached 7.42 %. An equal concentration of 7.09 % was reached for *S. cohnii* only after two hours of exposure. Nevertheless, further exposure of up to four hours led to a maximum cell associated copper ion concentration of 15.16 % for both strains.

Table 5: Percentage of cell associated copper after the exposure of 10^6 cells/cm² to pure copper surfaces (n = 3). SD indicates standard deviation.

	***E. coli* K12**	***S. cohnii***
Time [hour]	**% Cu/cell mean ± SD**	**% Cu/cell mean ± SD**
0	0.05 ± 0.02	0.07 ± 0.03
1	7.42 ± 0.86	5.84 ± 1.92
2	12.17 ± 2.86	7.09 ± 0.95
3	11.21 ± 6.06	10.91 ± 1.46
4	15.16 ± 0.92	15.16 ± 0.34

Figure 23 compares results of the total copper ion release after the exposure of buffer alone, cells exposed in buffer, the amount of copper ions in the supernatant and

associated with cells. As described above, the total copper ion release was 10 times higher after the exposure of cells in buffer compared to buffer alone. Additionally, an equal distribution of cell associated copper and copper ions in the supernatant is displayed. This indicates a more or less homogeneous distribution of copper ions associated with cells and in the supernatant.

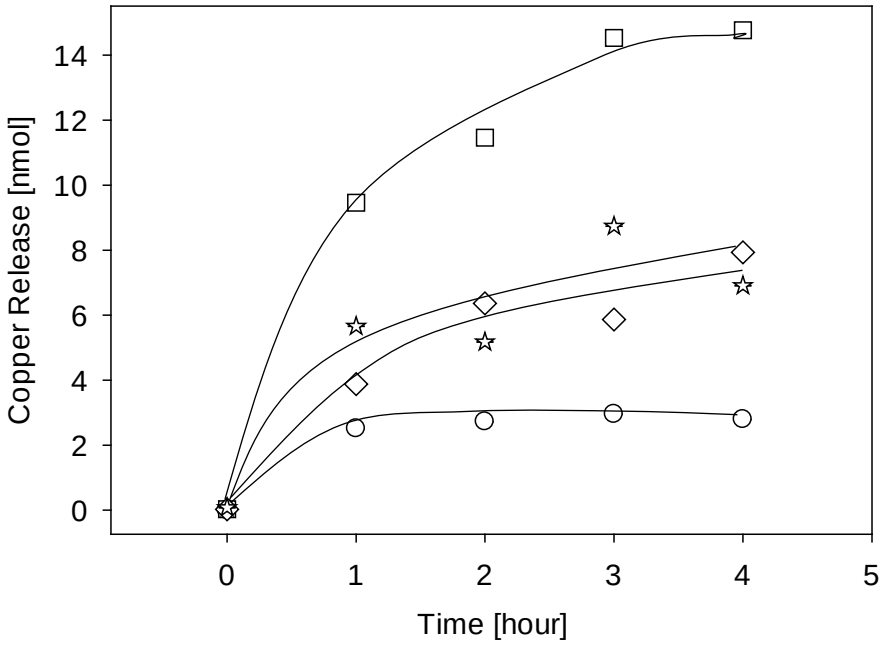


Figure 23: Comparison of the released copper ions after the exposure to pure copper surfaces (n = 3). The release was obtained after the exposure of 0.1x PBS (○) and 10^6 *E. coli* K12 cells/cm². Differences in the amount of copper ions of the supernatant (☆), cell associated (◇) and total amount of copper (□) were plotted.

3.1.5 Influence of $CuSO_4$ in solution and metallic surfaces

To determine whether a direct contact to pure copper surfaces was necessary for a successful killing, the following experiment with 10^7 *E. coli* K12 cells/ml, suspended in 0.1x PBS containing different $CuSO_4$ concentrations, was conducted. In Figure 24 different copper concentrations (from $CuSO_4$) with the corresponding survival data obtained after four hours of exposure are displayed. The investigated copper ion concentration ranged from 0.6 µmol/l, which is the concentration when no copper sulphate was added, to concentrations of 7083 µmol/l.

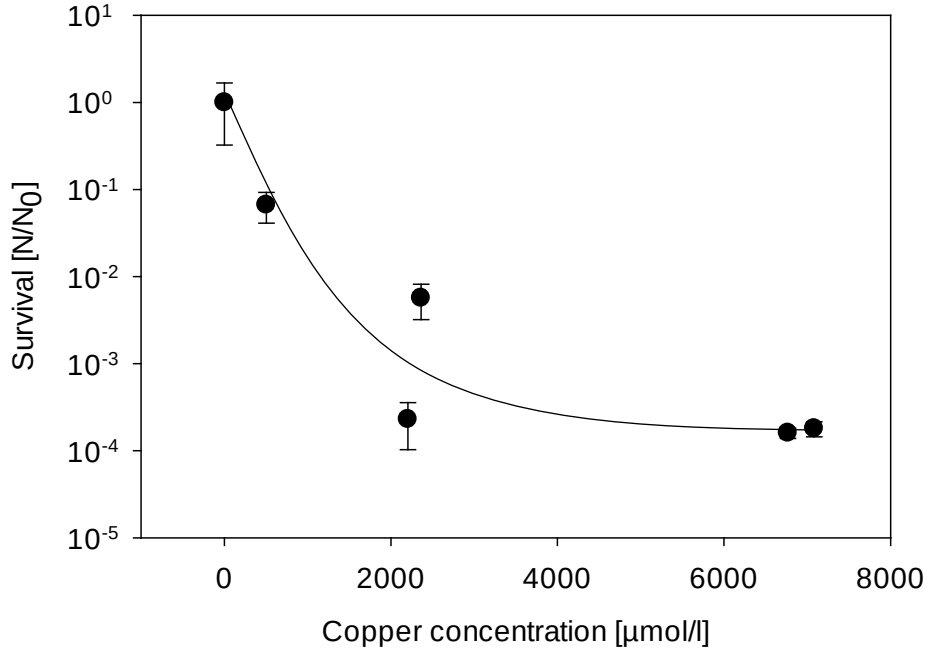


Figure 24: Survival of 10^7 *E. coli* K12 cells/ml after four hours of exposure to different concentrations of solved $CuSO_4$ in 0.1x PBS (n = 3). N are the CFUs obtained after exposure to $CuSO_4$ solutions, whereas N_0 is the starting cell concentration.

These results were compared with the results obtained after contact killing; the amount of released copper ions (paragraph 3.1.4) and the corresponding survival of cells after four hours of exposure (paragraph 3.1.1). After contact killing of 10^6 cells/cm² for four hours on pure copper surfaces, a decrease in cell survival by two orders of magnitude at a total copper ion concentration of 4 µmol/l was determined. The results obtained in this experiment (Figure 24) feature that a lower reduction of about one order of magnitude in cell survival was detected after an exposure of four hours to a higher copper ion concentration of 504 µmol/l. At copper concentrations of 6768 µmol/l and 7083 µmol/l the cell survival was decreased by about four orders of magnitude.

These experiments indicate that a direct contact to metallic surfaces led to higher inactivation rates of cells in comparison to copper solved in solution.

3.1.6 Influence of different buffer solutions

In the following chapter, experiments were conducted on different eutectic alloys. To analyse the potentially antimicrobial effect, cells were resuspended and exposed in 0.1 mol/l MOPS buffer (paragraph 2.10.1). This buffer was chosen to prevent reactions of eutectic alloys with components of the PBS buffer. PBS exposed on eutectic alloys led to

a quick reaction whereby an insoluble salt and a gas were produced. Analysis of the salt with SEM followed by an EDX measurement led to the following distribution of elements.

Table 6: Distribution of measured elements from the insoluble salt.

Chemical Element	Al	P	O	Na	Cl	K
	Mean ± SD					
Distribution [%]	16.12 ± 1.39	7.19 ± 1.79	68.43 ± 2.33	5.64 ± 1.20	1.21 ± 0.30	1.43 ± 0.45

The majority of elements was represented by oxygen with 68.43 %, followed by aluminium (16.12 %), phosphorus (7.19), sodium (5.64 %), potassium (1.43 %) and chlorine (1.21 %). The distribution of these elements could indicate which element was dissolved and which salt could have been formed.

To ensure the comparability of the previously obtained results in 0.1x PBS and the following results in 0.1 mol/l MOPS, additional contact killing analysis on pure copper surfaces with cells resuspended in 0.1x PBS and 0.1 mol/l MOPS was conducted. Further on, cells were exposed in 1x PBS to determine the effect of different salt concentrations on antimicrobial pure copper surfaces. The obtained results after the exposure of 10^6 cells/cm² of *E. coli* K12 and *S. cohnii* to stainless steel (N_0) and pure copper (N) surfaces are displayed in Figure 25.

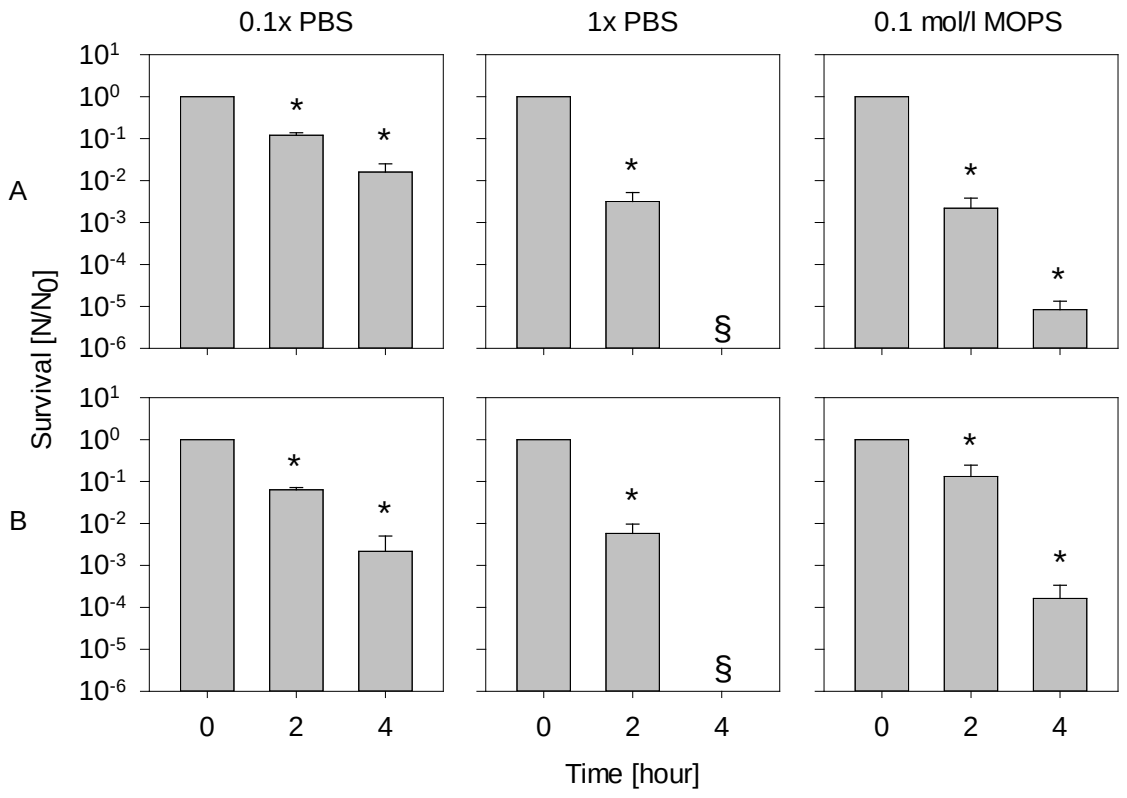


Figure 25: Survival of 10^6 cells/cm² of *E. coli* K12 (A) and *S. cohnii* (B) after the exposure to stainless steel (N_0) and pure copper (N) surfaces; * significant decrease in cell survival (p-value < 0.05), § below detection threshold.

Comparing these results, the highest toxic effect on *E. coli* K12 and *S. cohnii* was detected when exposed in 1x PBS. After four hours of exposure on pure copper surfaces in 1x PBS, no survival was determined anymore. This indicated that higher salt concentrations led to a decreased cell survival. Cells exposed in 0.1x PBS exhibited the lowest killing effects, followed by 0.1 mol/l MOPS exposed cells. After four hours of exposure, a reduction in cell survival by two orders of magnitude was determined for *E. coli* K12 exposed in 0.1x PBS and three orders of magnitude for *S. cohnii*. When exposed for four hours in 0.1 mol/l MOPS, *E. coli* K12 was reduced in cell survival by five and *S. cohnii* by four orders of magnitude.

Additional analysis was conducted to determine the release of copper ions after the exposure of 10^6 *E. coli* K12 cells/cm² in 0.1 mol/l MOPS of up to four hours. These results are listed in Table 7 and compared in Figure 26 with the results obtained in paragraph 3.1.4.

Table 7: Total amount of copper ions in 350 µl cell suspension and cell associated copper ion concentration after the exposure of 10^6 *E. coli* K12 cells/cm² in 0.1 mol/l MOPS to pure copper surfaces (n = 3). SD indicates standard deviation.

	Cu-surface	Cu-surface
Time [hour]	Cu ions [nmol] mean ± SD	% Cu/cell mean ± SD
0	0.33 ± 0.01	0.01 ± 0.01
1	17.42 ± 0.95	14.81 ± 0.80
2	21.37 ± 0.17	18.96 ± 0.38
3	24.93 ± 0.25	21.20 ± 1.91
4	26.84 ± 3.70	24.32 ± 7.06

Figure 26 displays comparisons of the total release of copper ions and the cell associated copper ion concentration after the exposure of *E. coli* K12 in 0.1x PBS and 0.1 mol/l MOPS.

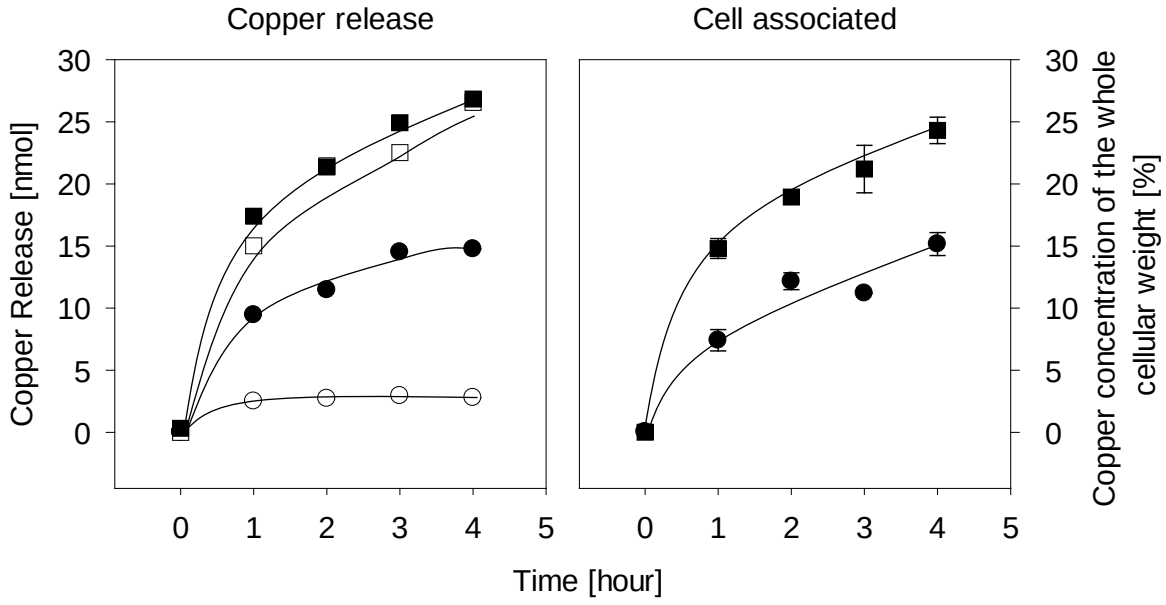


Figure 26: Left diagram: Amount of copper ions in 350 µl after the exposure of buffer alone and 10^6 *E. coli* K12 cells/cm² to pure copper surfaces (n = 3). Right diagram: Comparison of the cell associated copper concentration in 10^6 *E. coli* K12 cells/cm² after the exposure to pure copper surfaces (n = 3). Exposure of 0.1x PBS (○), cells exposed in 0.1x PBS (●), 0.1 mol/l MOPS (□) and cells exposed in 0.1 mol/l MOPS (■).

A comparison of the total release of copper ions of 0.1x PBS and 0.1 mol/l MOPS exposed cell suspensions displayed that the release increased higher for cells exposed in 0.1 mol/l MOPS (27 nmol) compared to 0.1x PBS (15 nmol) exposed samples. Comparing

the exposure of 0.1x PBS alone and cells suspended in 0.1x PBS, the release of copper ions was 10 times higher for exposed cells. In contrast to this, no significant difference in the release of copper ions was detected after the exposure of 0.1 mol/l MOPS buffer alone and cells suspended in 0.1 mol/l MOPS buffer. Additionally, the final cell associated copper concentration was compared. In case of 0.1 mol/l MOPS exposed samples, 24.3 % and for 0.1x PBS exposed samples 15.2 % of the whole cellular weight was composed of cell associated copper ions.

These results are comparable to the contact killing experiments described above. An exposure of cells in 0.1 mol/l MOPS resulted in less cell survival and additionally a higher release and cell associated copper ion concentration. Therefore, the following experiments were conducted with cells exposed in 0.1 mol/l MOPS on eutectic alloys, stainless steel and pure copper surfaces.

Summary for pure copper exposed samples

Summarizing this chapter, the results indicate that pure copper surfaces possess similar antimicrobial effects on both Gram-positive and Gram-negative strains, respectively. Furthermore, Cu^{+} and Cu^{2+} ions are released from pure copper surfaces while the most toxic effect was obtained by Cu^{+}. Considering damaging effects, ROS production and membrane damage was determined after the exposure of 10^5 cells/cm² but not at cell concentrations of 10^7 cells/cm². These concentrations confirmed results obtained by SEM analysis to possess a monolayer of cells.

Further experiments indicated that copper ions were released from pure copper surfaces whereas the amount of released copper ions was 10 times higher when cells were exposed compared to buffer alone. Additionally, the effect of different buffer solutions was determined by reference to cell survival and the release of copper ions. These results revealed a higher decrease in cell survival and release of ions in case of 0.1 mol/l MOPS exposed samples compared to 0.1x PBS exposed samples.

3.2 Contact killing on eutectic alloys

3.2.1 Survival efficiency

After the exposure of 10^5 and 10^6 cells/cm², the antimicrobial effect of the "fast" casted eutectic alloys (paragraph 2.4) and additionally stainless steel and pure copper surfaces was examined. Therefore, the survival and metabolic activity (paragraph 3.2.2) was determined. Figure 27 presents the obtained survival data after contact killing experiments were performed.

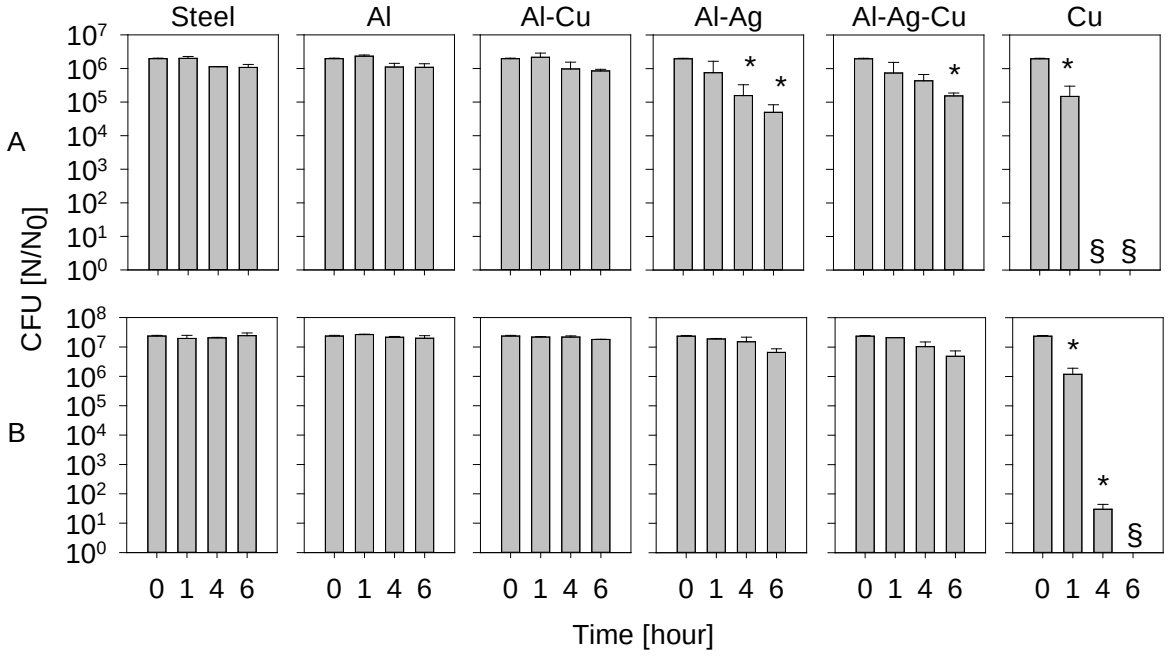


Figure 27: Contact killing of 10^5 (A) and 10^6 (B) *E. coli* K12 cells/cm² in 0.1 mol/l MOPS (n = 3). CFU obtained after the exposure to stainless steel, Al, Al-Cu, Al-Ag, Al-Ag-Cu and pure copper surfaces (N) were divided by the obtained CFU before exposure (N_0); * significant decrease in cell survival (p-value < 0.05), § below detection threshold.

The exposure of 10^6 *E. coli* K12 cells/cm² on eutectic alloys did not result in significant differences in cell survival. The only reduction in cell survival was determined for pure copper exposed samples. After four hours of exposure a decrease by 5.5 orders of magnitude and a complete reduction after six hours was detected. The investigation of 10^5 *E. coli* K12 cells/cm² led to higher decrease in cell survival. After four and six hours of exposure to pure copper surfaces, no survival of cells was examined. Additionally, an exposure of six hours on Al-Ag and Al-Ag-Cu surfaces led to a decrease in cell survival by one order of magnitude. The investigated materials seem to possess only slight or no antimicrobial effect. Therefore, further experiments were conducted to determine the

metabolic effect of the exposed samples. A decrease in the metabolic activity could indicate possible and even slight antimicrobial effects.

3.2.2 Influence on metabolic activity

The obtained survival data were supported by measurements of the metabolic activity. Exposed samples were incubated in medium (stained with Alamar Blue) and analysed spectrophotometrically. Cells with an active metabolism produce NADH and FADH which reduces Alamar Blue (resazurin) to the pink resofurin (paragraph 2.10.2). This shift can be displayed photographically (Figure 28) or monitored at 570 nm and 600 nm (Figure 29 and Table 8).

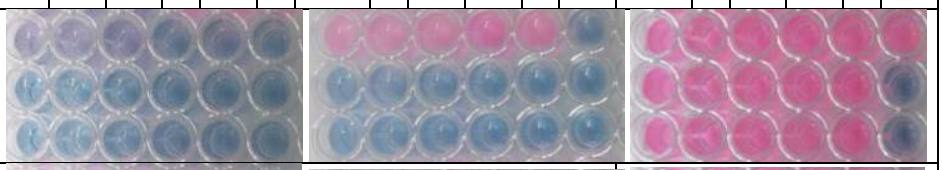
		Incubation [hour]																	
cells / cm²	Exposure [hour]	4						6						16					
		A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
10^5	1																		
	4																		
	6																		
10^6	1																		
	4																		
	6																		

Figure 28: The metabolic activity of 10^5 and 10^6 *E. coli* K12 cells/cm² is displayed by photographic images. Cells were exposed for one, four and six hours and subsequently incubated for up to 16 hours in medium which contained Alamar Blue. After four, six and 16 hours images were taken. Exposure on: A: Al; B: stainless steel; C: Al-Cu; D: Al-Ag; E: Al-Ag-Cu; F: pure copper.

The obtained relative OD_{600} results for *E. coli* K12 are presented in Figure 29.

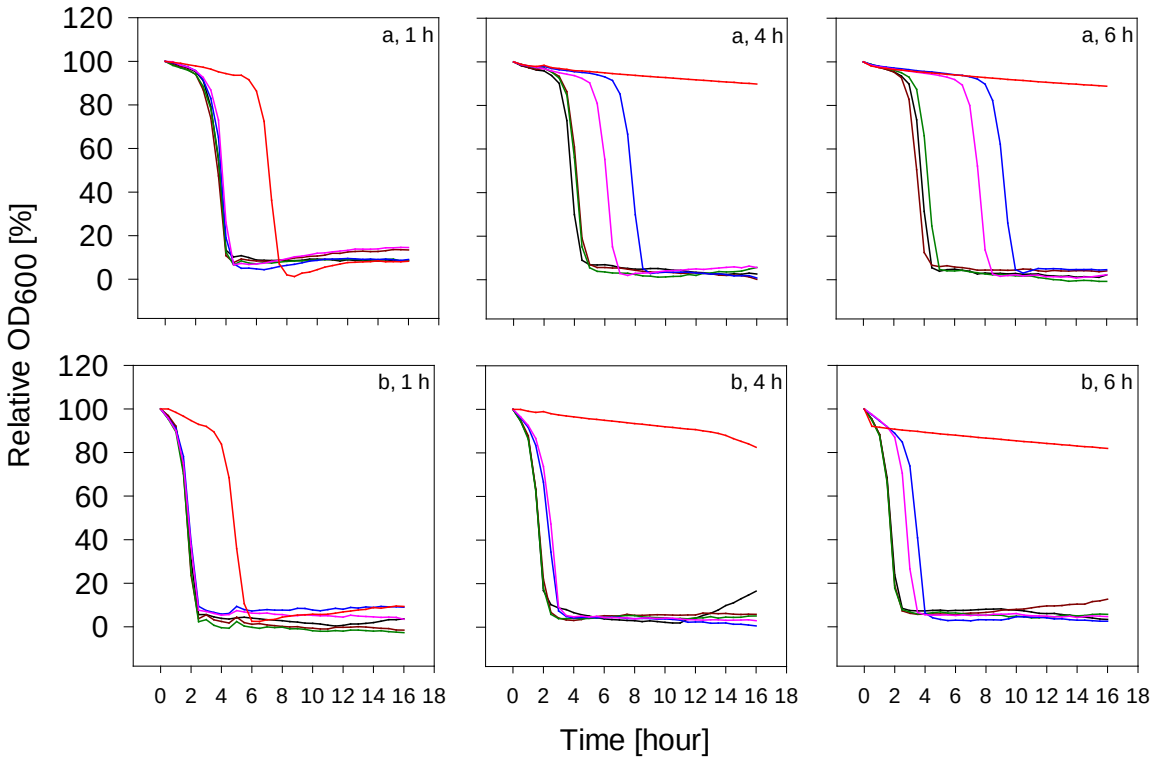


Figure 29: The metabolic activity was determined by measuring the decrease of the absorption at OD_{600}. Approximately, 10^5 (a) and 10^6 (b) *E. coli* K12 cells/cm² in 0.1 mol/l MOPS were exposed to Al: Black, stainless steel:Dk Red, Al-Cu: Dk Green, Al-Ag: Blue, Al-Ag-Cu: Pink and pure copper: Red.

Table 8 summarizes the time when 50 % of resazurin was reduced to resofurin.

Table 8: Halftime of the reduction of resazurin to resofurin after the exposure of 10^5 and 10^6 *E. coli* K12 cells/cm² to different surfaces. Cells were exposed for one, four and six hours and subsequently incubated for up to 16 hours in medium containing Alamar Blue.

Exposure [hour]	Material	10^5 cells/cm² Reduction$_{50}$ [hour]	10^6 cells/cm² Reduction$_{50}$ [hour]
1	St	3.8	1.9
	Al		
	Al-Cu		
	Al-Ag		
	Al-Ag-Cu		
	Cu	6.5	4.5
4	St	4	2
	Al		
	Al-Cu		
	Al-Ag	7.8	2.2
	Al-Ag-Cu	6	
	Cu	-	-
6	St	4	2
	Al		
	Al-Cu		
	Al-Ag	9	3.5
	Al-Ag-Cu	7.8	3
	Cu	-	-

Samples were exposed on potentially antimicrobial materials for one, four and six hours and incubated for 16 hours in medium stained with Alamar Blue to determine the metabolic activity. In case of stainless steel (control), Al (control) and Al-Cu exposed samples no inhibition of the metabolic activity was examined. The blue dye was reduced by 50 % after 3.8 hours of incubation when 10^5 cells/cm² were exposed and after 1.9 hours when 10^6 cells/cm² were exposed. This indicated that higher cell concentrations possess higher metabolic activity.

Exposure of 10^6 cells/cm² for one, four and six hours to Al-Ag and Al-Ag-Cu alloys did not result in significant decreases of the metabolic activity, compared to control samples. Whereby, a decrease in cell concentration of 10^5 cells/cm² exposed for four and six hours to Al-Ag and Al-Ag-Cu alloys led to a reduced metabolic activity. After the exposure for four hours to Al-Ag-Cu, the blue dye was reduced by 50 % within six hours and within 7.8 hours after the exposure of six hours to Al-Ag-Cu. In case of four hours Al-Ag exposed samples, the blue dye was reduced by 50 % after 7.8 hours of incubation and within nine hours after the exposure of six hours to Al-Ag. These results indicate that Al-Ag and Al-Ag-Cu alloys possess slight antimicrobial activities which were not significant by comparison of the survival of cells.

Exposure on pure copper surfaces led to the highest reduction in cell metabolism. After one hour of exposure of 10^5 cells/cm², 50 % of the blue dye was reduced after 6.5 hours and for 10^6 cells/cm² after 4.5 hours of incubation. Equal to the results obtained by the survival measurements, no reduction and therefore no metabolism was determined for samples which were exposed for four and six hours to pure copper surfaces.

3.2.3 Release of aluminium, copper and silver ions

As previously determined, the antimicrobial effect of the chosen eutectic alloys was negligible. To ascertain if this effect is caused by the release of ions from the materials, concentrations of free aluminium, copper and silver ions were determined. Therefore cell suspensions were exposed for several time points on materials with a subsequent ICP-MS analysis. The obtained results were summarized in Table 9 and Table 10.

Table 9 presents the obtained data of aluminium release.

Table 9: Total amount of aluminium ions in 350 µl suspension (10^6 *E. coli* K12 cells/cm² in 0.1 mol/l MOPS) after the exposure to Al, Al-Cu, Al-Ag and Al-Ag-Cu surfaces. SD indicates standard deviation. ND: Not determined.

	Al	Al-Cu	Al-Ag	Al-Ag-Cu
Time [hour]	**Al ions [nmol] mean ± SD**			
0	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
1	0.90 ± 0.20	0.68 ± 0.16	1.03 ± 0.18	0.94 ± 0.22
2	1.08 ± 0.12	0.76 ± 0.07	0.94 ± 0.14	1.40 ± 0.40
3	ND	ND	1.62 ± 0.22	1.27 ± 0.14
4	1.14 ± 0.06	0.87 ± 0.04	1.80 ± 0.45	1.11 ± 0.14

Comparing all materials, the aluminium release increased significantly after four hours of exposure. This increase was four-fold in case of Al-Cu alloys, five-fold for Al and Al-Ag-Cu alloys and eight-fold for Al-Ag alloys.

Equal results were received for the determination of the released copper and silver ions (Table 10).

Table 10: Total amount of copper and silver ions in 350 µl suspension (10^6 *E. coli* K12 cells/cm² in 0.1 mol/l MOPS) after the exposure to Al-Ag and Al-Ag-Cu surfaces. SD indicates standard deviation. ND: Not determined.

	Al-Cu	Al-Ag-Cu	Al-Ag	Al-Ag-Cu
Time [hour]	**Cu ions [nmol] mean ± SD**		**Ag ions [nmol] mean ± SD**	
0	0.33 ± 0.01	0.33 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
1	0.71 ± 0.13	0.00 ± 0.01	0.02 ± 0.02	2.29 ± 0.13
2	1.10 ± 0.35	0.07 ± 0.05	0.12 ± 0.04	4.28 ± 1.30
3	ND	0.28 ± 0.10	0.71 ± 0.45	4.09 ± 0.39
4	0.97 ± 0.37	0.42 ± 0.17	0.96 ± 0.32	3.59 ± 1.40

In case of Al-Ag-Cu alloys no significant release of copper ions was determined, whereas a significant 359-fold increase of the silver concentration was obtained after four hours of exposure. For Al-Cu exposed samples the copper concentration was significantly three-fold increased after four hours of exposure. After four hours of exposure on Al-Ag alloys the silver concentration was significantly 96-fold increased.

Summary for composite material exposed samples

In contrast to experiments on stainless steel and pure copper surfaces, 0.1 mol/l MOPS as opposed to 0.1x PBS was selected for contact killing experiments. Although promising, nearly no antimicrobial effect was determined after the exposure to “fast” casted eutectic alloys. Only small decreases of the cell metabolism and cell survival were detected after the exposure to Al-Ag and Al-Ag-Cu alloys. Further analysis with ICP-MS demonstrated that the ion release was negligible for eutectic alloys.

3.3 Genetic influences on copper response

In addition to experiments conducted with *E. coli* K12 and *S. cohnii*, experiments with selected strains of the Keio collection were conducted. These strains have knockouts in single genes which were either directly related to copper homeostasis or possess key roles in different cellular pathways. To ascertain the magnitude of importance of each knockout gene when cells get in contact to potentially antimicrobial surfaces such as pure copper or eutectic alloys, the following experiments were conducted.

3.3.1 Most important genes to survive copper stress

The identification of genes, which were of importance to survive copper stress, was investigated with all Keio strains mentioned in Table 1. In correspondence to the obtained MIC results for *E. coli* K12, all selected Keio strains were grown for up to 30 hours in similar concentrations of 0, 4 and 5 mmol/l $CuSO_4$ in medium. The growth was monitored by measurements at OD_{600}. Figure 30 displays the results of two selected strains.

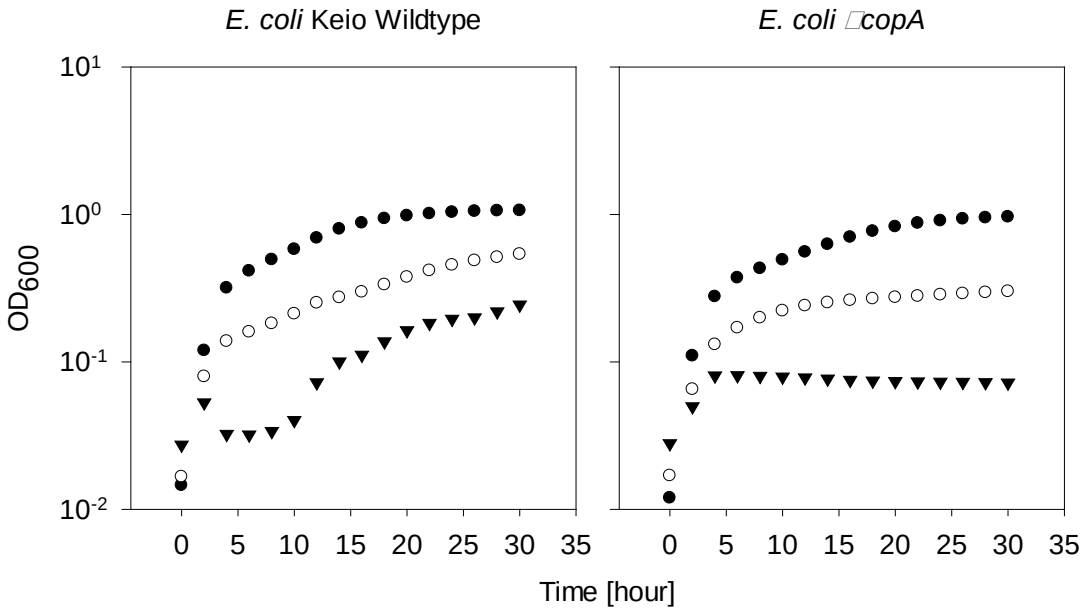


Figure 30: Growth curves of the *E. coli* Keio wildtype and *E. coli ΔcopA* in LB medium containing 0 mmol/l (●), 4 mmol/l (○) and 5 mmol/l (▼) $CuSO_4$ (n = 3).

At a concentration of 5 mmol/l $CuSO_4$, cell growth of the wildtype was not inhibited, whereas this concentration was sufficient to prevent cell growth of *E. coli ΔcopA, ΔcueO*, *ΔcueR*, *ΔcutA*, and *ΔrecA*. Further on, in another experiment cells were grown in 1.6 ml medium (with and without $CuSO_4$) and the OD determined after 23, 48 and 116 hours. Similar to the results of Figure 30, the five selected strains were highly sensitive to $CuSO_4$

in medium. The OD_{600} value at a $CuSO_4$ concentration of 4 mmol/l after 48 hours of incubation was under 0.4 (Table 11), except for *ΔcueR*. This strain was additionally selected due to the reason that the gene is responsible for the expression of CopA and CueO.

Table 11: OD_{600} values of selected strains of the Keio collection after the incubation of up to 116 hours in copper containing medium. Green highlighted values indicate cell growth, a decrease of green highlighted values to red highlighted values indicate inhibition of cell growth (red: no growth).

$CuSO_4$ conc.	0 mmol/l			1 mmol/l			2 mmol/l			3 mmol/l			4 mmol/l			5 mmol/l			6 mmol/l			7 mmol/l		
Time [hour]	23	48	116	23	48	116	23	48	116	23	48	116	23	48	116	23	48	116	23	48	116	23	48	116
WT	1.05	1.11	1.00	1.01	1.26	1.09	0.70	1.12	0.97	0.23	0.82	0.92	0.17	0.52	0.87	0.02	0.03	0.52	0.04	0.05	0.05	0.02	0.04	0.02
Δ *cusA*	0.89	0.98	0.84	0.74	1.00	0.93	0.65	0.96	0.88	0.46	0.73	0.83	0.08	0.56	0.73	-0.03	0.00	0.64	0.04	0.05	0.73	0.03	0.04	0.04
Δ *cusB*	0.93	0.92	0.78	0.79	1.02	0.88	0.57	0.92	0.88	0.40	0.67	0.99	0.11	0.44	0.78	0.01	0.03	0.49	0.04	0.05	0.06	0.00	0.05	0.03
Δ *cusC*	0.97	1.02	0.90	0.80	1.07	1.01	0.69	1.01	0.97	0.52	0.77	0.89	0.18	0.54	0.80	-0.03	0.00	0.75	0.03	0.03	0.48	0.02	0.03	0.03
Δ *cusF*	0.91	1.03	0.91	0.82	1.08	1.03	0.69	1.17	0.99	0.36	0.78	0.91	0.13	0.45	0.89	0.03	0.03	0.73	0.03	0.06	0.06	0.01	0.02	0.00
Δ *cusR*	0.78	1.05	0.88	0.60	0.96	0.96	0.50	0.83	1.02	0.44	0.80	0.99	0.48	0.70	0.98	-0.03	0.41	0.78	0.06	0.07	0.57	0.00	0.04	0.05
Δ *cusS*	0.94	1.07	0.93	0.81	1.11	1.12	0.70	1.06	1.05	0.43	0.91	1.00	0.18	0.54	0.91	0.02	0.02	0.62	0.03	0.05	0.27	0.00	0.06	0.03
Δ *copA*	0.91	0.96	0.83	0.41	0.73	0.68	0.27	0.40	0.44	0.14	0.24	0.23	0.04	0.12	0.24	-0.02	0.00	0.16	0.05	0.06	0.05	0.02	0.05	0.03
Δ *edd*	0.95	1.28	0.91	0.83	1.16	0.99	0.75	1.16	0.92	0.41	0.94	0.94	0.05	0.62	0.95	0.02	0.01	0.56	0.02	0.05	0.53	0.01	0.03	0.03
Δ *fumA*	0.79	0.99	0.87	0.69	1.04	1.06	0.66	1.11	1.15	0.50	0.79	0.97	0.05	0.55	1.00	-0.03	-0.01	0.54	0.03	0.05	0.07	0.00	0.04	0.05
Δ *leuC*	0.91	1.06	0.90	0.77	1.02	1.01	0.70	1.04	1.03	0.55	0.88	0.95	0.03	0.59	0.81	0.01	0.04	0.47	0.03	0.06	0.09	0.00	0.05	0.05
Δ *recA*	0.69	0.83	0.81	0.58	0.85	0.87	0.50	0.81	0.84	0.40	0.72	0.81	0.03	0.32	0.65	-0.03	-0.02	0.41	0.04	0.04	0.06	0.02	0.04	0.03
Δ *cutA*	1.06	1.24	0.96	0.92	1.22	0.93	0.60	1.05	0.95	0.33	0.77	0.82	0.03	0.04	0.60	0.03	0.03	0.00	0.03	0.05	0.05	0.03	0.03	0.03
Δ *cutC*	0.90	1.00	0.88	0.74	1.03	0.98	0.64	1.04	0.94	0.40	0.69	0.86	0.03	0.59	0.79	-0.03	-0.01	0.55	0.03	0.05	0.07	0.02	0.05	0.05
Δ *katE*	0.99	1.05	0.89	0.87	1.05	0.94	0.72	1.03	0.86	0.39	0.85	0.81	0.09	0.50	0.72	0.02	0.03	0.56	0.03	0.05	0.07	0.01	0.06	0.04
Δ *katG*	1.03	1.01	0.91	0.85	1.06	0.98	0.75	1.03	0.92	0.37	0.82	0.90	0.17	0.58	0.84	-0.03	-0.01	0.69	0.03	0.05	0.40	0.01	0.04	0.04
Δ *acrA*	0.95	1.13	0.98	0.85	1.11	1.03	0.68	1.25	0.96	0.42	0.91	0.91	0.18	0.72	0.92	0.03	0.03	0.58	0.03	0.05	0.06	0.01	0.05	0.04
Δ *acrB*	0.88	1.13	0.95	0.78	1.11	1.02	0.67	1.12	0.99	0.52	0.81	0.98	0.07	0.51	0.90	-0.03	-0.01	0.62	0.04	0.05	0.08	0.00	0.05	0.06
Δ *sodA*	0.88	1.00	0.94	0.74	1.06	1.00	0.65	1.00	0.98	0.41	0.71	0.92	0.11	0.62	0.81	0.02	0.04	0.61	0.03	0.07	0.08	0.02	0.07	0.06
Δ *rpoS*	0.94	1.06	0.98	0.78	1.05	1.00	0.68	1.03	0.94	0.46	0.89	0.86	0.14	0.50	0.81	-0.02	0.00	0.65	0.03	0.04	0.42	0.01	0.04	0.04
Δ *cueO*	0.83	1.18	1.01	0.68	0.96	0.94	0.53	0.81	0.90	0.45	0.60	0.73	0.07	0.37	0.73	0.02	0.00	0.42	0.03	0.03	0.03	0.03	0.03	0.01
Δ *cueR*	0.78	0.88	0.79	0.62	0.85	0.85	0.40	0.78	0.88	0.40	0.70	0.90	0.04	0.61	0.81	-0.03	-0.02	0.50	0.05	0.04	0.08	0.01	0.03	0.04

3.3.2 Survival efficiency

Cell survival after the exposure to potentially antimicrobial surfaces was determined for the five selected strains. These strains were either resuspended in 0.1x PBS and exposed to stainless steel and pure copper surfaces for up to four hours or in 0.1 mol/l MOPS and exposed on the “fast” casted eutectic alloys, stainless steel and pure copper surfaces for four hours. As described in paragraph 2.10.1, 10^6 cells/cm² were exposed and analysed subsequently.

Contact killing experiments in 0.1x PBS

The obtained results after exposure to stainless steel and pure copper surfaces are presented in Figure 31.

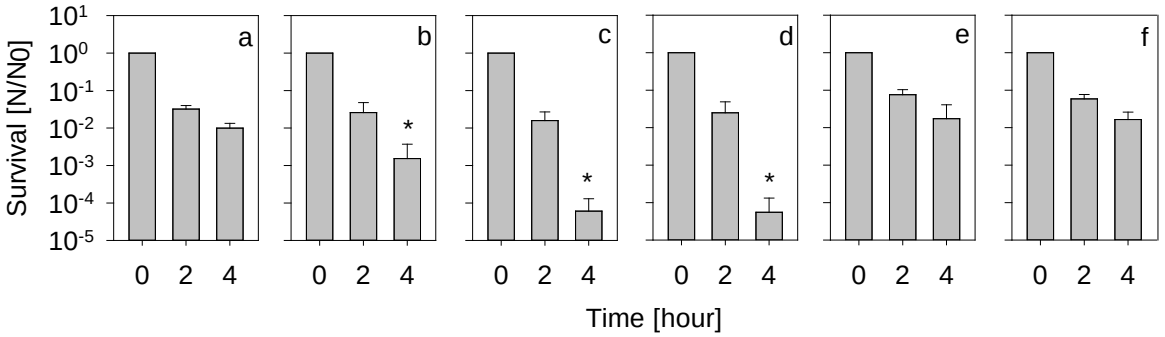


Figure 31: Survival of 10^6 cells/cm² of selected strains from the Keio collection exposed in 0.1x PBS to stainless steel (N_0) and pure copper (N) surfaces (n = 3). a: Wildtype, b: *ΔcutA*, c: *ΔcueR*, d: *ΔcueO*, e: *ΔcopA* and f: *ΔrecA*, * significant differences in cell survival in comparison to the wildtype (p-value < 0.05).

By comparing the obtained results of the Keio wildtype to the contact killing results of *E. coli* K12 in paragraph 3.1.1, equal results were obtained after four hours of exposure. Both strains exhibited a decrease in cell survival by two orders of magnitude. The Keio wildtype, *ΔcopA* and *ΔrecA* strains were the most resistant strains. Their cell survival decreased by two orders of magnitude after four hours of exposure. The most sensitive strains were *ΔcueO* and *ΔcueR* with a decrease in cell survival by 4.2 orders of magnitude after four hours of exposure. Cell survival of the *ΔcutA* strain decreased by 2.8 orders of magnitude and was therefore more sensitive than the wildtype but also more resistant compared to strains of *ΔcueO* and *ΔcueR*. Summarizing all obtained results a decreasing order of cell survival can be established. The most resistant strains were the Keio wildtype, *ΔcopA* and *ΔrecA* > *ΔcutA* > *ΔcueR* = *ΔcueO*.

Contact killing experiments in 0.1 mol/l MOPS

Figure 32 displays survival of the selected strains from the Keio collection after contact killing on potentially antimicrobial materials. As previously described in paragraph 3.1.6, different release of ions was determined after the exposure of cells in 0.1x PBS or 0.1 mol/l MOPS buffer. Therefore, differences in cell survival were assumed between Figure 31 and Figure 32.

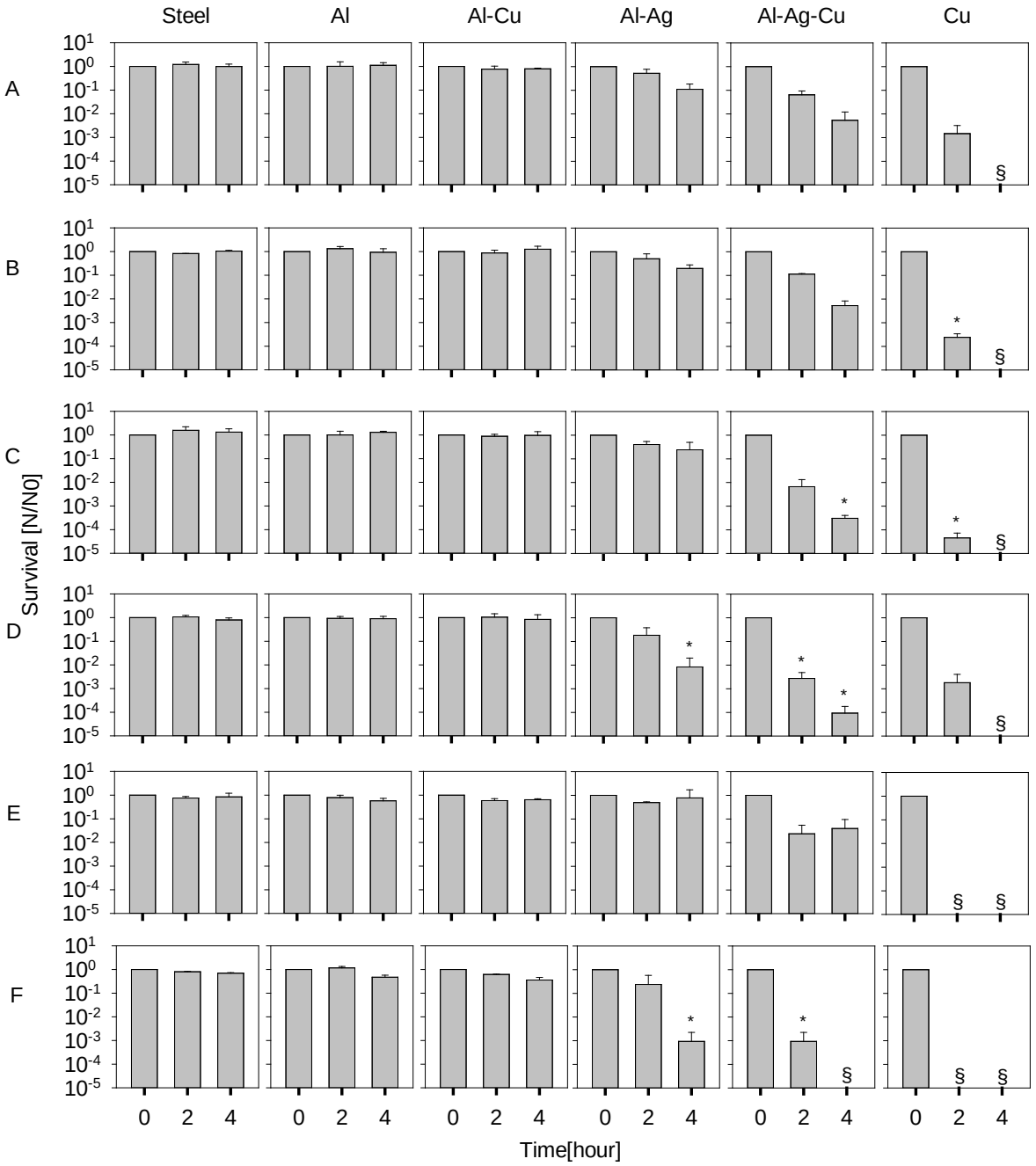


Figure 32: Survival of 10^6 cells/cm² of selected strains from the Keio collection exposed in 0.1 mol/l MOPS to stainless steel, pure copper (Cu) and eutectic alloys (Al, Al-Cu, Al-Ag and Al-Ag-Cu) (N) (n = 3). N_0: amount of the starting cell concentration. A: wildtype strain; B: *ΔcutA*, C: *ΔcueR*, D: *ΔcopA*, E: *ΔcueO* and F: *ΔrecA*; * significant decrease in cell survival in comparison to the wildtype (p-value < 0.05), § below detection threshold.

A comparison of all surfaces and selected strains indicated that no reduction in cell survival was detected when cells were applied to Al-Cu alloys, stainless steel and Al surfaces. Most of the tested strains also showed no reduction in cell survival when exposed to Al-Ag alloys, except *ΔcopA* and *ΔrecA*. In both cases a reduction in cell survival by one order of magnitude was measured after two hours of exposure. The cell

survival decreased further after four hours of exposure to Al-Ag alloys for *ΔcopA* by two and for *ΔrecA* by three orders of magnitude.

The most efficient materials were Al-Ag-Cu alloys and pure copper surfaces. After two hours of exposure to Al-Ag-Cu the survival was decreased by one order of magnitude for the wildtype strain and *ΔcutA*. The *ΔcueO* strain was decreased by 1.5, *ΔcueR* by two and *ΔrecA* and *ΔcopA* by three orders of magnitude. After four hours of exposure, the survival was further decreased by two (wildtype and *ΔcutA*), 3.5 (*ΔcueR*) and four orders of magnitude (*ΔcopA*). In case of *ΔcueO* the amount of survival was the same as obtained after 2 hours of exposure and no survival was determined after four hours of exposure in case of *ΔrecA*.

The highest reduction in cell survival was determined for cells exposed to pure copper surfaces. The exposure for two hours decreased the survival of the wildtype and *ΔcopA* by three, *ΔcutA* by four and *ΔcueR* by 4.5 orders of magnitude. In case of *ΔrecA* and *ΔcueO*, no cells were detected after two hours of exposure. Longer exposure of up to four hours on pure copper surfaces led to a total decrease in cell survival for all tested strains.

In comparison to the obtained results of *E. coli* K12 exposed in 0.1x PBS or 0.1 mol/l MOPS (paragraph 3.1.6), the reduction of cell survival was higher for the *E. coli* Keio wildtype strain suspended in 0.1 mol/l MOPS. After two hours on pure copper surfaces the survival of *E. coli* K12 and *E. coli* Keio wildtype cells exposed in 0.1x PBS was reduced by one and after four hours by two orders of magnitude. For 0.1 mol/l MOPS exposed *E. coli* K12 the survival was reduced by three after two hours and five orders of magnitude after four hours of exposure. In case of 0.1 mol/l MOPS exposure of the *E. coli* Keio wildtype a reduction in cell survival by three orders of magnitude was detected after the exposure of two hours. After the exposure of four hours on pure copper surfaces no cell survival was detected.

Summarizing all these results two decreasing orders depending on cell survival can be established. An exposure in 0.1 mol/l MOPS on pure copper surfaces led to the following order, starting with the most resistant strain: Keio wildtype = *ΔcopA* > *ΔcutA* > *ΔcueR* > *ΔcueO*, *ΔrecA*. The highest resistance after the exposure to silver containing alloys was obtained for the Keio wildtype = *ΔcueO* = *ΔcutA* > *ΔcueR* > *ΔcopA* > *ΔrecA*.

3.3.3 Release and uptake/binding of copper ions

Differences in the release of copper ions after the exposure of *E. coli* K12 and *ΔcopA* were determined by additional ICP-MS measurements. Therefore 0.1x PBS alone and 10^6 *ΔcopA* cells/cm², resuspended in 0.1x PBS, were exposed to pure copper surfaces. The obtained results were compared in Table 12 and Table 13 to the previously obtained *E. coli* K12 results (paragraph 3.1.4).

Table 12 presents the copper release after the exposure of *E. coli* K12 and *ΔcopA* to pure copper surfaces. The results for zero hours exposed cell suspension display the amount of copper ions which were present in cells under non-stressed conditions.

Table 12: Total amount of copper ions in 350 µl suspension (10^6 cells/cm²) after exposure to pure copper surfaces (n = 3). SD indicates standard deviation.

	***E. coli* K12 in 0.1x PBS**	***E. coli ΔcopA* in 0.1x PBS**
Time [hour]	**Cu ions [nmol] mean ± SD**	**Cu ions [nmol] mean ± SD**
0	0.03 ± 0.01	0.04 ± 0.013
1	9.46 ± 0.46	6.50 ± 0.69
2	11.46 ± 0.36	7.93 ± 0.18
3	14.53 ± 1.00	9.21 ± 0.81
4	14.77 ± 0.47	10.93 ± 1.10

As mentioned above, in case of *E. coli* K12, a maximal amount of 14.77 nmol copper ions was obtained even after three hours of exposure. Comparing the results for *E. coli* K12 and *ΔcopA*, the release of copper ions seems to be slower for *ΔcopA*. This was indicated by a similar amount of released copper ions (~ 11 nmol) for *E. coli* K12 after two hours of exposure and for *ΔcopA* after four hours of exposure. Comparing the final amount of copper ions after four hours of exposure a slightly lower but not significant release of copper ions was detected in case of *ΔcopA*.

Additionally, the cell associated copper concentration was determined in percent relating to the wet weight of cells and is displayed in Table 13. Under non-stress conditions the intracellular copper concentration was between 0.05 and 0.12 % of the whole cell weight.

Table 13: Percentage of cell associated copper after the exposure of 10^6 cells/cm² to pure copper surfaces (n = 3). SD indicates standard deviation.

	***E. coli* K12 in 0.1x PBS**	***E. coli ΔcopA* in 0.1x PBS**
Time [hour]	**% Cu/cell mean ± SD**	**% Cu/cell mean ± SD**
0	0.05 ± 0.02	0.12 ± 0.01
1	7.42 ± 0.86	4.70 ± 0.68
2	12.17 ± 2.86	5.71 ± 0.39
3	11.21 ± 6.06	7.99 ± 1.13
4	15.16 ± 0.92	19.52 ± 2.09

Similar to the results obtained for the total amount of copper ions, the increase of cell associated copper ions was higher for *E. coli* K12. After three hours of exposure the cell associated copper concentration of *ΔcopA* (7.99 %) was similar to the 7.42 % obtained for *E. coli* K12 after one hour of exposure. The final cell associated concentration of *E. coli* K12 was determined as 15.16 % and for *ΔcopA* as 19.52 %. Comparing the variance of error for both four hour exposed samples, no significant differences in the cell associated copper ion concentration could be determined. The obtained results indicated that no significant differences between *E. coli* K12 and *ΔcopA* in relation to the release of copper ions from the surfaces and the cell associated copper ion concentration was measurable.

Summary of the molecular processes

Summarizing the obtained contact killing results, three decreasing orders of cell survival can be established. After the exposure in 0.1x PBS on pure copper surfaces, *ΔcueO* and *ΔcueR* were identified as the most sensitive strains, whereas *ΔcopA* and *ΔrecA* exhibited the highest resistances. When exposed in 0.1 mol/l MOPS on pure copper surfaces, the *ΔrecA* was even more sensitive than *ΔcueO* and *ΔcueR*. In contrast to this, *ΔcueO* was the most resistant strain and *ΔcopA* and *ΔrecA* were the most sensitive strains when exposed to silver containing eutectic alloys.

Additionally, ICP-MS measurements were conducted to determine differences in the release and cell associated copper ion concentration after exposure of *ΔcopA* and *E. coli* K12. No significant differences of the copper ion release and the cell associated copper ion concentration was determined. Whereas in both experiments, a delay of copper release and cell associated copper was detected for *ΔcopA* exposed samples.

3.4 Impact of an alternative casting

Additionally to the “fast” casted eutectic alloys, “slow” casted Al-Ag-Cu alloys were examined. For these contact killing experiments, 10^6 cells/cm² of *E. coli* K12 and *S. cohnii* were suspended in 0.1x PBS and exposed to Al-Ag-Cu and pure copper surfaces.

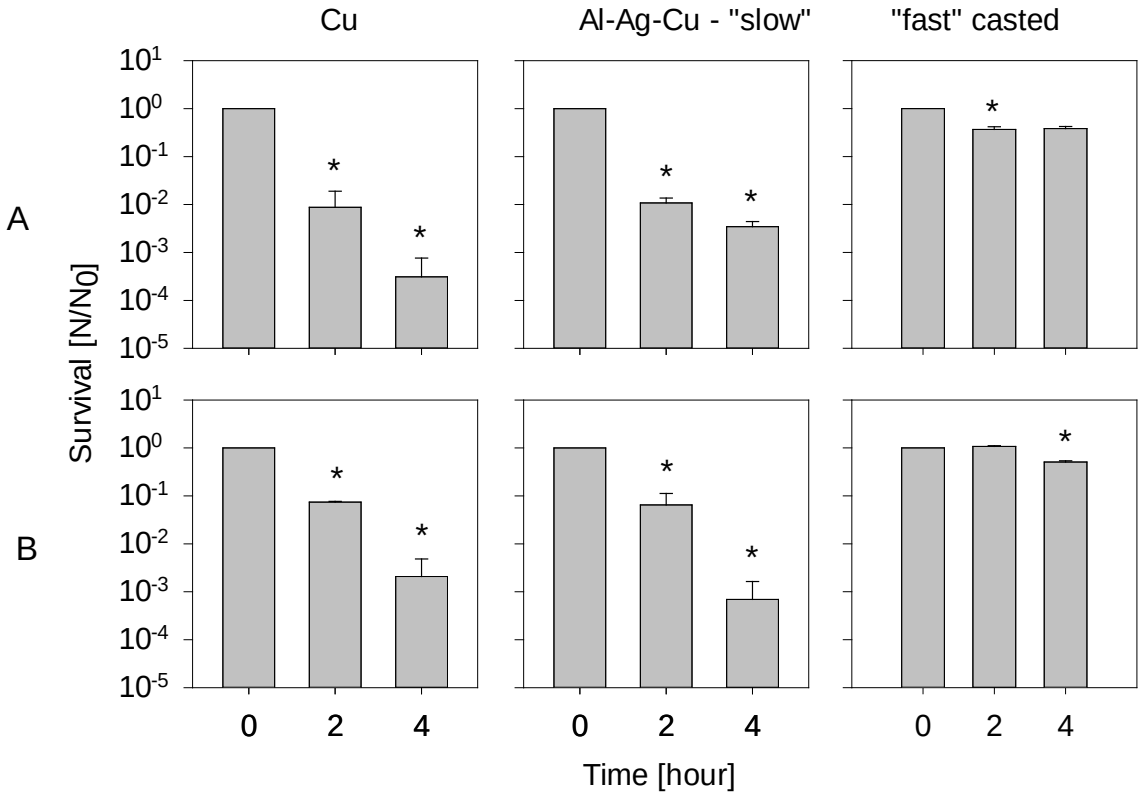


Figure 33: Survival of 10^6 cells/cm² of *E. coli* K12 (A) and *S. cohnii* (B) suspended in 0.1x PBS after the exposure to antimicrobial (N) and stainless steel (N_0) surfaces (n = 3), * significant decrease in cell survival (p-value < 0.05).

In case of *S. cohnii* a reduction in cell survival by three orders of magnitude was determined after the exposure of four hours to “slow” casted Al-Ag-Cu alloys and pure copper surfaces. By comparison, exposure of *E. coli* K12 for four hours to “slow” casted Al-Ag-Cu alloys led to a reduction in cell survival by 2.5 orders of magnitude and 3.5 after the exposure to pure copper surfaces. Contrary to these decreases in cell survival, only slight influences of both strains were determined after the exposure to “fast” casted Al-Ag-Cu alloys. Thereby, the effects on cell survival were negligible in comparison to both other tested materials.

These results indicate antimicrobial effects of the “slow” casted eutectic composite material (Al-Ag-Cu) and revealed further potentially deeply interesting experiments concerning antimicrobial studies on eutectic alloys.

3.5 Summary

Summarizing all these results, no significant differences were obtained for Gram-positive and Gram-negative strains. Both displayed membrane damages and a similar decrease in cell survival after contact killing on pure copper surfaces. Further experiments demonstrated damaging effects of the DNA and RNA and an increase in ROS production. Additional analysis conducted on different copper oxide surfaces pointed out that the most toxic copper ion is Cu^{+}.

Compared to 0.1x PBS exposed samples, less cell survival and a coincident higher release of copper ions was obtained for 0.1 mol/l MOPS exposed samples. Further analysis on “fast” casted eutectic alloys showed that only a slight decrease in cell survival and release of antimicrobial metals was detected. In contrast to this, a higher decrease in cell survival was detected for “slow” casted alloys.

The investigation of knockout mutant strains displayed *ΔcueO* und *ΔcueR* as the most sensitive strains when exposed to pure copper surfaces. As opposed to this, *ΔcopA* and *ΔrecA* were highly resistant when exposed in 0.1x PBS to pure copper surfaces. Beside this, *ΔcopA* and *ΔrecA* were the most sensitive strains when exposed to silver containing eutectic alloys. Contrary to the exposure on pure copper surfaces, *ΔcueO* exhibited the highest resistance after the exposure to silver containing eutectic alloys.

4. Discussion

Although antimicrobial metals are applied for centuries, the exact mechanisms of action are not fully understood. First promising investigations were performed in 1893 by von Nägeli. He discovered the oligodynamic properties of antimicrobial salts such as copper and silver [26]. However, the application of these metals as antimicrobial surfaces was first examined by Kuhn in 1982. In these experiments, the microbial diversity on brass and stainless steel doorknobs was investigated whereby a comparison of both pointed out that a diminished microbial contamination was verifiable on brass doorknobs [42]. From thereon, increasing investigations and applications of copper and silver as antimicrobials were conducted [28, 51, 52, 98, 99]. Nevertheless, the application of pure copper surfaces is difficult due to the chemical properties and costs. Therefore, many investigations were implemented with metallic alloys and their efficiency against microbial contaminations [57, 137, 138].

The current study was conducted on pure copper surfaces and eutectic alloys composed of mainly aluminium in addition to silver and/or copper. Additional experiments were performed on different copper oxide layers (Cu_2O and CuO) which were previously thermally prepared on pure copper surfaces. [127].

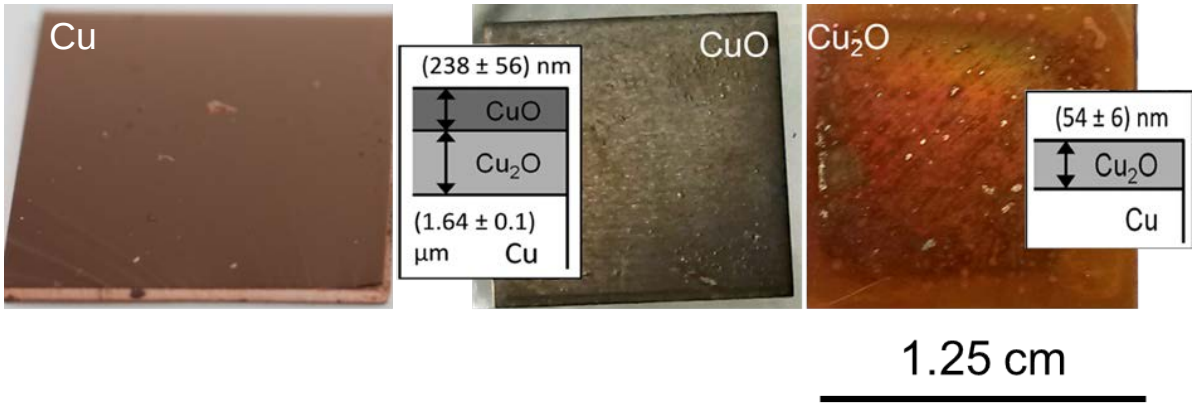


Figure 34: Photographic picture of a pure copper surface (Cu) and the copper oxide layers (CuO and Cu_2O). Cu_2O was produced after the exposure for 20 hours at 200 °C to air. The double layer of Cu_2O at the bottom and CuO on top was formed after the exposure to air for 150 minutes at 350 °C. The insets indicate schematically the layer dimensions; the CuO layer had a dimension of 238 nm ± 56 nm and was produced on top of the 1.64 µm ± 0.1 µm thick Cu_2O layer. In case of Cu_2O, a 54 nm ± 6 nm thick layer was produced [127].

Previous research has shown that cells in the stationary phase are more resistant to stress situations e.g. copper stress than cells from the logarithmic phase [83]. Supposing that environmental microorganisms are very resistant against stress conditions, cells were

routinely grown under optimal conditions until stationary phase was reached. With this experimental setup it can be ensured that conclusions can be drawn between laboratory experiments and microorganisms from the environment which might adhere to antimicrobial surfaces.

Initially to contact killing experiments, desiccation tolerance of several environmental isolates and human associated microorganisms was examined. On account of these measurements, the most interesting strains were to be determined and selected for further investigations. The presented results in this work were the well-known model organism *Deinococcus radiodurans* R1*, Escherichia coli* K12 and *Staphylococcus cohnii*. Less efficient strains related to cell survival under desiccation conditions and spore forming strains were excluded. Additionally, experiments with *D. radiodurans* R1 were not considered further due to its tetrad character; this complicated the determination of antimicrobial effects on single cells. The exclusion of spore forming cells is caused by the fact that spores were more resistant than vegetative cells. In experiments of Galeano *et al.*, they were not affected by antimicrobial silver- and zinc-coating, contrary to vegetative cells [139]. Another important reason for the exclusion was caused by the discovery of Kearns and Losick in 2005, that *B. subtilis* cells grew heterogeneously [140]. Therefore a reliable distinction of both cell forms was not trivial. By means of the obtained desiccation results, further investigations were performed with the Gram-negative *E. coli* K12 and Gram-positive *S. cohnii* [141, 142]. Both strains are human associated and were selected due to their high desiccation tolerance, similar growth behaviour and differences in the composition of the cell wall and membrane. Nevertheless, slight differences in their survival efficiency after the exposure to $CuSO_4$ in medium were observed. The obtained MIC results of $CuSO_4$ for *E. coli* K12 (5 mmol/l) showed comparable results to the data obtained by Tetaz and Luke (1983) (4 mmol/l) [143], whereby *S. cohnii* was slightly more resistant (7 mmol/l) than *E. coli* K12.

In comparison to $CuSO_4$ from which Cu^{2+} ions are solved in solution, the main experiments were conducted on pure copper surfaces. From pure copper surfaces both forms of copper ions Cu^{+} and Cu^{2+} are dissolved. To determine different contact killing effects caused by Cu^{+} and Cu^{2+}, investigations were conducted on copper oxide layers (Cu_2O and CuO) [127].

A comparison of the obtained results after the exposure of *E. coli* K12 and *S. cohnii* on copper oxide layers with the obtained results after exposure on pure copper surfaces demonstrated that the most efficient surface layer was composed of Cu^{+} followed by the pure copper surface which was able to release both forms of copper ions (paragraph 3.1.2). CuO layers were also efficient in contact killing of *E. coli* K12 and *S. cohnii* strains

but less efficient than the Cu_2O layer or pure copper surface. These results are supported by experiments with *Enterococcus hirae* previously described by Mathews *et al*. (2015) [41]. A possible explanation for the higher toxic effect of Cu^+ is that, in comparison to Cu^{2+}, Cu^+ penetrates intact cytoplasmic membranes. Once inside the cytoplasm, it leads to severe damages of DNA, RNA and proteins [86, 89]. Additionally, it reacts in a Fenton-like reaction and produces reactive oxygen species (ROS) [23, 74, 144]. ROS lead to further damages of the cell membrane, proteins, DNA and RNA. Similar to Cu^+, Cu^{2+} can also lead to lipid peroxidation, DNA and RNA degradation and protein dysfunction [79, 86-89] whereby Cu^{2+} is not available in the cytoplasm if the cytoplasmic membrane is intact. To cause damages in the cytoplasm, Cu^{2+} needs to be taken up by cells either actively via e.g. the Zn(II)-uptake system ZupT [145] or through lesions in the membrane. Additionally, Cu^{2+} can be transformed to Cu^+ by reducing mechanisms in the periplasm e.g. enterobactin which can act as a Cu^{2+} reductant [94]. Further investigations were conducted on pure copper surfaces due to the fact that thermally oxidized copper surfaces are not applicable in hospitals or else.

First contact killing experiments with *E. coli* K12 were conducted with cells grown overnight in NB medium. According to the DSMZ, NB is the optimal medium for *E. coli* K12. However, the nutrient composition of NB is much lower in comparison to TSY medium for *S. cohnii*. To ensure similar nutrient and therefore similar growth conditions, LB medium was used. It was already used by Macomber *et al.* (2007) [144] and contains an equal amount of nutrients compared to TSY medium. *E. coli* K12 cells grown overnight in LB medium showed higher copper tolerance in comparison to NB grown *E. coli* K12 cells (data presented in the supplements: Figure 36). Thereby the copper tolerance obtained for *E. coli* K12 cells grown in LB medium was comparable with *S. cohnii* cells grown in TSY medium. Both strains showed equal survivability after contact killing experiments [146]. For a better comparison of both strains and to minimize influences of different nutrient concentrations of the medium, further analysis with *E. coli* K12 was conducted with cells grown overnight in LB medium.

Additionally to the choice of medium, different buffers were examined. In many research publications 0.1 mol/l Tris HCl was used to investigate contact killing efficiencies of antimicrobial surfaces or alloys [41, 127, 147]. However, as previously shown by Hans *et al.* [127], 0.1 mol/l Tris HCl release more copper ions from pure copper surfaces than PBS. Higher release of copper ions means higher corrosion of the surface and a followed destabilization. To minimize these effects, cells of the current study were exposed in PBS to determine antimicrobial effects. Contact killing experiments with cells exposed in 1x PBS and 0.1x PBS showed that less cell survival was obtained for cells exposed in

1x PBS. This is caused by the higher salt concentration of 1x PBS which led to an increased corrosion of surfaces. As a consequence more ions are available in suspension, followed by an increased killing effect [110]. Therefore further experiments on pure copper surfaces were conducted in 0.1x PBS to minimize corrosion of the surfaces. Additionally, to counteract the issue of increased corrosion on eutectic alloys, 0.1 mol/l MOPS was used for contact killing experiments. MOPS is a so called “Good buffer” and does neither play a role in cell metabolism nor is it toxic [148]. It was chosen because of the reactive features of eutectic alloys with PBS. PBS consists of three different components (Na_2HPO_4, KH_2PO_4 and NaCl) whereby the dissolved ions react under gas-forming conditions and the production of an insoluble salt after exposure to eutectic alloys. SEM-EDX measurements pointed out that the salt consisted of three major elements (aluminium, phosphorus and oxygen) and inferior concentrations of sodium, chlorine and potassium. Due to this distribution it could be assumed that aluminium reacted with PBS to insoluble aluminium salts, certainly $AlPO_4$ and/or $Al(OH)_3$. Thereby possibly H_2 was additionally formed. This reaction did not take place when MOPS was exposed to eutectic alloys.

For a better comparability of both buffers, experiments on pure copper surfaces with *E. coli* K12 and *S. cohnii* cells exposed in either 0.1x PBS or 0.1 mol/l MOPS were investigated. The comparison of results showed that the survival of cells exposed in 0.1 mol/l MOPS decreased much higher in comparison to 0.1x PBS exposed cells. To determine the reason for the different toxic effects the total release and cell associated copper ion concentration after the exposure to pure copper surfaces in 0.1x PBS and 0.1 mol/l MOPS was determined. Thereby the results pointed out that a higher release of copper ions and a higher cell associated copper ion concentration was detected for 0.1 mol/l MOPS exposed samples. Taken into account what Montigny and Champeil postulated in 2007, a possible reason for the higher release of copper ions could be that MOPS is able to chelate copper ions [149]. This decreases the concentration of free copper ions in suspension which in turn can lead to a further release of copper ions from the surface. By reference to Montigny and Champeil the higher release of copper ions can be explained. However, the higher cell associated copper ion concentration cannot be explained by this assumption but possibly by researches of Mash and Chin (2003). They postulated that no binding of copper would be formed with MOPS buffer even if MOPS is a chelating agent [150]. The results of both studies can only explain one of both measured results; therefore further investigations need to be conducted.

4.1 Survival after contact killing

Further on, contact killing experiments were conducted. Therefore, the application of cells in a monolayer was of major concern. A monolayer ensures that every cell gets in direct contact to the surface and obtains equal stress conditions. The results obtained by SEM analysis revealed that a multilayer of cells was formed for cell concentrations of 10^8 and 10^7 cells/cm² (Figure 15). Concerning the outer and inner regions of the multilayer, it is estimated that the majority of cells is located between the outer layer and adhered cells with a supplemental lower copper ion concentration. Therefore, these cells were protected against the toxic effect of free copper ions. Additionally, cells which were directly exposed to antimicrobial surfaces protect upper cells from toxic effects of the surface through shielding. These shielding effects could explain the high survivability of cells at high cell concentrations, even after four hours of exposure. The optimal cell concentration was determined to be ranged between 10^5 and 10^6 cells/cm². At a cell concentration of 10^6 cells/cm² only a few cells were detected to lie on top of other cells whereas a complete monolayer was obtained after the exposure of 10^5 cells/cm² (Figure 15). Significant decreases in cell survival were detected at a cell concentration of 10^5 cells/cm². At this concentration, all cells were in direct contact to the antimicrobial surface and therefore no shielding effects existed. Additionally, at cell concentrations of 10^5 cells/cm² or higher, most of the cells were in direct contact to other cells and therefore able to interact and communicate. In general, communication of bacteria is caused by quorum sensing which includes e.g. conjugation, motility or antibiotic production. For bacterial survival, it is of major importance to interact with their environment, for example when they are exposed to antimicrobial surfaces [151, 152]. At lower cell concentrations, a monolayer was exposed where every cell was in direct contact to the surface. Furthermore, an interaction of cells was no longer possible which could led to a higher reduction in cell survival.

The similar survivability of *E. coli* K12 and *S. cohnii* after exposure to antimicrobial surfaces could indicate that the influence of differences in the composition of the cell wall and membrane is negligible. Based on these results, contact killing experiments were conducted either with 10^5 or 10^6 cells/cm² when experiments were conducted with cells in a monolayer or 10^7 cells/cm² if the difference to a multilayer of cells was investigated.

In addition to contact killing experiments on pure copper surfaces, the contact killing effect was investigated on eutectic alloys. They were partitioned into “slow” and “fast” casted alloys whereby the majority of experiments, including the molecular analysis experiments, were conducted on “fast” casted alloys.

Initial experiments were conducted to determine the effect of pure aluminium. Due to the fact that no antimicrobial effect was observed for samples exposed to Al, the effect of copper and silver in combination with aluminium was examined. Therefore two alloys were casted; both contained aluminium and additionally either silver or copper. Synergistic effects of copper and silver were examined with an alloy which contained all three elements. All alloys were casted as eutectic mixtures to guarantee a consistent distribution of metals. In case of Al-Cu alloys the material was composed of two crystals; Al and Al_2Cu. Al-Ag was composed of Al and Ag_2Al and Al-Ag-Cu of Al, Al_2Cu and Ag_2Al crystals. The structure of Al-Ag-Cu alloys were analysed by Dennstedt and Ratke in 2012. They could show that different solidification rates led to different constellations of the crystal structure. Thereby a quicker solidification led to a decrease in ordering from brick-like to irregular structures (Figure 35) [153].

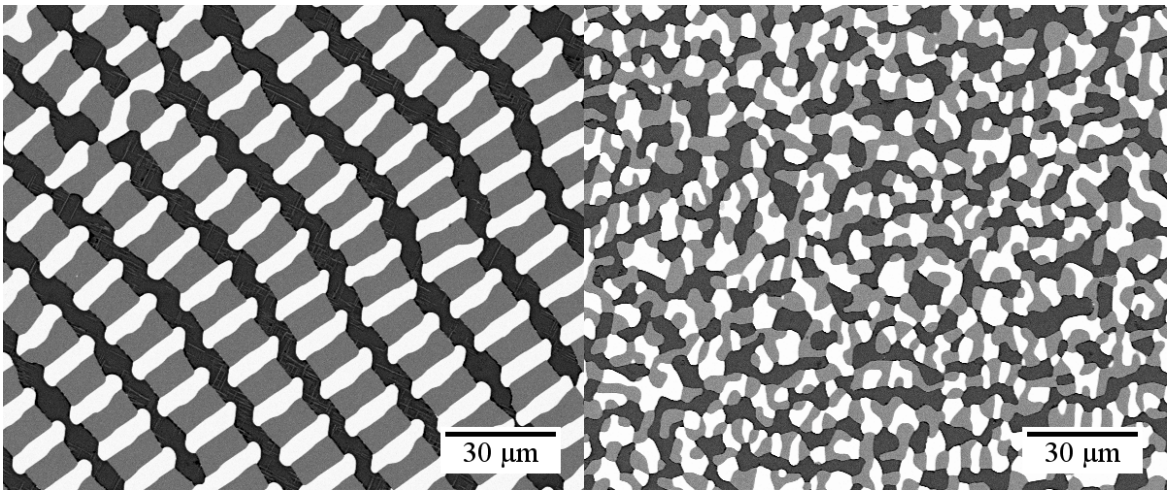


Figure 35: SEM images of the brick like structure (left panel) after slow solidification and irregular structure (right panel) after fast solidification of Al-Ag-Cu alloys. Dark: Al, grey: Al_2Cu, white: Ag_2Al. [153].

The crystal structure of both “fast” and “slow” eutectic alloys in the current work was obtained after quick solidification. All casts were poured at room temperature and cooled down in cool water. This ensured a very quick but not controlled cool down which induced a higher chaotic structure of crystals. Due to the higher chaotic structure every cell was in direct contact to antimicrobial metals.

The “slow” casted alloy was composed of Al-Ag-Cu. After four hours of exposure a decrease in cell survival which was similar to the survival after the exposure to pure copper surfaces was obtained. Possible reasons why the “slow” batch of alloys behaved different to the “fast” batch of alloys was ascribed to the different casting procedures. In general, the composition was equal but contrary to the “fast” batch of alloys which were molten, mixed and casted in a mould of 15 mm, the “slow” casted Al-Ag-Cu alloy was

produced in two melting processes. The first melting was casted in a quadratic form of 16 cm *16 cm length with a height of approximately one – two cm. Afterwards, smaller pieces of this cast were molten and mixed again followed by a cast in a 12 mm mould. The casting in the quadratic form and the smaller mould diameter led to a quicker cool down of the material which led to a more irregular structure with a higher distribution of smaller crystals (Figure 35). The higher irregular structure of the “slow” batch of alloys led to a higher antimicrobial effect. This experiment indicated that the processing and solidification rate is highly important to obtain antimicrobial effects of the alloy. Therefore the protocol and thickness of the material need to be optimized. Possibly, the casting of thin materials is better suited in comparison to huge blocks which were eroded into smaller pieces afterwards. Such a thin surface will solidify quicker and could be applicable for the exchanging of plastic light switches or other surface areas for example in hospitals.

The survivability of Gram-positive and Gram-negative strains after the exposure on “fast” casted eutectic alloys was not diminished when exposed to Al and stainless steel, which both were used as controls, and Al-Cu alloys. In case of Al-Ag and Al-Ag-Cu exposed samples a slight reduction in cell survival was determined, whereas higher reduction was obtained after the exposure to pure copper surfaces. For a more precise investigation of the possible antimicrobial effect, the metabolic activity of 10^5 and 10^6 exposed *E. coli* K12 cells/cm² was examined. Therefore, the reduction of resazurin (blue) to resofurin (red) was analysed spectrophotometrically [129, 130]. Similar to the obtained results after contact killing, no inhibition of the metabolic activity was determined for Al, stainless steel and Al-Cu exposed samples. However, a diminished but not inactive metabolism was detected for 10^5 cells/cm² exposed to Al-Ag and Al-Ag-Cu alloys. Thereby the negative effect on the metabolic activity was higher for Al-Ag alloys. Possible reasons for the less antimicrobial effects could be caused by the alloy composition. In case of eutectic Al-Cu alloys only 17.5 mol% (33.3 weight%) copper was present. This concentration was much lower compared to pure copper surfaces (99.9 weight%). Therefore it was expected that the antimicrobial effect was lower, but unfortunately the copper concentration seems to be too low to exhibit antimicrobial effects. In case of Al-Ag alloys, the silver concentration was 34.3 mol% (67.6 weight%) and of Al-Ag-Cu alloys 18.1 mol% (42.2 weight%) with additional 12.8 mol% (17.6 weight%) copper. In sum, around 30 mol% (~ 60 weight%) of both materials was composed of antimicrobial metals. Comparing the antimicrobial metal concentration of Al-Ag and Al-Ag-Cu alloys, a slightly higher percentage was present in Al-Ag alloys. This could cause these small differences in the metabolic activity. On the basis of the EPA registered copper alloys, the Copper Development Association (CDA) concluded that most of them are composed of about 60 weight% copper [154]. Taken into account that silver atoms are much heavier than copper atoms, it can be assumed that the

metal concentration used in this study could be too low to possess antimicrobial efficiency. Therefore, in further analysis the concentration of antimicrobial metals could be increased or the casting optimized.

An increase of copper and/or silver results in the loss of the eutectic composition. This in turn leads to an irregular distribution and regions of crystallised antimicrobial metals which were not composed of aluminium. This in turn can lead to separated regions which possibly possess higher antimicrobial effects on the survival of microorganisms. Contrary to this, copper or silver crystals which were not associated with aluminium could lead to a higher corrosion and therefore a loss in stability of the surfaces. In case of silver, this effect is possibly negligible due to the fact that silver is not as reactive as copper and slower oxidized. These facts indicate that the optimization of the casting protocols is more feasible than changing the metal composition.

4.2 Damaging effects after contact killing

Additionally to the determination of cell survival, damaging effects to cellular compartments caused by copper ions were investigated for *E. coli* K12 exposed to pure copper surfaces. When exposed in a multilayer of cells, no increase in ROS production or membrane damage was obtained. Contrary to the non-existent decrease in cell survival after two hours of exposure, experiments of cells exposed in a monolayer indicated ROS production and membrane damage already after one hour of exposure. This led to the following conclusions. On the one hand, ROS production and membrane damage occurred directly after contact to antimicrobial surfaces, but on the other hand the impact of these effects needed some time to develop to cause cell death. One explanation for this could be that free copper ions were bound to the cell membrane or sequestered via complexation or chelation by ligands as previously shown by Borkow and Gabbay (2005) and Lemire *et al.* (2013) [71, 93]. Thereby, intracellular free copper ions were held at a minimum and further toxic effects prevented. Another possible explanation for the delayed cell death could be caused by the fact that cells were grown overnight in a medium with a high nutrient composition and exposed in buffer solution. Therefore they possess slight residual energy (personal communication: Prof. Dr. Reinhard Wirth, Regensburg University, Germany) which could be used to repair damages caused by copper ions at the beginning of the experiment. Further exposure of up to four hours without energy led to a rapid decrease in cell survival, possibly caused by the constantly high ROS concentration, decreased membrane integrity and higher concentration of free copper ions. Additionally, after longer exposure to free copper ions, the buffering capacity of

sequestration molecules could be depleted. Thereby no further buffering of copper ions was possible.

Further on, the effect of copper ions to *in vivo* and *ex vivo* exposed DNA and RNA was examined to clarify if damages occurred. Copper has two binding sites in the DNA helix. One is present every four nucleotides; the other is an intercalating site which is present in every base pair [90]. Both could lead to mutation, disordering of the helical structure, DNA strand breakage and crosslinking [33, 35, 72, 89-92]. For *in vivo* experiments, no changes in the DNA and RNA integrity were detected even after four hours of exposure. This could be explained by the fact that DNA and RNA are present in the cytoplasm and therefore shielding effects occur through the cell wall and membrane. Secondly, copper ions can be sequestered extra- and intracellularly through siderophores like enterocholin or enterobactin [94]. Last but not least, until now it is unclear whether copper ions appear primarily in the periplasm instead of in the cytoplasm [144]. If they were primarily present in the periplasm, where no DNA or RNA is located, then the diminished fragmentation of *in vivo* exposed DNA and RNA could be explained by this. On the contrary, the *ex vivo* experiments indicated that a decrease in the DNA and RNA integrity was detected as early as after one hour of exposure. Thereby, RNA seems to be quicker fragmented compared to DNA. This can be attributed to the fact that the measured RNA was composed of smaller fragments (2900 nt) in comparison to genomic DNA (>10000 nt) which could make the detection of fragmentation easier.

The results of the current study indicate that membrane damage and ROS production are major effects caused by contact killing on pure copper surfaces. Comparing these results with the published data of Espirito Santo *et al.* (2011) similar conclusions were drawn. Contrary to this, Warnes *et al.* (2011, 2012) postulated that DNA damage is the primary effect of contact killing [83-85]. The contradicting results of different researchers require further mechanistic analysis of the contact killing effect.

Experiments on pure copper surfaces and eutectic alloys were conducted with selected strains of the Keio collection. The Keio Collection comprises of single-gene knockout mutant strains of the *E. coli* K12 derivate BW25113. Each mutant was constructed through exchange of single genes by a kanamycin cassette [111]. By reference to this library the importance of single genes in molecular pathways can be examined. In previous experiments of e.g. Outten *et al*. (2000, 2001) or Rensing *et al.* (1999), several *E. coli* mutant strains with deletions in single genes were examined in the presence of $CuSO_4$ in solution [112, 114, 155]. The experiments conducted in the current work were performed on pure copper surfaces and different potentially antimicrobial alloys containing

aluminium, silver and/or copper. After growing in medium which contained $CuSO_4$ concentrations of 4 or 5 mmol/l, the most important genes were determined and these knockout mutant strains selected for further analysis. All selected strains had either a direct link to copper and/or silver transport mechanisms e.g. Cu^+-translocating P-type ATPase (CopA) [156] or a general function in the microorganisms e.g. Fumarase A, participates in the TCA cycle (FumA) [119].

The following deleted genes in *E. coli* mutant strains have a direct link to copper and silver transport mechanisms:

ΔcueR (Keio:JW0476), *ΔcopA* (Keio:JW0473), *ΔcueO* (Keio:JW0119), *ΔcusR* (Keio:JW0560), *ΔcusS* (Keio:JW5082), *ΔcusA* (Keio:JW0564), *ΔcusB* (Keio:JW0563), *ΔcusC* (Keio:JW0561), *ΔcusF* (Keio:JW0562), *ΔcutA* (Keio:JW4097) and *ΔcutC* (Keio:JW1863)

CueR is a dual regulator, binds Cu^+ or Ag^+ and regulates the expression of CopA and CueO in the presence of these ions [157]. CopA is a P-type ATPase which translocates Cu^+ and probably Ag^+ from the cytoplasm to the periplasm under energy consumption [94]. CueR and CopA are responsible for a rapid diminishing of free Cu^+ and Ag^+ ions inside the cytoplasm and a transport to the periplasm [156]. CueO, a multi-copper oxidase, is present in the periplasm. It oxidizes the catechol siderophore enterobactin and Cu^+ to the less toxic Cu^{2+}. The enterobactin in the reduced form reduces Cu^{2+} to Cu^+, the oxidized form sequesters copper and might additionally decrease the copper concentration by exporting copper ions outside of the cell [94]. Another very important transporter is the Resistance-Nodulation-Cell Division (RND)-efflux complex of the CusCFBA system. The two component system of CusSR is responsible for the regulation of the CusCFBA in the presence of copper and silver ions [112]. CusA is located in the cytoplasmic membrane and associated with the membrane fusion protein CusB which is located in the periplasm. Both are associated with the outer membrane factor CusC. The small periplasmic copper binding protein CusF binds Cu^+ and Ag^+ and transfers these metals directly to CusB which exports them outside cells [158]. Until now, it is unclear whether the RND mediated copper efflux is only accomplished by the periplasmic transport or additionally the cytoplasmic transport via the CusA [30]. CutA and CutC are located in the cytoplasm and induced in the presence of silver or copper ions. These proteins decrease the cytoplasmic copper and silver concentration through binding of free silver or copper ions [115]. As mentioned before, under conditions where cells exhibit intact cytoplasmic membranes, Cu^{2+} is not available in the cytoplasm. Therefore, all

analysed knockout genes are based on the export of Cu^{+} from the cytoplasm and periplasm or the transformation of Cu^{+} to the less toxic Cu^{2+}.

The following deleted genes in *E. coli* mutant strains have a general function in the microorganism:

ΔrpoS (Keio:JW5437), *ΔacrA* (Keio:JW0452), *ΔacrB* (Keio:JW0451), *ΔsodA* (Keio:JW3879), *ΔkatE* (Keio:JW1721), *ΔkatG* (Keio:JW3914) *ΔrecA* (Keio:JW2669), *ΔleuC* (Keio:JW0071), *ΔfumA* (Keio:JW1604) and *Δedd* (Keio:JW1840)

RpoS encodes for the RNA polymerase subunit, the sigma factor σ^{S} and regulates the general stress response in *E. coli* [122]. AcrA and AcrB are multidrug efflux pumps located in the periplasm (AcrA) and the cytoplasmic membrane (AcrB). These three genes play a role in promoting the SIM response of *E. coli* [126, 159]. Superoxide radicals are catalysed to molecular oxygen or hydrogen peroxide by SodA [123]. However, hydrogen peroxide is also toxic and causes DNA damage via Fenton reaction. At high hydrogen peroxide concentration it is scavenged by KatE and at low concentration by KatG [160, 161]. A knockout of SodA, KatE and KatG means higher stress levels and consequently more damaging effects of aerobic living microorganisms. To reduce the level of damaging effects via repair, cells synthesize RecA. This protein is responsible for homologous recombination and therefore DNA repair [162]. The other chosen genes were involved in specific pathways of the cells. LeuC is necessary for the amino acid biosynthesis of leucine [163] and FumA is one of three fumarase enzymes in the TCA cycle [119]. Edd is a phosphogluconate dehydratase and part of the Entner-Doudoroff pathway (KDPG: 2-keto-3-deoxy-6-phosphogluconate) where pyruvate is synthesized from glycose [120].

As written above, previous experiments were conducted with selected strains in a $CuSO_4$ solution. Thereby Outten *et al.* (2001) tested the strains of *E. coli ΔcopA* and *E. coli ΔcueO*, as the most sensitive strains [112]. Similar to these results, the most sensitive strains to copper sulphate in solution (in the current study) were *ΔcopA*, *ΔrecA*, *ΔcutA,* and *ΔcueO*. With these strains and additionally the strain of *ΔcueR*, contact killing effects of different antimicrobial surfaces were observed.

Exposure of the Keio wildtype strain to pure copper surfaces led to similar results compared to *E. coli* K12 cells. Therefore, a comparison of *E. coli* K12 and the Keio wildtype strain was possible.

Similar to survival experiments conducted before, no decrease in survival was determined after the exposure to stainless steel surfaces, Al and Al-Cu alloys. Stainless steel and

aluminium were used as control surfaces while Al-Cu was a eutectic composite material which seems to obtain a too low concentration of copper to receive antimicrobial effects.

In all experiments, the *ΔcutA* and *ΔcueR* strain were in between the most sensitive and resistant strains, whereby *ΔcutA* was always more resistant compared to *ΔcueR*. CueR is responsible for the expression-regulation of CueO and CopA. In case of pure copper exposed samples, *ΔcueO* was always more sensitive and *ΔcopA* more resistant to copper stress than both other mentioned strains. CueO detoxifies copper ions and leads to the sequestration of them by oxidizing the siderophore enterobactin [94]. These mechanisms are very important for cells to diminish free copper ions and the production of ROS through the Fenton like reaction with Cu^{+}. Additionally, this oxidization of Cu^{+} to Cu^{2+} minimizes the penetration of Cu^{+} to the cytoplasm, which is not possible for Cu^{2+}. CopA in turn is responsible for the translocation of copper ions from the cytoplasm to the periplasm and therefore important for the diminishing of free copper ions in the cytoplasm. The relatively high tolerance of *ΔcopA* and low tolerance of *ΔcueO* possibly indicate that copper ions were primarily present in the periplasm and not in the cytoplasm [144]. If this is the case, the transport of copper ions via CopA was not as important as the detoxification of copper ions through CueO in the periplasm.

As opposed to the obtained results for *ΔcueO* and *ΔcopA* exposed on pure copper surfaces, resistances were reversed for cells exposed on silver containing surfaces. Here, the highest resistance was observed for *ΔcueO*. As mentioned above the amount of silver ions in Al-Ag alloys was about 34 mol% and 18.1 mol% in Al-Ag-Cu alloys with additional 12.8 mol% copper. CueO is not implicated in silver resistance which could be explained by its purpose [164]. It oxidizes Cu^{+} to Cu^{2+} but in comparison to copper, it cannot oxidize the noble metal silver. Additionally, silver cannot be sequestered by the CueO oxidized siderophore, enterobactin [94]. Therefore the presence of CueO is not essential when exposed to silver containing surfaces. Contrary to this, *ΔcopA* showed high sensitivity to the exposure on silver containing surfaces. Until now, it remains unclear whether CopA is involved [165] in silver resistance or not [166] but the obtained results of the current study may indicate that CopA is involved in silver resistance. However further in-depth investigations need to be conducted to verify this assumption.

RecA is responsible for DNA repair after damaging effects occurred through e.g. copper or silver ions. The *ΔrecA* strain showed the highest tolerance, similar to the wildtype strain when exposed in 0.1x PBS on pure copper surfaces. Contrary to this, it was the most sensitive strain after the exposure in 0.1 mol/l MOPS on pure copper surfaces and silver alloys. The reason for the reverse resistances after the exposure to pure copper surfaces could be caused by the different release rates of copper ions. In case of 0.1 mol/l MOPS

exposed samples, the release was much higher in comparison to 0.1x PBS exposed samples. As mentioned above, until now, it is unclear whether copper ions are primarily in the periplasm or cytoplasm [144] but the results of 0.1x PBS exposed samples indicate that copper ions were primarily in the periplasm. If this was the case, the presence of RecA was not that important due to the fact that only a few DNA damaging effects occur through copper stress. An increased copper ion release after the exposure of cells in 0.1 mol/l MOPS led to higher copper ion availability and a possible increase in the cytoplasm. Therefore more damaging effects of the DNA could occur. This in turn, has consequences regarding to cell survival of *ΔrecA* strains. They did no longer have the ability to repair and were therefore highly sensitive. Similar results were obtained after the exposure on silver containing surfaces (Al-Ag and Al-Ag-Cu). For both alloys, a significant release of silver ions was determined. Additionally, in case of Al-Ag exposed samples, a slight increase of cell associated silver ions was determined whereas no significant increase was obtained for Al-Ag-Cu exposed samples. Furthermore, the cell associated copper ion concentration for Al-Ag-Cu exposed samples also increased significant (data presented in the supplements: Table 14 and Table 15). Comparing the results of cell associated ions, the silver ion concentration for Al-Ag alloys was smaller in comparison to the sum of copper and silver ions from Al-Ag-Cu. Despite the higher concentration of antimicrobial metals, the toxic effect of Al-Ag was slightly higher compared to Al-Ag-Cu. This indicated a higher toxic effect of the non-essential metal silver [36]. These higher cell associated concentration of ions in turn can lead to more damaging effects of the DNA and a higher sensitivity of *ΔrecA* strains.

4.3 Release of antimicrobial ions

Further analysis of the release rate of ions from pure copper surfaces and eutectic alloys completed the contact killing experiments. Previous experiments by Molteni *et al.* (2010) and Espirito Santo *et al.* (2011) indicated that the release of copper ions could be of major importance for contact killing [83, 110]. To further investigate this hypothesis, the release after exposure of buffer alone, cells exposed in buffer and additionally the cell associated copper ion concentration were determined with ICP-MS measurements. The magnitude of released and cell associated copper ions was compared to the obtained results by Espirito Santo *et al*. (2011) [83]. Thereby, the concrete amount of copper atoms/ml could not be directly compared to the results of Espirito Santo *et al.* due to the reason that they exposed a higher cell concentration in 1x PBS. Nevertheless, comparing the magnitudes of release after three hours of exposure it becomes obvious that similar dimensions were

obtained. The release of copper ions after the exposure of buffer alone increased 10-fold and after the exposure of cells in buffer 100-fold after three hours of exposure. Contrary to these results the amount of copper atoms/cell was vice versa. In case of 0.1x PBS exposed samples, the starting cell associated copper concentration was 10^7 atoms/cell, increasing of up to 10^9 atoms/cell after three hours of exposure. For 1x PBS exposed samples the intracellular copper concentration was diminished by 10^4 atoms/cell at the beginning and 10^8 atoms/cell after three hours of exposure. Comparing both results three major differences in the experimental setup need to be adduced. First of all, the exposed cell and salt concentration in experiments conducted by Espririto Santo *et al.* was much higher compared to 0.1x PBS exposed samples. Higher salt concentrations lead to an increase in corrosion and therefore higher copper ion concentrations in suspension. While the higher salt concentration led to an increased corrosion, the application of higher cell concentration led to a diminishing of the copper concentration. This is explained by the fact that the obtained copper concentration was distributed by more cells which resulted in a smaller amount of copper ions per cell. Additionally, samples analysed by Espririto Santo *et al.* were washed after exposure with an EDTA containing buffer. EDTA chelated membrane bound copper ions and removed them from cells. All mentioned points could lead to a smaller intracellular copper concentration of 1x PBS exposed samples.

Comparing the release of copper ions for the Gram-positive *S. cohnii* and the Gram-negative *E. coli* K12 strain, the release increased slower after the exposure of *S. cohnii*. The slower release and as explained later, slower uptake (cell association) of copper ions could be caused by differences in the availability of transporters. Another reason for the different release of copper ions could be caused by variances in the medium composition. Copper is an essential metal and therefore added in nutrient media with different concentrations, depending on the manufacturing processes. The assumption that one medium could obtain higher copper concentrations was disproven by ICP-MS measurements; in LB 15 ng/ml and 8 ng/ml in TSY was determined. Considering the very low amount it can be assumed as similar.

Comparing the results of total copper ion release, it becomes apparent that the release was much higher when cells were exposed to pure copper surfaces in comparison to buffer alone. These results indicated that cells were either directly or indirectly responsible for this effect. Mathews *et al*. examined in 2013 the release of copper ions and the toxic effect for two differently treated pure copper surfaces. One surface was a normal pure copper surface and the other was coated with a thin plastic layer which was perforated by laser beam. The lateral spacing of the originated holes was 770 nm. Because of this treatment, cells were not able to adhere directly to the surface. After exposure, the

survival and release of copper ions were determined. For both experimental conditions the release of copper ions was similar but the cell survival on coated surfaces was not decreased in comparison to pure copper exposed samples [147]. These results indicate that cells dissolve copper ions indirectly from surfaces by e.g. excretion of complexing agents. Thereby a complexing agent which contains functional groups like -NH_2 (amino), -O- (ether) or -S- (thioether) can form a chemical compound with metals [167].

Similar results were shown for the cell associated copper concentration, which was a rapid increase of cell associated copper ions. One % of the whole cell weight is composed of trace elements, whereas 0.01 % of the whole cellular weight is represented by copper ions [136]. This intracellular copper concentration was determined for both, Gram-positive (0.07 % ± 0.03 %) and Gram-negative (0.05 % ± 0.02 %) strains. From thereon, a rapid increase after exposure to pure copper surfaces was determined. While the cell associated copper concentration increased faster for *E. coli* K12, the final cell associated copper concentration after four hours of exposure reached the same level for both strains. Depending on the starting intracellular copper concentration the amount increased 200-fold (*S. cohnii*) and 300-fold (*E. coli* K12) (paragraph 3.1.4). This could lead to the rapid decrease in cell survival after four hours of exposure (two (*E. coli* K12) or 2.6 (*S. cohnii*) orders of magnitude). The obtained results for the cell associated copper ion concentration were comparable to the results for the total release of copper ions. The lack of significant distinctions of cell associated copper ions indicated that differences in the cell wall and membrane did not account for these measurements.

A comparison of cell associated copper ions and copper ions in the supernatant showed that equal amounts were obtained. This indicated that no active transport of copper ions inside cells were in progress under these experimental conditions. Equilibrium was reached through the osmotic flux of copper ions from the supernatant to (at least) the periplasm of cells. Additionally, these results indicate that the return transport of copper ions outside of the cell is too slow to maintain a reduced intracellular copper level. Further analysis is necessary to determine if copper ions are primarily present in the periplasm, cytoplasm or adhered to cell membrane. To achieve this goal, different follow up strategies were described in paragraph 5.

Mathews *et al*. (2013) postulated that a direct contact to a metallic surface was of major importance to obtain an antimicrobial effect [147]. To verify this, a different experimental setup was implemented with *E. coli* K12 cells. They were exposed for four hours to different copper sulphate concentrations suspended in 0.1x PBS with subsequent analysis of cell survival and ICP-MS measurement to determine the copper concentration. The

obtained results were compared with the release of copper ions and survival after four hours of contact killing on pure copper surfaces.

In solution, the cell survival was decreased by 1.1 orders of magnitude at a copper ion concentration of 503.7 µmol/l. In comparison to this, the final copper concentration after contact killing on pure copper surfaces was only 4.2 µmol/l, while the cell survival was decreased by two orders of magnitude. This result indicated that the toxic effect of copper ions was more intense in contact killing experiments with a direct contact to a metallic antimicrobial surface. Cells suspended in solution are surrounded by a homogenous distribution of copper ions whereas cells in contact killing experiments adhere to the surface and release copper ions over time. In contrast to the measured cells in suspension, the surrounding copper ion concentration is lower but the local concentration is much higher, compared to the measured concentration in cell suspension. This local copper ion concentration cannot be determined with my experimental setup but could lead to higher toxic effects on cells, especially when nearly every cell got in contact to the metallic surface (a monolayer was exposed) [147]. Another possible reason for the higher toxic effect of pure copper surfaces in comparison to higher concentrations of $CuSO_4$-solutions is caused by the charge of solubilised copper ions. In $CuSO_4$-solutions, only Cu^{2+} ions are available. As mentioned before, these ions were not able to penetrate cytoplasmic membranes or lead to ROS production. Therefore they are less toxic compared to Cu^{+}. Samples exposed to pure copper surfaces are in contrast to this exposed to Cu^{+} and Cu^{2+} which can be dissolved from the surface in addition to a high local copper concentration.

The release of copper ions after the exposure of *E. coli ΔcopA* was investigated. This strain does not synthesize the cytoplasmic membrane protein CopA and was selected due to different sensitivities against copper ions in solution and pure copper surfaces. Without CopA a transport via this protein is not possible which could lead to a higher cytoplasmic concentration of copper ions. This in turn could lead to a delayed release of copper ions caused by *ΔcopA* cells. The obtained results of the release of copper ions and the cell associated copper ion concentration after the exposure of *ΔcopA* to pure copper surfaces were compared to the obtained results after exposure of *E. coli* K12. By contrast with *E. coli* K12, the copper ion release of *ΔcopA* increased a bit slower; after three hours of exposure 9.2 nmol ± 0.8 nmol copper was released in case of *ΔcopA* exposed samples. This amount was reached after one hour of exposure of *E. coli* K12 (9.5 nmol ± 0.5 nmol). The final amount of copper was determined after four hours with 14.8 nmol ± 0.5 nmol (*E. coli* K12) and 10.9 nmol ± 1.1 nmol (*ΔcopA*) and was determined as similar due to the variance of error. Comparing the intracellular copper concentration, a higher percentage

with 0.12 % ± 0.01 % was obtained for *ΔcopA* (0.05 % ± 0.02 % for *E. coli* K12). Further results pointed out that the cell associated copper concentration was diminished. Similar to the total release of copper ions, after three hours of exposure it reached the level which was reached for *E. coli* K12 after one hour of exposure. Further exposure of up to four hours led to a maximum cell associated copper concentration for *E. coli* K12 of 15.2 % ± 0.9 % and 19.5 % ± 2.1 % for *ΔcopA*. Taken into account the variance of error, no significant difference was determined for these strains. This indicated that knockouts of the cytoplasmic membrane protein CopA did not influence the overall release and cell associated copper ion concentration. Nonetheless, the release and cell associated copper ion concentration were delayed in the beginning.

Additionally, the release of aluminium, silver and copper ions from eutectic alloys were determined. The results indicated that the total ion concentration did significantly increase, except the copper concentration of Al-Ag-Cu alloys. However, this release was much lower in comparison to the pure copper exposed samples which could explain why the antimicrobial effect of eutectic alloys was not as high as the antimicrobial effect of pure copper surfaces. The inferior release could be caused by the fact that both antimicrobial metals were only available as compounds with aluminium which could possibly decrease the release of these ions and additionally minimizes corrosion of the surface. Nevertheless, it was mentioned before that possibly the direct contact to the metallic surface is of major importance to obtain toxic effects. To ensure that every cell is exposed to equal stress conditions and gets in contact to antimicrobial spots on the alloy each cast was cooled down quickly after casting. Due to this, a chaotic structure was formed without bigger single spots of aluminium alone or aluminium-copper/silver crystals. Further investigations on antimicrobial eutectic compositions with an optimized casting protocol need to be conducted. On the basis of the obtained results it is assumed that the release of ions would be held at a minimum while the toxicity to microorganisms will be increased.

5. Conclusion and Outlook

Under wet, aerobic conditions, copper reacts with oxygen whereby dissolution of copper ions occurs. The release is important for cellular uptake to produce ROS and cause damaging effects to the cell membrane, DNA and RNA. Nonetheless, experiments showed that a direct contact to copper surfaces is more important than the presence of free copper ions. Oxidised copper surfaces still exhibit antimicrobial effects but change the surface roughness, stability and appearance. Therefore pure copper surfaces are difficult for the application in hospitals e.g. as doorknobs or light switches.

On the basis of these results, subsequent experiments were conducted on new, potentially antimicrobial alloys to investigate if these have antimicrobial properties. These alloys were composed of the antimicrobial metals silver and/or copper and are crystallized as compounds with aluminium. This could lead to a minimised release of ions and a longer stability of the alloys. Contrary to measurements with “fast” casted alloys, the antimicrobial effects of “slow” casted Al-Ag-Cu alloys was as high as after the exposure to pure copper surfaces. This indicated that the casting procedure is of major importance. Different casting protocols and solidification rates of the samples could lead to higher distribution of crystals and therefore higher antimicrobial effects. The reason why eutectic alloys were chosen is accounted by the fact that the application of alloys is much better in comparison to pure copper surfaces in terms of the prize and corrosive features. They possess a lower antimicrobial metal concentration and exhibit lower corrosive effects when they were applied to moist conditions or to oxygen atmosphere. Therefore, they possibly possess longer lifetimes and are better to applicate.

In molecular studies with different knockout genes of *E. coli*, CopA and CueO were identified as the most important genes when cells were exposed to antimicrobial surfaces. This indicated that, except for the direct contact, the decrease of free copper/silver ions in the cytoplasm and the transformation of Cu^{+} to Cu^{2+} is of major importance for cells to survive stress conditions on antimicrobial surfaces.

Results from this study raise some very intriguing questions for further experiments. Depending on the cell associated copper ion concentration, very high levels of copper ions were obtained. Therefore further analysis with TEM and ICP-MS could be conducted to determine if these ions were primarily present in the cytoplasm, periplasm or adhered to the cell membrane.

Additional studies on double mutant strains e.g. CopA/CueO or else exposed to antimicrobial surfaces/alloys can be conducted. By means of these mutant strains, more detailed molecular analysis of the importance of several genes in correspondence to specific metal ions like silver and/or copper can be conducted.

Further on, follow-up experiments with eutectic alloys should be performed, whereby two different strategies can be pursued. First of all, the composition of antimicrobial metals can be increased. This would lead to non-eutectic compositions with crystals of pure antimicrobial metals which possibly possess higher antimicrobial effects. Another, perhaps more interesting strategy is concerned by alternative casting protocols. Thereby, the eutectic character of the surfaces are maintained and additional intermediate steps as described for “slow” casted alloys could be integrated.

Last but not least with this experimental setup several other potentially antimicrobial surfaces can be examined for new applications.

6. References

1 Nilsson, MP, and Fielden, FJ, *Primitive time-reckoning; a study in the origins and first development of the art of counting time among the primitive and early culture peoples*. Lund: CWK Gleerup. 1920.

2 Ruggles, C, *Ancient astronomy*. ABC-CLIO, Inc. 2005.

3 Horneck, G, and Rettberg, P, *Complete course in astrobiology*. Wiley-VCH. 2007.

4 Horneck, G, Klaus, DM, and Mancinelli, RL, *Space microbiology*. Microbiol Mol Biol Rev. 2010. **74**: 121-56.

5 Groß, U, *Kurzlehrbuch medizinische Mikrobiologie und Infektiologie*. Georg Thieme. 2013.

6 Van Houdt, R, and Leys, N, *Monitoring the microbial burden in manned space stations*. Infect Drug Resist. 2012. **8**: 299-308.

7 International Space Station medical operations reqierements documents, Revision B. May 2003.

8 NASA SBIR 2016 Phase I Solicitation, H3 Life support and habitation systems. 2016. 1-4.

9 NASA Technology Roadmaps, TA 6: Human health, life support, and habilitation systems. July 2015.

10 Gorbach, SL, *Medical microbiology, microbiology of the gastrointestinal tract*. University of Texas Medical Branch at Galveston. 1996.

11 Singleton, P, *Bacteria in biology, biotechnology and medicine*. Wiley-VCH. 1999.

12 Bentley, R, and Meganathan, R, B*iosynthesis of vitamin K (menaquinone) in bacteria.* Microbiol Rev. 1982. **46**: 241-80.

13 Kenneth, JR, and Ray, CG, *Sherris medical microbiology*. McGraw Hill. 2004.

14 Roth, RR, and James, WD, *Microbial ecology of the skin.* Annu Rev Microbiol. 1988. **42**: 441-64.

15 Hudault, S, Guignot, J, and Servin A, *Escherichia coli strains colonising the gastrointestinal tract protect germfree mice against Salmonella typhimurium infection*. Gut. 2001 **49**: 47-55.

16 Reid, G, Howard, J, and Gan, BS, *Can bacterial interference prevent infection?* Trends Microbiol. 2001. **9**: 424-8.

17 Fernandes, AP, Perl, TM, and Herwaldt LA, *Staphylococcus cohnii: a case report on an unusual pathogen*. Clin Perform Qual Health Care. 1996. **4**: 107-9.

18 Edmond, MB, Wallace, SE, McClish, DK, Pfaller, MA, Jones, RN, and Wenzel, RP, *Nosocomial bloodstream infections in United States hospitals: a three-year analysis*. Clin Infect Dis. 1999 **29**: 239-44.

19 European Centre for Disease Prevention and Control, Healthcare-associated infections. http://ecdc.europa.eu/en/healthtopics/Healthcare-associated_infections/Pages/index.aspx. 13.06.2016.

20 Becker, W, *Die Entdeckungsgeschichte des Staphylococcus aureus – Ein historischer Überblick* –. Orthopedic dynamic Heft 2015. **2**: 7-13.

21 European Centre for Disease Prevention and Control, *Annual epidemiological report Reporting on 2010 surveillance data and 2011 epidemic intelligence data*. 2012.

22 Alexander, JW, *History of the Medical use of silver*. Surg Infect (Larchmt). 2009. **10**: 289-92.

23 Grass, G, Rensing, C, and Solioz, M, *Metallic copper as an antimicrobial surface*. Appl Environ Microbiol. 2011. **77**: 1541-7.

24 Dollwet, HHA, and Sorenson JRJ, *Historic uses of copper compounds in medicine*. J Trace Elem Med Biol. 1985. **2**: 80-7.

25 Siebeneicher, H, *Oligodynamische Wirkung der Metalle und Metallsalze*. Kolloid-Zeitschrift, 1932. **2**: 243–53

26 Von Nägeli, KW, *Über die oligodynamischen Erscheinungen an lebenden Zellen*. Neue Denkschr Allg Schweiz ges Naturwiss. 1893. **33**: 174–82.

27 Kawakami, H, Yoshida, K, Nishida, Y, Kikuchi, Y, and Sato, Y, *Antibacterial properties of metallic elements for alloying evaluated with application of JIS Z 2801:2000*. ISIJ International. 2008. **48**: 1299-1304.

28 Gabbay, J, Borkow, G, Mishal, J, Magen, E, Zatcoff, R, and Shemer-Avni, Y, *Copper oxide impregnated textiles with potent biocidal activities*. J Ind Text. 2006. **35**: 323-35.

29 Emsley, J, *Nature's building blocks: an A-Z guide to the elements*. New Rev Up Edition. 2011. 124.

30 Rensing, C, and Grass, G, *Escherichia coli mechanisms of copper homeostasis in a changing environment*. FEMS Microbiol Rev. 2003. **27**: 197-213.

31 Frausto da Silva, JJR, and Williams, RJP, *The biological chemistry of the elements*. Clarendon Press, Oxford, 2001.

32 Puig, S, and Thiele, DJ, *Molecular mechanisms of copper uptake and distribution*. Curr Opin Chem Biol. 2002 **6**: 171-80.

33 Dupont, CL, Grass, G, and Rensing, C, *Copper toxicity and the origin of bacterial resistance--new insights and applications*. Metallomics. 2011. **3**: 1109-18.

34 Grass, G, and Rensing, C, *Genes involved in copper homeostasis in Escherichia coli*. J Bacteriol. 2001. **183**: 2145-7.

35 Festa, RA, and Thiele, DJ, *Copper: an essential metal in biology*. Curr Biol. 2011. **21**: R877-83.

36 Nies, DH, *Microbial heavy-metal resistance*. Appl Microbiol Biotechnol. 1999. **51**: 730-50.

37 Kim, EH, Rensing, C, and McEvoy, MM, *Chaperone-mediated copper handling in the periplasm.* Nat Prod Rep. 2010. **27**: 711-9.

38 Franke, S, Grass, G, Rensing, C, and Nies, DH, *Molecular analysis of the copper-transporting efflux system CusCFBA of Escherichia coli*. J Bacteriol. 2003. **185**: 3804-12.

39 Abou-Shanab, RA, van Berkum, P, and Angle, JS, *Heavy metal resistance and genotypic analysis of metal resistance genes in gram-positive and gram-negative bacteria present in Ni-rich serpentine soil and in the rhizosphere of Alyssum murale*. Chemosphere. 2007. **68**: 360-7.

40 Waldron, KJ, and Robinson, NJ, *How do bacterial cells ensure that metalloproteins get the correct metal?* Nat Rev Microbiol. 2009. **7**: 25-35.

41 Mathews, S, Kumar, R, and Solioz, M, *Copper reduction and contact killing of bacteria by iron surfaces*. Appl Environ Microbiol. 2015. **81**: 6399-403.

42 Kuhn, PJ, *Doorknobs: a source of nosocomial infection*. Diagnostic Med. 1983. 62-3.

43 United States Environmental Protection Agency, *EPA registers copper-containing alloy products*. The National Academic Press, 2008.

44 Casey, AL, Adams, D, Karpanen, TJ, Lambert, PA, Cookson, BD, Nightingale, P, Miruszenko, L, Shillam, R, Christian, P, and Elliott, TS, *Role of copper in reducing hospital environment contamination*. J Hosp Infect. 2010. **74**: 72-7.

45 Lin, Y-SE, Vidic, RD, Stout, JE, and Yu, VL, *Individual and combined effects of copper and silver ions on inactivation of Legionella pneumophila*. Wat Res. 1996. **8**: 1905-13.

46 Cassells, JM, Yahya, MT, Gerba, CP, and Rose, JB, *Efficacy of a combined system of copper and silver and free chlorine for inactivation of Naegleria fowleri amoebas in water*. Water Sci Technol. 1995. **31**: 119–22.

47 Mietzner, SSR, Farley, A, Wald, ER, Ge, JH, States, SJ, Libert, T, Wadowsky, RM, *Efficacy of thermal treatment and copper-silver ionization for controlling Legionella pneumophila in high-volume hot water plumbing systems in hospitals*. Am J Infect Control. 1997. **25**: 452-7.

48 Faúndez, G, Troncoso, M, Navarrete, P, and Figueroa, G, *Antimicrobial activity of copper surfaces against suspensions of Salmonella enterica and Campylobacter jejuni*. BMC Microbiol. 2004. **4**: 19.

49 Zhu, L, Elguindi, J, Rensing, C, and Ravishankar, S, *Antimicrobial activity of different copper alloy surfaces against copper resistant and sensitive Salmonella enterica*. Food Microbiol. 2012. *30*: 303-10.

50 Noyce, JO, Michels, H, and Keevil, CW, *Use of copper cast alloys to control Escherichia coli O157 cross-contamination during food processing*. Appl Environ Microbiol. 2006. **72**: 4239-44.

51 Anita, S, Ramachandran, T, Rajendran, R, Koushik, CV, and Mahalakshmi, M, *A study of the antimicrobial property of encapsulated copper oxide nanoparticles on cotton fabric*. Text Res J. 2011. **81**: 1081-8.

52 Cady, N, Behnke, J, and Strickland, A, *Copper-based nanostructured coatings on natural cellulose: nanocomposites exhibiting rapid and efficient inhibition of a multi-drug resistant wound pathogen*. Adv Funct Mater. 2011. **21**: 2506-14.

53 Anyaogu, KC, Fedorov, AV, and Neckers, DC, *Synthesis, characterization, and antifouling potential of functionalized copper nanoparticles*. Langmuir. 2008. **24**: 4340-6.

54 Chapman, J, Le Nor, L, Brown, R, Kitteringham, E, Russell, S, Sullivan, T, and Regan, F, *Antifouling performances of macro- to micro- to nano-copper materials for the inhibition of biofouling in its early stages*. J Mater Chem B. 2013. **1**: 6194.

55 Lewis, MJ, and Young, TW, *Brewing*. Aspen Publishers, Inc. 2002.

56 Marais, F, Mehtar, S, and Chalkley, L, *Antimicrobial efficacy of copper touch surfaces in reducing environmental bioburden in a South African community healthcare facility*. J Hosp Infect. 2010. **74**: 80-2.

57 Mikolay, A, Huggett, S, Tikana, L, Grass, G, Braun, J, and Nies, DH, *Survival of bacteria on metallic copper surfaces in a hospital trial*. Appl Microbiol Biotechnol. 2010. **87**: 1875-9.

58 Laitinen, K, Yliopiston, H, Instituutti, H, and Voutilainen, P, *Clinical trial on using copper and brass surfaces in a hospital in West-Finland using microbiological assessment*. Intern Rep. 2010.

59 Karpanen TJ1, C.A., Lambert PA, Cookson BD, Nightingale P, Miruszenko L, Elliott TS, The antimicrobial efficacy of copper alloy furnishing in the clinical environment: a crossover study. Infect Control Hosp Epidemiol, 2012. 33(1): p. 3-9.

60 Schmidt, MG, Attaway, HH, Sharpe, PA, John Jr., J, Sepkowitz, KA, Morgan, A, Fairey, SE, Singh, S, Steed, LL, Cantey, JR, Freeman, KD, Michels, HT, and Salgado, CD, *Sustained reduction of microbial burden on common hospital surfaces through introduction of copper*. J Clin Microbiol. 2012. **50**: 2217-23.

61 Schmidt, MG, Attaway, HH, Fairey, SE, Steed, LL, Michels, HT, and Salgado, CD, *Copper continuously limits the concentration of bacteria resident on bed rails within the intensive care unit*. Infect Control Hosp Epidemiol. 2003. **5**: 1-5.

62 Salgado, CD, Sepkowitz, KA, John, JF, Cantey, JR, Attaway, HH, Freeman, KD, Sharpe, PA, Michels, HT, and Schmidt, MG, *Copper surfaces reduce the rate of healthcare-acquired infections in the intensive care unit*. Infect Control Hosp Epidemiol. 2013. **34**: 479-86.

63 O'Gorman, J, and Humphreys, H, *Application of copper to prevent and control infection. Where are we now?* J Hosp Infect. 2012. **81**: 217-23.

64 Visurraga, JD, Gutierrez, C, von Plessing, C, and Garcia, A, *Metal nanostructures as antibacterial agents.* Science against microbial pathogens. 2011. 2010-9.

65 Bondarenko, O, Ivask, A, Käkinen, A, and Kahru, A, *Sub-toxic effects of CuO nanoparticles on bacteria: kinetics, role of Cu ions and possible mechanisms of action*. Environ Pollut. 2012. **169**: 81-9.

66 Lee, YJ, Kim, S, Park, S-H, Park, H, and Huh, Y-D, *Morphology-dependent antibacterial activities of Cu_2O*. Materials Letters. 2011. **65**: 818-20.

67 Vargas-Reus, MA, Memarzadeh, K, Huang, J, Ren, GG, and Allaker, RP, *Antimicrobial activity of nanoparticulate metal oxides against peri-implantitis pathogens*. Int J Antimicrob Agents. 2012. **40**: 135-9.

68 Christy, AJ, Nehru, LC, and Umadevi, M, *A novel combustion method to prepare CuO nanorods and its antimicrobial and photocatalytic activities*. Powder Technology. 2013. **235**: 783-6.

69 Ren, G, Hu, D, Cheng EWC, Vargas-Reus, MA, Reip, P, and Allaker, RP, *Characterisation of copper oxide nanoparticles for antimicrobial applications*. Int J Antimicrob Agents. 2009. **33**: 587-90.

70 Elguindi, J, Moffitt, S, Hasman, H, Andrade, C, Raghavan, S, and Rensing, C, *Metallic copper corrosion rates, moisture content, and growth medium influence survival of copper ion-resistant bacteria*. Appl Microbiol Biotechnol. 2011. **89**: 1963-70.

71 Borkow, G, and Gabbay, J, *COPPER As a biocidal tool*. Curr Med Chem. 2005 **12**: 2163-75.

72 Sagripanti, JL, Routson, LB, and Lytle, CD, *Virus inactivation by copper or iron ions alone and in the presence of peroxide*. Appl Environ Microbiol. 1993. **59**: 4374-6.

73 Huang, HI, Shih, HY, Lee, CM, Yang, TC, Lay, JJ, and Lin, YE, *In vitro efficacy of copper and silver ions in eradicating Pseudomonas aeruginosa, Stenotrophomonas maltophilia and Acinetobacter baumannii: implications for on-site disinfection for hospital infection control*. Water Res. 2008. **42**: 73-80.

74 Halliwell, B, and Gutteridge, JM, *Oxygen toxicity, oxygen radicals*. Biochem J. 1984. **219**: 1-14.

75 Fenton, HJH, *Oxidation of tartaric acid in presence of iron*. J Chem Soc, Trans. 1894. **65**: 899-910.

76 Halliwell, B, and Gutteridge, JM., *Role of free radicals and catalytic metal ions in human disease: an overview*. Methods Enzymol. 1990. **186**: 1-85.

77 Hong, R, Kang, TY, Michels, CA, Gadura, N, *Membrane lipid peroxidation in copper alloy-mediated contact killing of Escherichia coli*. Appl Environ Microbiol. 2012. **78**: 1776-84.

78 Espirito Santo, C, Taudte, N, Nies, DH, and Grass, G, *Contribution of copper ion resistance to survival of Escherichia coli on metallic copper surfaces*. Appl Environ Microbiol. 2008. **74**: 977-86.

79 Yoshida, Y, Furuta, S, and Niki, E, *Effects of metal chelating agents on the oxidation of lipids induced by copper and iron*. Biochim Biophys Acta. 1993. **1210**:81-8.

80 Van den Berg, BM, Nieuwdorp, M, Stroes, ES, and Vink, H, *Glycocalyx and endothelial (dys) function: from mice to men*. Pharmacol Rep. 2006. **58**: 75-80.

81 Jung, WK, Koo, HC, Kim, KW, Shin, S, Kim, SH, and Park, YH, *Antibacterial activity and mechanism of action of the silver ion in Staphylococcus aureus and Escherichia coli*. Appl Environ Microbiol. 2008. **74**: 2171-8.

82 Yamanaka, M, Hara, K, and Kudo, J, *Bactericidal actions of a silver ion solution on Escherichia coli, studied by energy-filtering transmission electron microscopy and proteomic analysis*. Appl Environ Microbiol. 2005. **71**: 7589-93.

83 Espirito Santo, C, Lam, EW, Elowsky, CG, Quaranta, D, Domaille, DW, Chang, CJ, and Grass, G, *Bacterial killing by dry metallic copper surfaces*. Appl Environ Microbiol. 2011. **77**: 794-802.

84 Warnes, SL, and Keevil, CW, *Mechanism of copper surface toxicity in vancomycin-resistant enterococci following wet or dry surface contact*. Appl Environ Microbiol. 2011. **77**: 6049-59.

85 Warnes, SL, Caves, V, and Keevil, CW, *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. 2012. **14**: 1730-43.

86 Macomber, L, and Imlay, JA, T*he iron-sulfur clusters of dehydratases are primary intracellular targets of copper toxicity*. Proc Natl Acad Sci U S A. 2009. **106**: 8344-9.

87 OÌsterberg, R, *Study of the biochemistry of copper (INCRA research report)*. International Copper Research Association. 1980. 1-30.

88 Ortiz de Orue Lucana, D, Wedderhoff, I, and Groves, MR, *ROS-mediated signalling in bacteria: zinc-containing cys-x-x-cys redox centres and iron-based oxidative stress*. J Signal Transduct. 2012. **2012**: 605905.

89 Dowjat, WK, Kharatishvili, M, and Costa, M, *DNA and RNA strand scission by copper, zinc and manganese superoxide dismutases*. Biometals. 1996. **9**: 327-35.

90 Sagripanti, JL, Goering, PL, and Lamanna, A, *Interaction of copper with DNA and antagonism by other metals*. Toxicol Appl Pharmacol. 1991. **110**: 477-85.

91 Rifkind, JM, Shin, YA, Heim, JM, and Eichhorn, GL, *Cooperative disordering of single stranded polynucleotides through copper crosslinking*. Biopolymers. 1976. **15**: 1879-1902.

92 Borkow, G, *Using copper to fight microorganisms*. Curr Chem Biol. 2012. **6**: 93-103.

93 Lemire, JA, Harrison, JJ, and Turner, RJ, *Antimicrobial activity of metals: mechanisms, molecular targets and applications*. Nat Rev Microbiol. 2013. **11**: 371-84.

94 Grass, G, Thakali, K, Klebba, PE, Thieme, D, Müller, A, Wildner, GF, and Rensing, C, *Linkage between catecholate siderophores and the multicopper oxidase CueO in Escherichia coli*. J Bacteriol. 2004. **186**: 5826-33.

95 Kim, EH, Nies, DH, McEvoy, MM, and Rensing, C, *Switch or funnel: how RND-type transport systems control periplasmic metal homeostasis*. J Bacteriol. 2011. **193**: 2381-7.

96 Gibbard, J, *Public health aspects of the treatment of water and beverages with silver*. Am J Public Health Nations Health. 1937. **27**: 112-9.

97 Birmele, MN, McCoy, LE, and Roberts, MS, *Disinfection of spacecraft potable water systems by passivation with ionic silver*. American Institute of Aeronautics and Astronautics. 1-8.

98 Goodman, SB, Yao, Z, Keeney, M, and Yang, F, *The future of biologic coatings for orthopaedic implants*. Biomaterials. 2013. **34**: 3174-83.

99 Saint, S, Elmore, JG, Sullivan, SD, Emerson, SS, and Koepsell, TD, *the efficacy of silver alloy-coated urinary catheters in preventing urinary tract infection: a meta-analysis*. Am J Med. 1998. **105**: 236-41.

100 Monteiro, DR, Gorup, LF, Takamiya, AS, Ruvollo-Filho, AC, de Camargo, ER, and Barbosa, DB, *The growing importance of materials that prevent microbial adhesion: antimicrobial effect of medical devices containing silver*. Int J Antimicrob Agents. 2009. **34**: 103-10.

101 Roe, D, Karandikar, B, Bonn-Savage, N, Gibbins, B, and Roullet, JB, *Antimicrobial surface functionalization of plastic catheters by silver nanoparticles*. J Antimicrob Chemother. 2008. **61**: 869-76.

102 Kumar, R, and Munstedt, H, *Silver ion release from antimicrobial polyamide/silver composites*. Biomaterials. 2005. **26**: 2081-8.

103 Allaker, RP, Vargas-Reus, MA, and Ren, GG, *Nanometals as antimicrobials*. Antimicrobial Polymers. Wiley-VCH. 2011.

104 Silvestry-Rodriguez, N, Sicairos-Ruelas, EE, Gerba, CP, and Bright, KR, *Silver as a disinfectant*. Rev Environ Contam Toxicol. 2007. **191**: 23-45.

105 Xu, FF, and Imlay, JA, *Silver(I), mercury(II), cadmium(II), and zinc(II) target exposed enzymic iron-sulfur clusters when they toxify Escherichia coli*. Appl Environ Microbiol. 2012. **78**: 3614-21.

106 Yoon, KY, Hoon Byeon, J, Park, JH, Hwang, J, *Susceptibility constants of Escherichia coli and Bacillus subtilis to silver and copper nanoparticles*. Sci Total Environ. 2007. **373**: 572-5.

107 Fisher, K, Pope, M, and Phillips, C, *Combined effect of copper and silver against Pseudomonas aeruginosa*. J Hosp Infect. 2009. **73**: 180-2.

108 Domke, W, *Werkstoffkunde und Werkstoffprüfung*. Cornelsen Verlag. 1994

109 Hötzer, J, Jainta, M, Steinmetz, P, Nestlera, B, Dennstedt, A, Genau, A, Bauer, M, Köstler, H, Rüde, U, *Large scale phase-field simulations of directional ternary eutectic solidification*. Acta Materialia. 2015. **93**: 194-204.

110 Molteni, C, Abicht, HK, and Solioz, M, *Killing of bacteria by copper surfaces involves dissolved copper*. Appl Environ Microbiol. 2010. **76**: 4099-101.

111 Baba, T, Ara, T, Hasegawa, M, Takai, Y, Okumura, Y, Baba, M, Datsenko, KA, Tomita, M, Wanner, BL, and Mori, H, *Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection*. Mol Syst Biol. 2006. **2**: 2006 0008.

112 Outten, FW, Huffman, DL, Hale, JA, and O'Halloran, TV, *The independent cue and cus systems confer copper tolerance during aerobic and anaerobic growth in Escherichia coli*. J Biol Chem. 2001. **276**: 30670-7.

113 Munson, GP, Lam, DL, Outten, FW, and O'Halloran, TV, *Identification of a copper-responsive two-component system on the chromosome of Escherichia coli K-12*. J Bacteriol. 2000. **182**: 5864-71.

114 Outten, FW, Outten, CE, Hale, J, and O'Halloran, TV, *Transcriptional activation of an Escherichia coli copper efflux regulon by the chromosomal MerR homologue, cueR*. J Biol Chem. 2000. **275**: 31024-9.

115 Fong, ST, Camakaris, J, and Lee, BT, *Molecular genetics of a chromosomal locus involved in copper tolerance in Escherichia coli K-12*. Mol Microbiol. 1995. **15**: 1127-37.

116 Li, J, Ji, C, Chen, J, Yang, Z, Wang, Y, Fei, X, Zheng, M, Gu, X, Wen, G, Xie, Y, and Mao, Y, *Identification and characterization of a novel Cut family cDNA that encodes human copper transporter protein CutC*. Biochem Biophys Res Commun. 2005. **337**: 179-83.

117 Somers, JM, Amzallag, A, and Middleton, RB, *Genetic fine structure of the leucine operon of Escherichia coli K-12*. J Bacteriol. 1973. **113**: 1268-72.

118 Yang, HL, and Kessler, DP, *Genetic analysis of the leucine region in Escherichia coli B-r: gene-enzyme assignments*. J Bacteriol. 1974. **117**: 63-72.

119 Flint, DH, Emptage, MH, and Guest, JR, *Fumarase a from Escherichia coli: purification and characterization as an iron-sulfur cluster containing enzyme*. Biochemistry. 1992. **31**: 10331-7.

120 Egan, SE, Fliege, R, Tong, S, Shibata, A, Wolf, RE Jr, Conway, T, *Molecular characterization of the Entner-Doudoroff pathway in Escherichia coli*. J Bacteriol. 1992. **174**: 4638-46.

121 Courcelle, J, and Hanawalt, PC, *RecA-dependent recovery of arrested DNA replication forks*. Annu Rev Genet. 2003. **37**: 611-46.

122 Maciag, A, Peano, C, Pietrelli, A, Egli, T, De Bellis, G, and Landini, P, *In vitro transcription profiling of the sigmaS subunit of bacterial RNA polymerase: re-definition of the sigmaS regulon and identification of sigmaS-specific promoter sequence elements*. Nucleic Acids Res. 2011. **39**: 5338-55.

123 Britton, L, and Fridovich, I, *Intracellular localization of the superoxide dismutases of Escherichia coli: a reevaluation.* J Bacteriol. 1977. **131**: 815-20.

124 Loewen, PC, and Switala, J, *Purification and characterization of catalase HPII from Escherichia coli K12*. Biochem Cell Biol. 1986. **64**: 638-46.

125 Seaver, LC, and Imlay, JA, *Hydrogen peroxide fluxes and compartmentalization inside growing Escherichia coli*. J Bacteriol. 2001. **183**: 7182-9.

126 Al Mamun, AA, Lombardo, MJ, Shee, C, Lisewski, AM, Gonzalez, C, Lin, D, Nehring, RB, Saint-Ruf, C, Gibson, JL, Frisch, RL, Lichtarge, O, Hastings, PJ, and Rosenberg, SM, *Identity and function of a large gene network underlying mutagenic repair of DNA breaks*. Science. 2012. **338**: 1344-8.

127 Hans, M, Erbe, A, Mathews, S, Chen, Y, Solioz, M, and Mücklich, F, *Role of copper oxides in contact killing of bacteria*. Langmuir. 2013. **29**: 16160-6.

128 Von Borries, B, and Ruska, E, *Das Übermikroskop als Fortsetzung des Lichtmikroskops*. 72-77.

129 O'Brien, J, Wilson, I, Orton, T, and Pognan, F, *Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity*. Eur J Biochem. 2000. **267**: 5421-6.

130 Nicholson, WL, Ricco, AJ, Agasid, E, Beasley, C, Diaz-Aguado, M, Ehrenfreund, P, Friedericks, C, Ghassemieh, S, Henschke, M, Hines, JW, Kitts, C, Luzzi, E, Ly, D, Mai, N, Mancinelli, R, McIntyre, M, Minelli, G, Neumann, M, Parra, M, Piccini, M, Rasay, RM, Ricks, R, Santos, O, Schooley, A, Squires, D, Timucin, L, Yost, B, and Young, A, The O/OREOS mission: first science data from the space environment survivability of living organisms (SESLO) payload. Astrobiology. 2011. **11**: 951-8.

131 LIVE/DEAD BacLight bacterial viability kits. Molecular Probes. 15.7.2004.

132 CellROX oxidative stress reagents. Molecular Probes by life technologies. 6.5.2012.

133 Lindsay, JA, and Sung, JM, *The RM test for determining methicillin-resistant Staphylococcus aureus lineages*. Methods Mol Biol. 2010 **642**: 3-11.

134 T009-NanoDrop 1000-&-NanoDrop 8000-Nucleic-Acid-Purity-Ratios. Thermo Scientific. 4.2008.

135 Thomas, R, *A beginner's guide to ICP-MS*. Spectroscopy. 2001. **16**-**18**.

136 Neidhardt, FC, *Chemical composition of Escherichia coli*. *Escherichia coli* and *Salmonella typhimurium* - cellular and molecular biology. ASM Press. 1987. 3-6.

137 Mehtar S, Wiid, I, Todorov, SD, *The antimicrobial activity of copper and copper alloys against nosocomial pathogens and Mycobacterium tuberculosis isolated from healthcare facilities in the Western Cape: an in-vitro study*. J Hosp Infect. 2008. **68**: 45-51.

138 Michels, HT, Wilks, SA, Noyce, JO, and Keevil, CW, *Copper alloys for human infectious disease control*. Presented at Materials Science and Technology Conference. 2005. 1-11.

139 Kearns, DB, and Losick, R, *Cell population heterogeneity during growth of Bacillus subtilis*. Genes Dev. 2005. **19**: 3083-94.

140 Galeano, B, Korff, E, and Nicholson, WL, Inactivation of vegetative cells, but not spores, of Bacillus anthracis, B. cereus, and B. subtilis on stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. Appl Environ Microbiol. 2003. **69**: 4329-31.

141 Stefano, M, Del Rosso, A, Saldutto, P, Galatioto, GP, and Vincentini, C, *Intrascrotal abscess, Propionibacterium acnes and Staphylococcus cohnii ssp. cohnii: a case report and review of the literature*. Case Rep Urol. 2012. **2012**: 313694.

142 Zarger, MSS, and Khatoon, F, *Phytochemical, GC-MS analysis and antibacterial activity of bioactive compounds of Petroleum ether leaf extracts of Salix viminalis*. IJSR. 2013. 494-500.

143 Tetaz, TJ, and Luke, RK, *Plasmid-controlled resistance to copper in Escherichia coli*. J Bacteriol. 1983. **154**: 1263–8.

144 Macomber, L, Rensing, C, and Imlay, JA, *Intracellular copper does not catalyze the formation of oxidative DNA damage in Escherichia coli*. J Bacteriol. 2007. **189**: 1616-26.

145 Nies DH, and Herzberg, M, *A fresh view of the cell biology of copper in enterobacteria*. Mol Microbiol. 2013. **87**: 447-54.

146 Rojan, A-L, *Der Einfluss von antimikrobiellen Kupferwerkstoffen auf Bakterien – Eine elektronenmikroskopische Strukturanalyse*. 2013.

147 Mathews, S, Hans, M, Mücklich, F, Solioz, M, *Contact killing of bacteria on copper is suppressed if bacterial-metal contact is prevented and is induced on iron by copper ions*. Appl Environ Microbiol. 2013. **79**: 2605-11.

148 Good, NE, Winget, GD, Winter, W, Connolly, TN, Izawa, S, and Singh, RM. *Hydrogen ion buffers for biological research*. Biochemistry. 1966. **5**: 467-77.

149 Montigny, C, and Champeil, P, *Use of metallochromic dyes and potentiometric pH-meter titration to detect binding of divalent cations to "Good's" buffers: 4-morpholinepropanesulfonic acid (MOPS) does not bind Mg^{2+}*. Anal Biochem. 2007. **366**: 96-8.

150 Mash, HE, and Chin, Y-P, *Complexation of copper by zwitterionic aminosulfonic (Good) buffers*. Anal Chem. 2003. **75**: 671-7.

151 Voloshin, SA, and Kaprelyants, AS, *Cell-cell interactions in bacterial populations*. Biochemistry (Mosc). 2004. **69**: 1268-75.

152 Miller, MB, and Bassler, BL, *Quorum sensing in bacteria*. Annu Rev Microbiol. 2001. **55**: 165-99.

153 Dennstedt, A, and Ratke, L. *Microstructures of directionally solidified Al–Ag–Cu ternary eutectics*. Trans Indian Inst Met. 2012. **65**: 777-82.

154 Proper use and care of antimicrobial copper alloys. 2014. 1-7.

155 Rensing, C, Fan, B, Sharma, R, Mitra, B, and Rosen, BP, *CopA: An Escherichia coli Cu(I)-translocating*. Proc Natl Acad Sci U S A. 2000. **97**: 652-6.

156 Changela, A, Chen, K, Xue, Y, Holschen, J, Outten, CE, O'Halloran, TV, and Mondragón, A, *Molecular basis of metal-ion selectivity and zeptomolar sensitivity by CueR*. Science. 2003. **301**:1383-7.

157 Stoyanov, JV, Hobman, JL, and Brown, NL, *CueR (YbbI) of Escherichia coli is a MerR family regulator controlling expression of the copper exporter CopA*. Mol Microbiol. 2001. **39**: 502-11.

158 Bagai, I, Rensing, C, Blackburn, NJ, and McEvoy, MM, *Direct metal transfer between periplasmic proteins identifies a bacterial copper chaperone*. Biochemistry. 2008. **47**: 11408-14.

159 Foster, PL, *Stress-induced mutagenesis in bacteria*. Crit Rev Biochem Mol Biol, 2007. **42**: 373-97.

160 Park, S, You, X, and Imlay, JA, *Substantial DNA damage from submicromolar intracellular hydrogen peroxide detected in Hpx- mutants of Escherichia coli*. Proc Natl Acad Sci U S A. 2005. **102**: 9317-22.

161 Seaver, LC, and Imlay, JA, *Alkyl hydroperoxide reductase is the primary scavenger of endogenous hydrogen peroxide in Escherichia coli*. J Bacteriol. 2001. **183**: 7173-81.

162 Kuzminov, A, *Recombinational repair of DNA damage in Escherichia coli and bacteriophage lambda*. Microbiol Mol Biol Rev. 1999. **63**: 751-813.

163 Yang, HL, and Kessler, DP, *Genetic analysis of the leucine region in Escherichia coli B-r: gene-enzyme assignments*. J Bacteriol. 1974. **117**: 63-72.

164 Delmar, JA, Su, CC, and Yu, EW, *Bacterial multidrug efflux transporters*. Annu Rev Biophys. 2014. **43**: 93-117.

165 Stoyanov, JV, Magnani, D, and Solioz, M, *Measurement of cytoplasmic copper, silver, and gold with a lux biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of Escherichia coli*. FEBS Lett. 2003. **546**: 391-4.

166 Franke, S, Grass, G, and Nies, DH. *The product of the ybdE gene of the Escherichia coli chromosome is involved in detoxification of silver ions*. Microbiology. 2001. **147**: 965-72.

167 Birch, L, and Bachofen, R, *Complexing agents from microorganisms*. Experientia. 1990. **46**: 827-34.

7. Acknowledgements

At this point I would like to thank all the people who made this thesis possible.

First of all, I would like to express my sincere gratitude to Prof. Dr. Reinhard Wirth, who made this work possible and who guided me during my PhD time. Furthermore I would like to thank my second and third corrector, PD Dr. Christine.Hellweg and Prof. Dr. Reinhard Rachel for all their support and willingness to value my thesis.

Additional thanks goes to Dr. Günther Reitz and PD Dr. Christine Hellweg, who gave me the opportunity to carry out my thesis at the German Aerospace Center in the Radiation Biology Department and in particular to Dr. Petra Rettberg. I am very grateful that I could write my thesis in this Department and got the possibility to present my work at conferences and attended summer schools in order to establish a scientific network.

Special thanks goes to Dr. Ralf Möller who originated the great cooperation with Prof. Dr. Frank Mücklich (Saarland University) and Prof. Dr. Lorenz Radtke (DLR, Cologne) and for his support during the final stage of the writing process. These cooperations gave me the opportunity to broaden my horizon in the field of material sciences and made my research possible. With this I would also like to thank Prof. Dr. Frank Mücklich and Prof Dr. Lorenz Radtke for the great support and cooperation. Further on, thank you Dr. Michael Hans (formerly Saarland University) and Anne Dennstedt (formerly DLR, Cologne) for the great and enjoyable support and reliable production of materials. Additionally, I would like to thank Dr. Christina Hein (Saarland University) for the rapid processing of my ICP-MS measurements. I would like to thank all of you for your kindness and friendly discussions and additionally patience when a microbiologist wanted to know more about eutectic compositions or asked other questions related to the chemistry of materials.

Further on, I would like to thank Dr. Stefan Leuko, Dr. Elke Rabbow, Dr. Kristina Beblo-Vranesevic, Dr. Ralf Möller and Dr. Petra Rettberg for the possibility to critically discuss all my questions. Particularly, I would like to thank Dr. Stefan Leuko for correcting my thesis from the very beginning, for all his patience and good comments.

For all the expedient discussions and helpful advices during my thesis, I would like to thank my mentor Dr. Harald Huber (Regensburg University) and for his technical support corresponding to my experiments, I would like to thank Prof. Dr. Reinhard Rachel (Regensburg University). Additionally, I would like to thank Prof. Dr. Uwe Deppenmeier for mentoring me during my PhD.

Finally, I would like to acknowledge the SpaceLife graduate program (Helmholtz Space Life Sciences Research School) for funding the thesis and providing insights into a diverse field of space-related topics. As part of SpaceLife I am furthermore grateful to Claudia Schmitz, PD Dr. Christine Hellweg, Dr. Luis Spitta, and Anna-Maria Trautmann for their workshop organization and great support.

The "Regensburg International Graduate School of Life Sciences" (RIGeL) from the University of Regensburg is also acknowledged.

In addition I would like to thank all my colleagues at the German Aerospace Center in Cologne who provided a wonderful working atmosphere. Thank you very much for the nice time we had in the laboratories and in the office and the expedient discussions. Especially I would like to thank Andre Parpart, Simon Barczyk, Britta Rowehl and Thomas Urlings for their technical support and Dagmar Koschnitzki, Felix Fuchs, Bernd Henschenmacher, Dr. Corinna Panitz, Maria Bohmeier and Andrea Schröder for the nice talks, comments and appreciations during my thesis.

Last but not least, I would like to thank Andre and my family, especially my mother. She always supported me and gave me the opportunity to find my own way.

Supplementary

Contact killing on pure copper surfaces

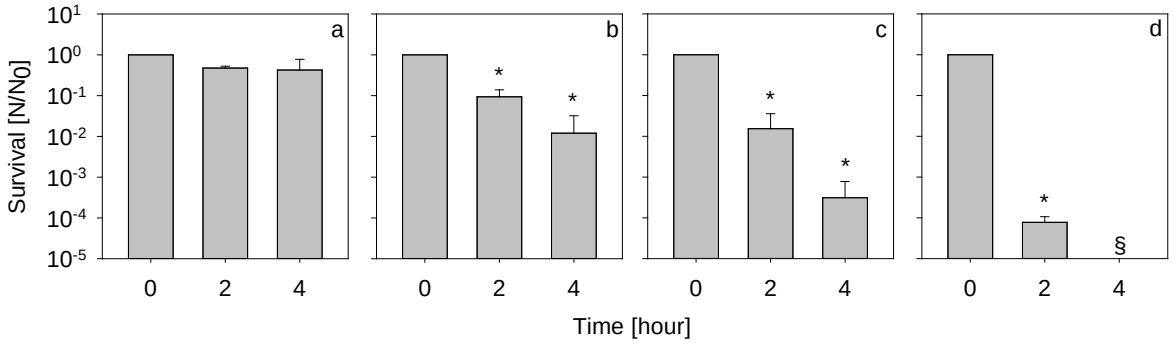


Figure 36: Survival of *E. coli* K12 grown overnight in NB medium and suspended in 0.1x PBS after the exposure to stainless steel (N_0) and pure copper surfaces (N) (n = 3). a: 10^8 cells/cm², b: 10^7 cells/cm², c: 10^6 cells/cm² and d: 10^5 cells/cm²; * significant decrease in cell survival (p-value < 0.05), § below detection threshold.

Nucleic acid fragmentation (RNA)

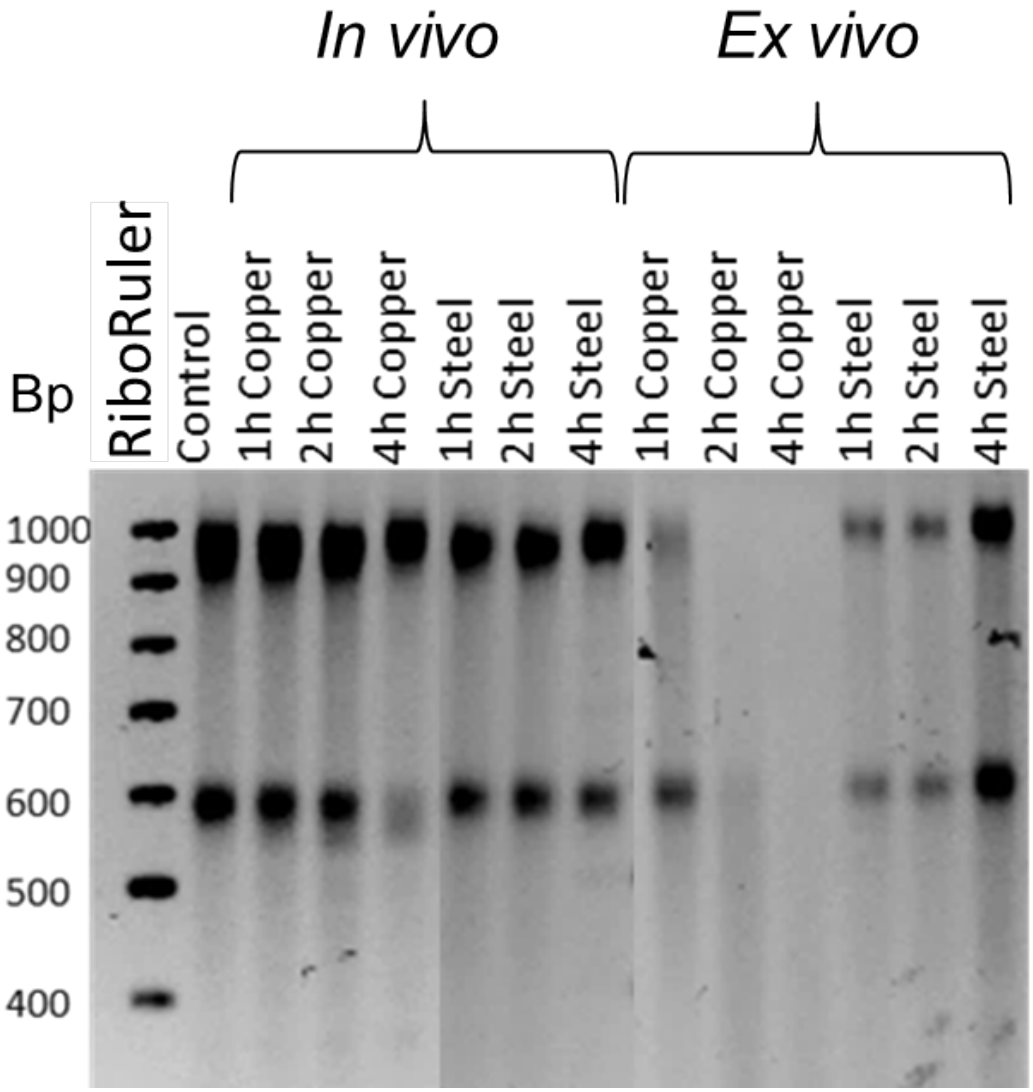


Figure 37: A typical gel electrophoresis of *in vivo* and *ex vivo* exposed RNA separated in a 2 % agarose gel. For *in vivo* analysis, 10^7 cells/cm² were exposed to stainless steel and pure copper surfaces. For *ex vivo* analysis, 15 µl DNA solution was exposed to 0.12 cm² stainless steel and pure copper surfaces. After the exposure and isolation 300 ng of each solution was separated on the agarose gel.

SEM images of eutectic alloys

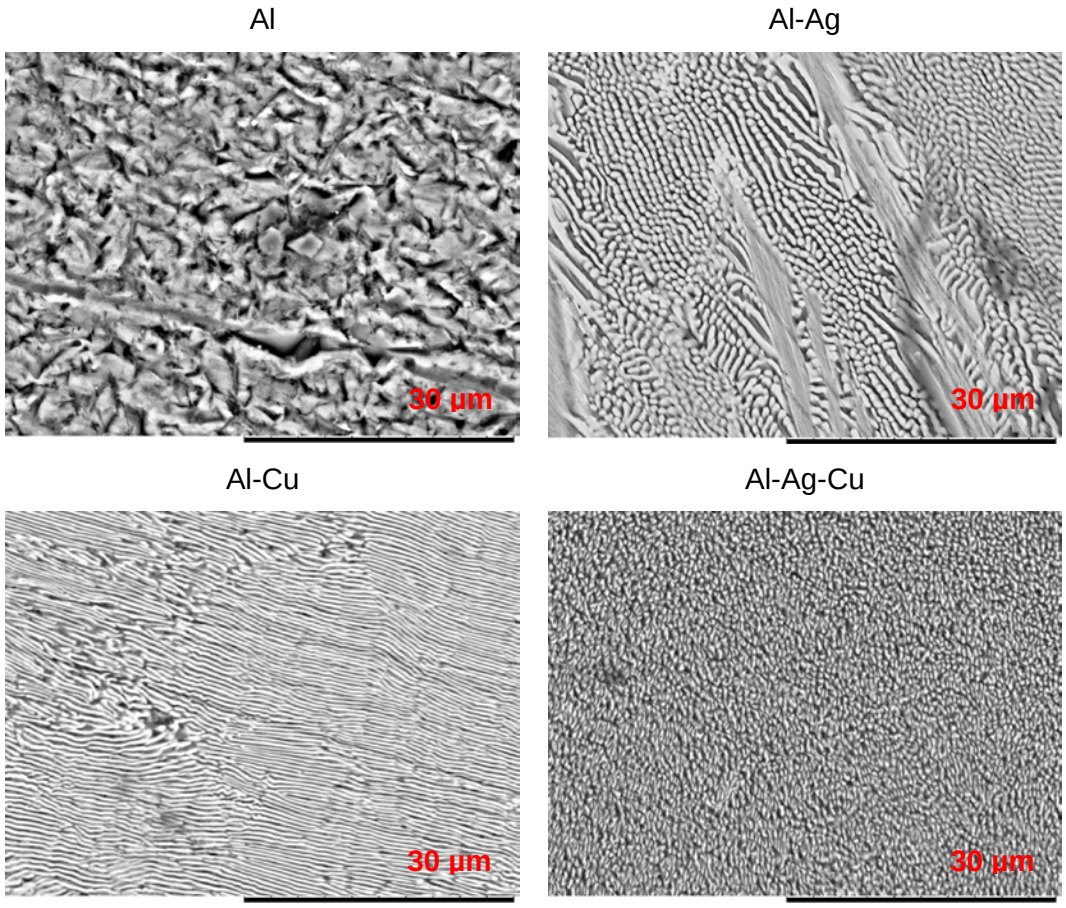


Figure 38: Surface structure of "fast" casted alloys after sanding received with the SEM-EDX.

Contact killing of *S. cohnii* on potentially antimicrobial surfaces

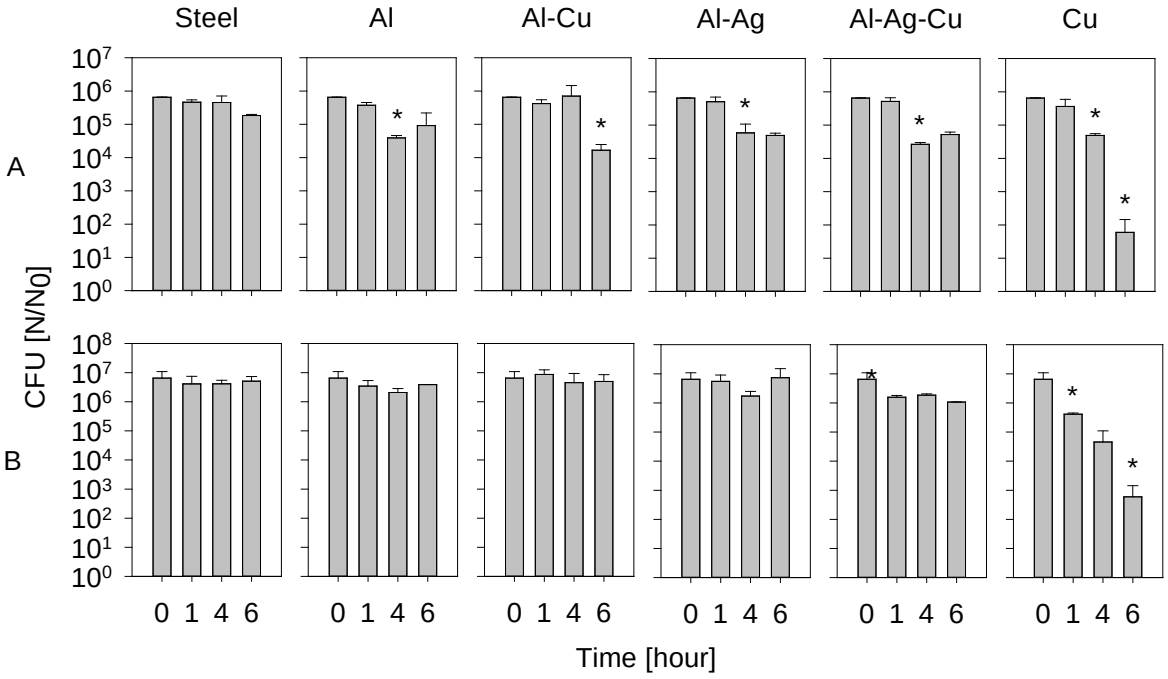


Figure 39: Contact killing of 10^5 (A) and 10^6 (B) *S. cohnii* cells/cm² in 0.1 mol/l MOPS (n = 3). CFU obtained after the exposure to stainless steel, Al, Al-Cu, Al-Ag, Al-Ag-Cu and pure copper surfaces (N) were divided by the obtained CFU before exposure (N_0); * significant decrease in cell survival (p-value < 0.05).

Keios – most important genes to survive copper stress

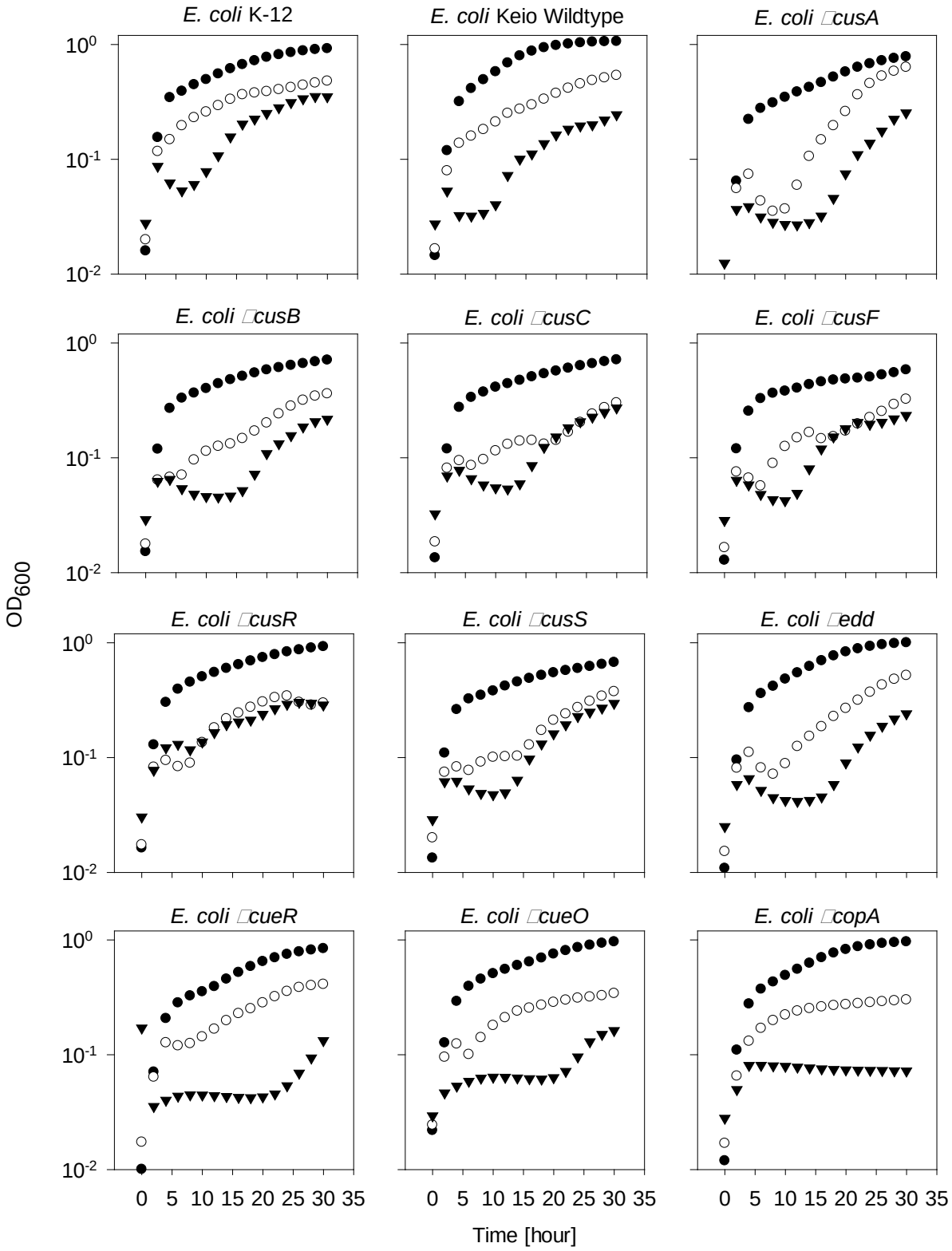


Figure 40: Growth curves of selected Keio strains in LB medium containing 0 mmol/l (●), 4 mmol/l (○) and 5 mmol/l (▼) $CuSO_4$ (n = 3).

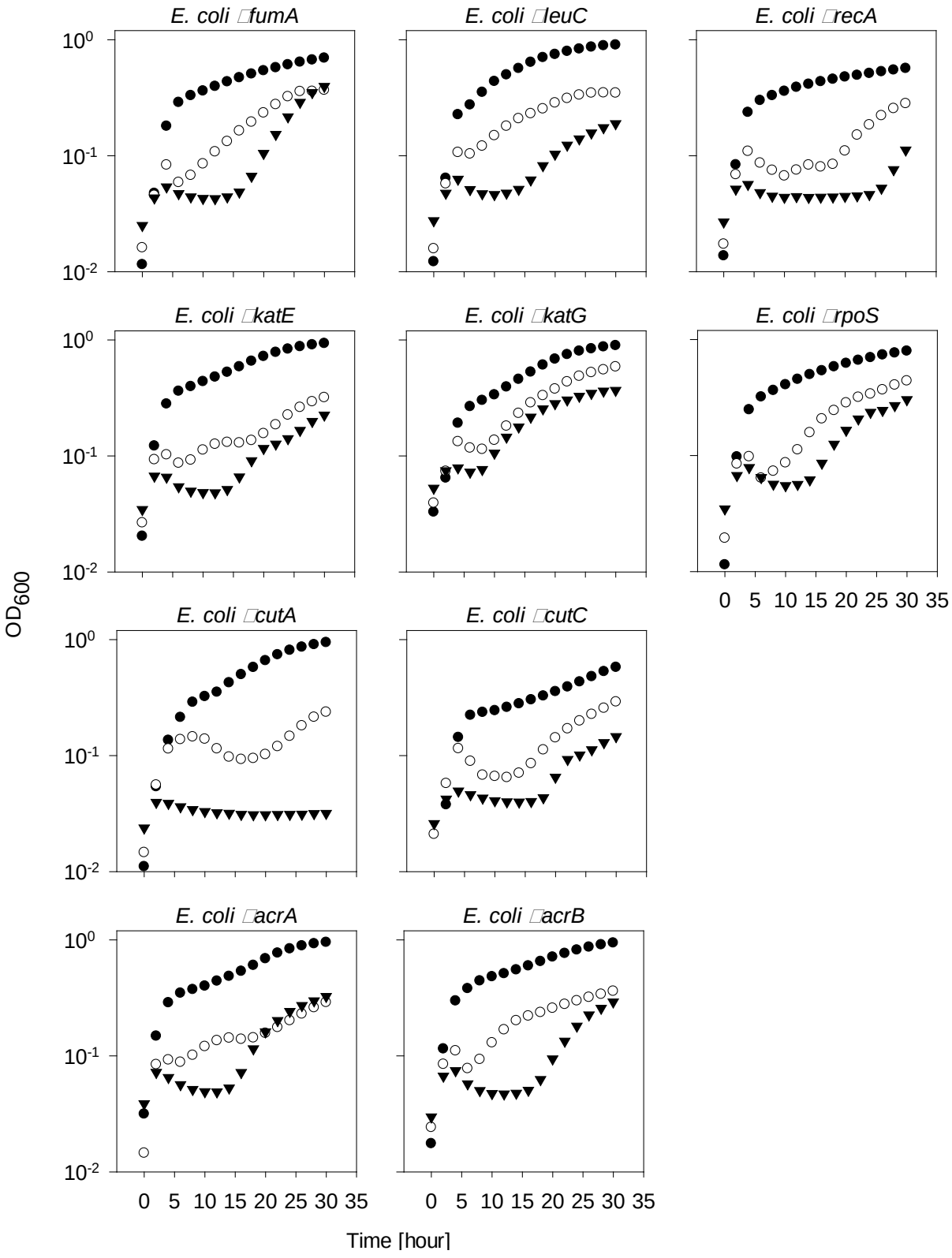


Figure 41: Growth curves of selected Keio strains in LB medium containing 0 mmol/l (●), 4 mmol/l (○) and 5 mmol/l (▼) $CuSO_4$ (n = 3) (continued).

Table 14: Percentage of cell associated silver after the exposure of 10^6 *E. coli* K12 cells/cm² to Al-Ag and Al-Ag-Cu alloys (n = 3). SD indicated standard deviation.

	Al-Ag-surface	Al-Ag-Cu-surface
Time [hour]	**% Ag/cell Mean ± SD**	
0	0.00 ± 0.00	0.00 ± 0.00
1	0.00 ± 0.00	0.00 ± 0.00
2	0.00 ± 0.00	0.00 ± 0.00
3	0.83 ± 0.71	0.14 ± 0.20
4	1.24 ± 0.56	0.37 ± 0.31

Table 15: Percentage of cell associated copper after the exposure of 10^6 *E. coli* K12 cells/cm² to Al-Cu and Al-Ag-Cu alloys (n = 3). SD indicated standard deviation, ND: not determined.

	Al-Cu-surface	Al-Ag-Cu-surface
Time [hour]	**% Cu/cell Mean ± SD**	
0	0.01 ± 0.01	0.01 ± 0.01
1	0.21 ± 0.06	1.48 ± 0.15
2	0.45 ± 0.21	3.46 ± 1.03
3	ND	3.78 ± 0.45
4	0.37 ± 0.21	3.19 ± 1.33

Phase diagram

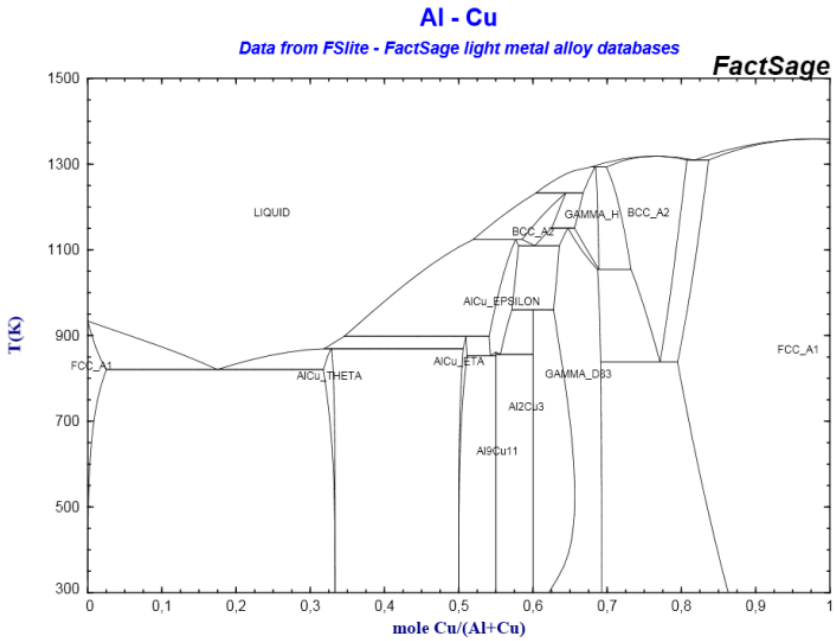


Figure 42: Phase diagram of a Al-Cu.

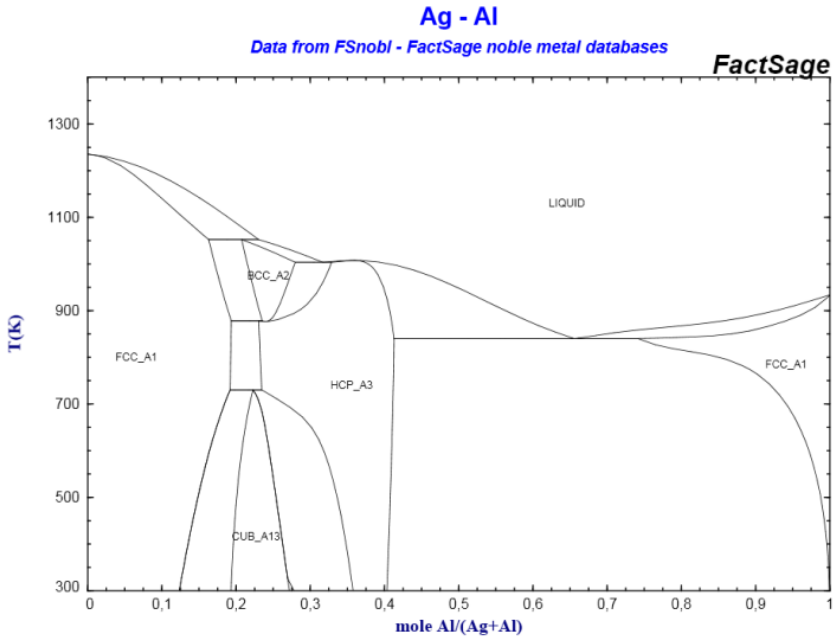


Figure 43: Phase diagram of Al-Ag.