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Influence of copper ions on the viability and cytotoxicity of *Pseudomonas aeruginosa* under conditions relevant to drinking water environments

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ABSTRACT

Copper plumbing materials can be the source of copper ions in drinking water supplies. The aim of the current study was to investigate the influence of copper ions on the viability and cytotoxicity of the potential pathogen *Pseudomonas aeruginosa* that presents a health hazard when occurring in building plumbing systems. In batch experiments, exposure of *P. aeruginosa* (10^6 cells/mL) for 24 h at 20 °C to copper-containing drinking water from domestic plumbing systems resulted in a loss of culturability, while total cell numbers determined microscopically did not decrease. Addition of the chelator diethyldithiocarbamate (DDTC) to copper-containing water prevented the loss of culturability. When suspended in deionized water with added copper sulfate (10 μM), the culturability of *P. aeruginosa* decreased by more than 6 log units, while total cell counts, the concentration of cells with intact cytoplasmic membranes, determined with the LIVE/DEAD BacLight kit, and the number of cells with intact 16S ribosomal RNA, determined by fluorescent in situ hybridization, remained unchanged. When the chelator DDTC was added to copper-stressed bacteria, complete restoration of culturability was observed to occur within 14 d. Copper-stressed bacteria were not cytotoxic towards Chinese hamster ovary (CHO-9) cells, while untreated and resuscitated bacteria caused an almost complete decrease of the concentration of viable CHO-9 cells within 24 h. Thus, copper ions in concentrations relevant to drinking water in plumbing systems seem to induce a viable but non-culturable (VBNC) state in *P. aeruginosa* accompanied by a loss of culturability and cytotoxicity, and VBNC cells can regain both culturability and cytotoxicity, when copper stress is abolished.

Introduction

The potentially pathogenic bacterium *P. aeruginosa* is regarded as a contaminant of drinking water environments, where it can present a hazard to human health, especially when occurring in building plumbing systems (Cunliffe et al., 2011; Mena and Gerba, 2009). As an opportunistic pathogen, *P. aeruginosa* is involved in nosocomial infections of immunocompromised patients, and its occurrence in water supplies of hospitals and other health-care facilities has been the cause of water-associated disease and outbreaks (Anaissie et al., 2002; Exner et al., 2005). The main water-related routes of transmission are exposure of damaged skin, ears and eyes to contaminated water, and inhalation of *P. aeruginosa*-containing aerosols, while the risk of gastrointestinal infection via water ingestion is low (Mena and Gerba, 2009).

The detection of hygienically relevant bacteria in drinking water is routinely performed by standardized methods based on the cultural detection of the organisms. However, bacteria can lose culturability without being dead. This physiological condition has been designated as the viable but non-culturable (VBNC) state in which bacteria fail to grow on traditional bacteriological media under routine cultivation conditions, but are still alive and demonstrate low metabolic activity (Oliver, 2005, 2010). Bacteria enter the VBNC state in response to environmental stresses such as nutrient limitation, temperatures outside the favourable range of growth or the presence of disinfectants. On resuscitation under favourable conditions, VBNC cells can again become culturable. Growth-independent assays based on molecular methods are employed to assess bacterial viability (Keer and Birch, 2003). Indicators of bacterial viability are respiratory activity, cytoplasmic membrane integrity, maintenance of membrane potential and the presence of messenger RNA or ribosomal RNA (rRNA) that is detected by fluorescence in situ hybridization (FISH) with oligonucleotide probes targeted at specific sequences of 16S rRNA molecules.

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Oliver (2005, 2010) listed more than 60 bacterial species capable of entering the VBNC state, including water-associated opportunistic pathogens. At least some pathogens retain their infectious potential in the VBNC state or can regain infectivity after resuscitation. The VBNC state of pathogens in drinking water systems may present a potential public health hazard. Thus, it is important to identify environmental factors which control the transition into the VBNC condition and the resuscitation to the culturable and infectious state.

Recent studies demonstrated the induction of the VBNC state by copper ions (Cu^{2+}) in several gram-negative bacteria (Alexander et al., 1999; Del Campo et al., 2009; Ghezzi and Steck, 1999; Grey and Steck, 2001a,b; Ordax et al., 2006). For *P. aeruginosa* inactivation of planktonic bacteria as well as of biofilms by copper ions was shown in a number of studies (Huang et al., 2008; Teitzel and Parsek, 2003; Teitzel et al., 2006) based exclusively on cultural methods, so that it remained unknown if copper ions might induce a VBNC state in this bacterial species.

Sources of copper ions in drinking water can be copper materials which are widely used for pipes or fixtures of plumbing systems (WHO, 2006). The basis of the present study was the observation that exposure of *P. aeruginosa* to copper-containing drinking water resulted in a loss of culturability, whereas the bacteria remained culturable in drinking water without copper ions. The aims of the present study were to investigate (i) the possibility that copper ions in concentrations relevant to drinking water systems induced the VBNC state in *P. aeruginosa*, (ii) the resuscitation of the bacteria by abolishing copper stress after addition of a copper chelator, and (iii) the influence of copper on the cytotoxicity of *P. aeruginosa* towards mammalian cells.

Materials and methods

Sampling of drinking water

Sampling of drinking water was performed according to the standard ISO 19458 (2006), using sterile (180 °C, 4 h) 1-L glass bottles. Water samples were collected from taps of two buildings (building A and building B), which were served by water supply A (Duisburg, Germany) and water supply B (Mülheim an der Ruhr, Germany), respectively. The plumbing systems of both buildings were made of copper. Samples from water supply A were collected within the drinking water distribution system at a tap of a water storage tank. Water from water supply B (finished water) was collected from a tap at the outlet of a waterworks. Samples from water supplies A and B were taken from a continuously running sampling tap; water from buildings was collected at taps after flushing for 30 min. Free chlorine concentration of drinking water was determined by the N,N-diethyl-*p*-phenylenediamine colorimetric method, using a portable spectrophotometer. All water samples were sterile-filtered through a cellulose acetate filter (0.2 μm pore size) for further use. Copper analysis of filter-sterilized water samples was performed by the IWW Water Centre (Mülheim an der Ruhr, Germany), using inductively coupled plasma optical emission spectrometry according to ISO 11885 (2007). A preliminary experiment had shown that filtration through 0.2 μm pore size membrane filters did not cause a significant change in the copper content of tap water.

Preparation of bacterial suspensions

The environmental strain *P. aeruginosa* AdS originally isolated from water of a plumbing system (Moritz et al., 2010) was used in all experiments. Additionally, type strain *P. aeruginosa* DSM 50071 and wild-type strain *P. aeruginosa* PAO1 were employed in some experiments. The bacteria were cultivated on nutrient agar (Merck) for 24 h at 36 °C. Single colonies from this culture were inoculated into 20 mL Lenox broth (per L: 10 g tryptone, 5 g NaCl, 5 g yeast extract, pH 7.0) in a 100-mL Erlenmeyer flask and incubated for 24 h at room temperature (approximately 24 °C) with agitation (shaking water bath, 180 rpm). The cells were harvested by centrifugation (15 min, 1912 × *g*, 10 °C), washed twice in 20 mL water and finally resuspended in 20 mL water. Depending on the experiments, filter-sterilized drinking water, finished water from the waterworks or deionized water were used for the washing and resuspension procedures. The bacterial suspensions were adjusted to a cell density of approximately 2×10^6 cells/mL.

Testing of bacterial copper sensitivity

Batch sensitivity tests were performed in sterile 250-mL Erlenmeyer flasks. 20 mL bacterial suspension were mixed with 20 mL of test medium. Test waters were filter-sterilized finished water, drinking water, deionized water with added copper sulfate ($CuSO_4 \times 5H_2O$; final concentrations ranging from 0.01 μM to 100 μM), or in some experiments copper nitrate and copper chloride, and deionized water without added copper as a control. In some experiments a solution of the chelator sodium diethyldithiocarbamate trihydrate (DDTC; Fluka) was prepared in 20 mL of the test medium before mixing with 20 mL of the bacterial suspension (final concentration of DDTC 100 μM). The final cell density was approximately 1×10^6 cells/mL. The suspensions were incubated statically at 20 °C in the dark for up to 24 h.

Resuscitation experiments

20 mL of bacterial test suspensions (cell density of approximately 2×10^6 cells/mL) were prepared in deionized water with or without 10 μM copper sulfate and incubated at 20 °C for 24 h. Then the suspensions were mixed with 20 mL of deionized water or deionized water supplemented with DDTC (final concentration 100 μM) in 250-mL Erlenmeyer flasks. The bacterial suspensions were incubated at 20 °C. At various times (days 0, 1, 4, 7 and 14), aliquots of 3 mL were withdrawn for further analysis.

Determination of colony counts

Undiluted test suspensions and decimal dilutions of test suspensions prepared in sterile deionized water were plated in triplicate on non-selective nutrient agar (Merck). Colonies were enumerated after 2 d of incubation at 36 °C. Results were expressed as colony forming units (cfu) per mL. Agar plates with colony numbers between 30 and 300 were considered for enumeration.

Determination of total and viable cell counts

For total cell count determination, bacteria were stained with the fluorochrome 4′,6-diamidino-2-phenylindole (DAPI). 1 mL of DAPI solution (25 μg/mL in 2% (v/v) formaldehyde) was added to 4 mL of diluted test suspension. After incubation at room temperature for 20 min in the dark the solution was filtered through a black polycarbonate membrane filter (Millipore, 0.2 μm pore size).

For the determination of viable cells the LIVE/DEAD *Bac*Light bacterial viability kit (Molecular Probes) was used. 1.5 μl SYTO 9 and 1.5 μl propidium iodide, both dissolved in dimethyl sulfoxide (DMSO), were mixed. Propidium iodide was pre-diluted 1:200 in DMSO. 3 μl of the mixed stains were added to 1 mL of bacterial test suspension. After incubation of the mixture in the dark at room temperature for 20 min, 4 mL sterile particle-free deionized water were added and the suspension was filtered through a black polycarbonate filter (Millipore, pore size 0.2 μm).

Cells on membrane filters were enumerated under an epifluorescence microscope at 1000-fold magnification with immersion oil. 20 randomly selected fields of view were examined for each filter with the help of a counting grid (100 μm × 100 μm).

FISH analysis

FISH of *P. aeruginosa* cells was performed, using probe Psae16S-182 labeled with Cy3 (Wellinghausen et al., 2005). 4 mL of bacterial suspension (1×10^6 cells/mL) were filtered on a black polycarbonate filter (2.5 cm diameter, 0.2 μm pore size) that was transferred to a Petri dish. The cells were fixed by adding 2 mL 4% paraformaldehyde in phosphate-buffered saline, pH 7.2 (PBS) to the filter surface. After 15 min at 4 °C, the paraformaldehyde solution was removed and the filter surface was washed once in PBS. Subsequent dehydration was performed by placing the filter in 50%, 80% and 96% ethanol (3 min for each step). For hybridization, 1 mL of hybridization buffer (0.9 M NaCl, 20 mM Tris [pH 8.0], 0.01% SDS, 40% (v/v) formamide and 10 ng/μL oligonucleotide probe Psae16S-182) was added to the filter that was incubated at 46 °C for 90 min. After removal of the buffer, 2 mL washing buffer (56 mM NaCl, 20 mM Tris [pH 8.0], 0.01% SDS and 5 mM EDTA for probe Psae16S-182) were added to the filter surface. After incubation at 46 °C for 15 min, the filter was rinsed with sterile particle-free deionized water. Bacterial cells were counterstained by adding 1 mL DAPI solution (10 μg/mL) to the filter surface. After incubation in the dark for 20 min, the filter was washed in deionized water and stored at 4 °C until enumeration. Cells were counted using an epifluorescence microscope at 1000-fold magnification. 20 randomly selected fields of view or at least 400 cells were enumerated for each filter with the help of a counting grid (100 μm × 100 μm).

Cytotoxicity testing

Chinese hamster ovary (CHO-9) cells were cultured in Ham's F-12 medium (c.c.pro GmbH) supplemented with 10% fetal calf serum and 0.5% L-glutamine at 37 °C under 5% CO_2. 4 mL medium containing approximately 1×10^6 CHO-9 cells were placed into each well of a 6-well plate and incubated at 37 °C for 24 h. For exposure to bacteria, the medium was removed and 3.8 mL fresh medium and 0.2 mL of bacterial suspension (approximately 10^6 cells/mL; 14 d after resuscitation) or 0.2 mL deionized water (control) were added to the cells which were then further incubated at 37 °C for up to 24 h. After exposure for different times, the medium was removed and the cells were washed once with 3 mL phosphate-buffered saline, pH 7.4 (PBS). Then the cells were trypsinized with 1 mL 0.05% trypsin for 20 s. After removing the trypsin, the cells were incubated for 3 min at 37 °C and then resuspended in 0.5 mL PBS. For cell staining, 0.5 mL trypan blue were then added to the cell suspension and the mixture was incubated for 3 min at 37 °C. Immediately after this time, stained cells were counted microscopically (10-fold magnification) and the viability was determined in relation to controls (CHO-9 cells with medium instead of bacterial suspension).

Results

To investigate the influence of copper ions on the survival of environmental strain *P. aeruginosa* AdS in drinking water, the bacteria were exposed to water samples with varying levels of copper. The test waters were tap water from two buildings (buildings A and B) with copper plumbing systems, and drinking water from two different water supplies A and B that served buildings A and B, respectively. Water supply A was weakly chlorinated with the concentration of free chlorine in water from the distribution system and building A at or below the detection limit of 0.01 mg/L. Water supply B was non-chlorinated. The pH values in water samples from supply A, building A, supply B and building B were 8.8 ± 0.1 ($n = 3$), 8.7 ± 0.2 ($n = 7$), 8.3 ± 0.2 ($n = 3$) and 8.2 ± 0.2 ($n = 4$), respectively The copper content of drinking water from water supply A and finished water taken at the outlet of a waterworks serving water supply B were below or near the detection limit (≤0.002 mg/L), while the mean copper levels in tap water from the buildings were significantly higher with mean concentrations of 1.04 μM (0.066 ± 0.018 mg/L; $n = 7$) and 0.33 μM (0.021 ± 0.008 mg/L; $n = 4$) in buildings A and B, respectively.

Suspensions of *P. aeruginosa* AdS were prepared in the water samples and incubated at 20 °C for 24 h. Total cell counts and colony counts were determined at the start and the end of the incubation period. In Fig. 1a exemplary results are shown for exposure of the bacteria to water samples from building A and water supply A. The total cell counts of approximately 10^6 cells/mL at the start of the experiments did not significantly change during 24 h of exposure in the different types of drinking water. The colony counts in tap water from building A decreased by approximately 5 log units (Fig. 1a) and in tap water from building B by approximately 3.5 log units (not shown), whereas no decline of culturability was observed in samples from the water supplies. Addition of the copper chelator DDTC prevented the loss of culturability of *P. aeruginosa* in tap water from building A (Fig. 1a) and B (not shown). These observations indicated that copper ions were involved in the loss of *P. aeruginosa* culturability.

To exclude the interference of other substances in drinking water, experiments were performed with bacterial suspensions in deionized water with or without added copper sulfate. Loss of culturability was not observed when the bacteria were incubated for 24 h in deionized water, but at a concentration of 1 μM copper, which fell in the range of copper levels of the tap water samples, a decrease of colony counts by several log units was observed with total cell counts remaining nearly constant, whereas in the presence of the copper chelator DDTC culturability of the bacteria was not affected (Fig. 1b). All further experiments were performed with deionized water as the test medium.

Copper ions impaired culturability of *P. aeruginosa* AdS in a concentration- and time-dependent manner. A decrease of colony counts by approximately 1 log unit and 3.5 log units was observed at a copper concentration of 0.1 μM and 1 μM, respectively, and a complete loss of culturability occurred at 10 μM (Fig. 2a). A slow decrease of colony counts was observed at the beginning of exposure of the bacteria to copper (10 μM), followed by a more rapid decline after 7 h of incubation to undetectable levels after 10 h (Fig. 2b). In all experiments, the initial total cell counts of approximately 10^6 cells/mL remained unchanged over the experimental period of 24 h. In the presence of 10 μM copper a decrease of culturability by more than 6 log units over 24 h and prevention of loss of culturability by the addition of DDTC was also observed for type strain *P. aeruginosa* DSM 50071 and wild-type *P. aeruginosa* PAO1, demonstrating that the inhibitory effect of copper ions on culturability was not restricted to *P. aeruginosa* AdS. Copper chloride and copper nitrate at a concentration of 10 μM caused a decrease of colony counts by more than 6 log units in three independent experiments (data not shown) as observed for copper sulfate, while total cell counts remained unchanged and addition of DDTC prevented loss of culturability, indicating that copper, and not the anion, was responsible for the inhibition of culturability of *P. aeruginosa*.

The observed lack of culturability could be due to cell death or to entry of the bacteria into a viable state without being able to form colonies on nutrient agar under the cultivation conditions used. To differentiate between viable and dead cells, LIVE/DEAD *Bac*Light Bacterial Viability Kit was used. Using a mixture of SYTO 9 and propidium iodide, bacteria with intact membranes fluoresce bright green, whereas in bacteria with damaged membranes, propidium

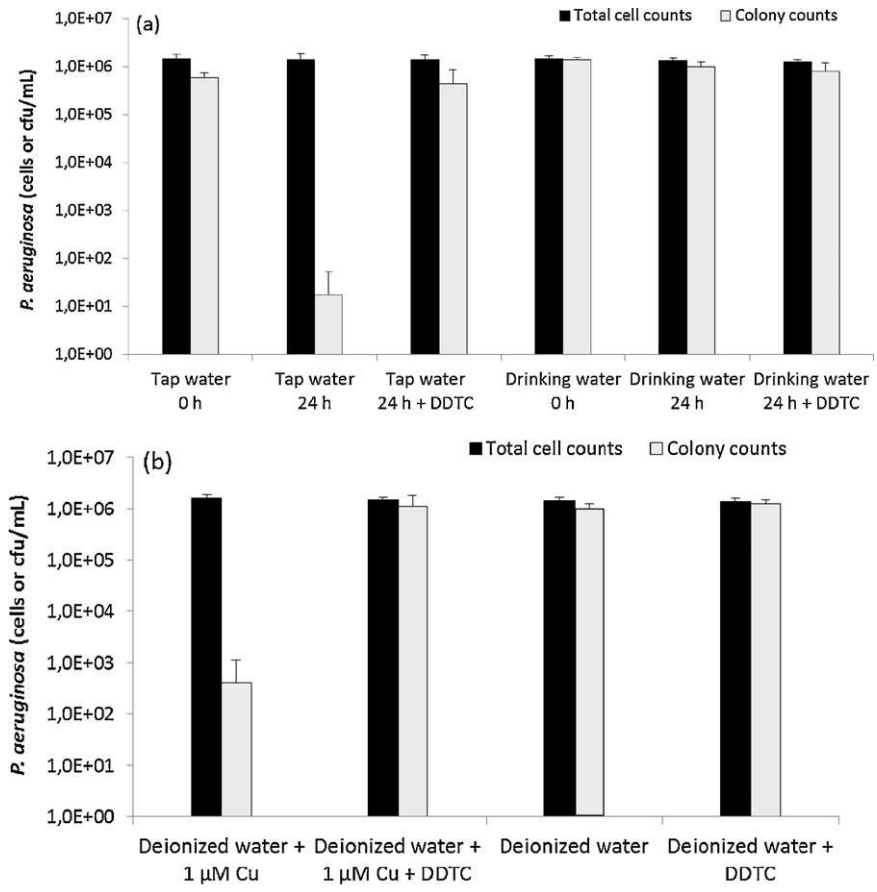

Fig. 1. Total cell counts and colony counts of *P. aeruginosa* AdS exposed to (a) tap water from building A and drinking water from water supply A, and (b) to deionized water with or without added copper sulfate (1 μM) at 20 °C for 24 h in the absence and presence of the copper chelator DDTC (100 μM). Total cell counts were determined by epifluorescence microscopy of DAPI-stained bacteria and colony counts were determined on nutrient agar after incubation at 36 °C for 2 d. Error bars indicate standard deviation (n = 2–7).

iodide abolishes the green fluorescence of SYTO 9 by displacing this dye from complexes with DNA and causes these cells to fluoresce red (Haugland, 2005). Thus, using a combination of SYTO 9 and propidium iodide allows distinguishing between live and damaged or dead bacterial cells. According to the manufacturer, equal volumes of SYTO 9 and propidium iodide solutions are recommended for viability staining. However, this mixture was not suitable in the present study, since in preliminary experiments suspensions of unstressed *P. aeruginosa* AdS in copper-free deionized water revealed a relatively high proportion (approximately 96%) of red-fluorescent cells, although the total cell counts and colony counts were nearly identical, indicating that almost all bacterial cells were culturable. Using a diluted (1:200) propidium iodide solution resulted in an appropriate staining mixture, yielding a high proportion (approximately 94%) of green-fluorescent cells of unstressed *P. aeruginosa* cells. In a control experiment, *P. aeruginosa* AdS (approximately 10^6 cells/mL) suspended in deionized water was incubated with 1 g/L hydrogen peroxide at 20 °C for 24 h. This treatment resulted in a complete loss of culturability (decrease of colony counts by more than 6 log units) and a proportion of 96% of red-fluorescent (membrane-damaged) cells, while in the absence of hydrogen peroxide, no decrease of colony counts and the number of green-fluorescent cells was observed, confirming that the modified SYTO 9/propidium iodide mixture was suitable for monitoring membrane damage or death of *P. aeruginosa* AdS in response to a bactericidal agent.

Preliminary experiments had shown that exposure of *P. aeruginosa* in copper-containing tap water revealed an almost unchanged level of green fluorescent cells during incubation at 20 °C for 24 h, while culturability decreased by several orders of magnitude (data not shown). In deionized water that contained copper at a concentration (10 μM) resulting in complete loss of culturability the fraction of green fluorescent cells remained almost unchanged (Fig. 3), indicating that even at an elevated copper concentration, membrane integrity of the bacteria was not impaired.

In parallel, FISH was used to quantitate the concentration of bacteria with intact rRNA as an indicator of cell viability. Using *P. aeruginosa*-specific oligonucleotide probe Psae16S-182 targeted at 16S rRNA, no significant decrease of the concentration of FISH-positive cells occurred upon exposure to 10 μM copper for 24 h (Fig. 3).

To investigate the potential restoration of culturability by abolishing copper stress, suspensions of *P. aeruginosa* which had been exposed to 10 μM copper for 24 h were incubated at 20 °C for 14 d in the presence or absence of the copper chelator DDTC, and total cell counts and colony counts were monitored over 14 days. In control suspensions without added copper, the total cells counts and colony counts did not change significantly over the whole experimental period (Fig. 4). However, in suspensions of copper-treated cells, bacteria were not detected culturally, while the total cell counts of approximately 10^6 cells/mL remained constant. When DDTC had been added to suspensions of copper-stressed cells, a slow increase in colony counts was observed and after 14 d the concentrations of culturable cells of approximately 10^6 cfu/mL corresponded to those in bacterial suspensions without added copper

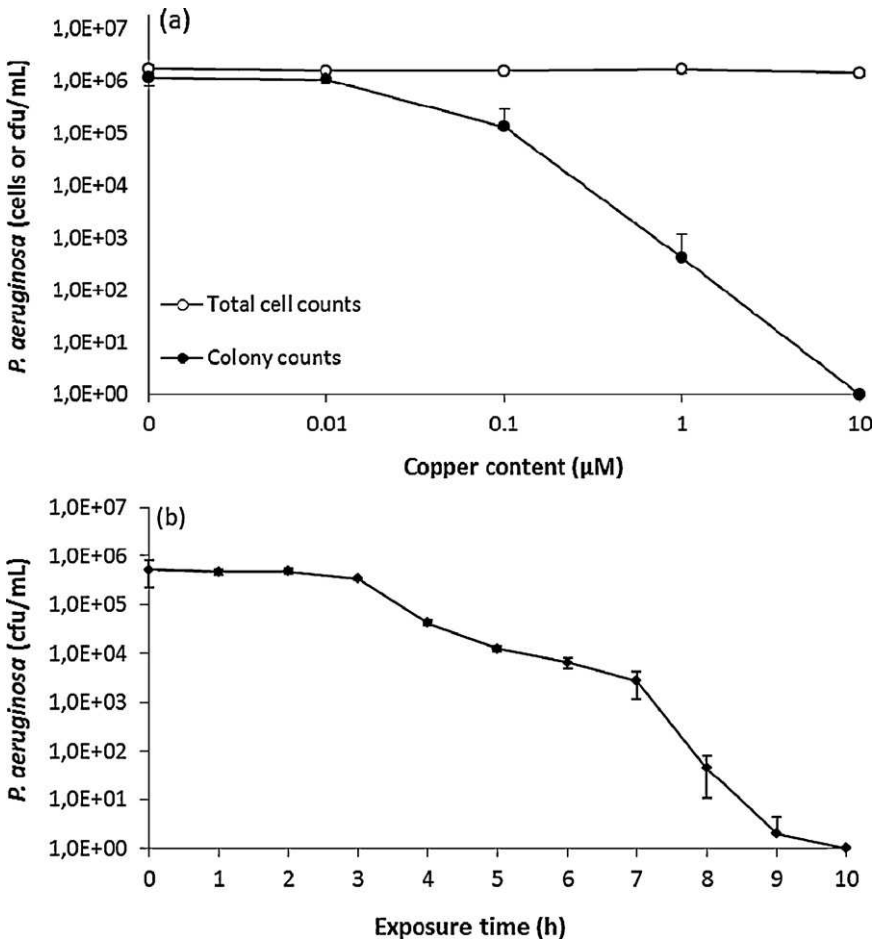

Fig. 2. Total cell counts and colony counts of *P. aeruginosa* AdS after incubation in deionized water at 20 °C at various concentrations of copper sulfate after incubation for 24 h (a) and for various incubation times at a concentration of 10 μM copper sulfate (b). Total cell counts were determined by epifluorescence microscopy of DAPI-stained bacteria and colony counts were determined on nutrient agar after incubation at 36 °C for 2 d. Error bars indicate standard deviation (n = 2–8).

(Fig. 4). The increase in culturability did not seem to be due to multiplication, since the total cells counts (approximately 10^6 cells/mL) remained unchanged over the whole incubation period. These results indicated resuscitation of copper-stressed *P. aeruginosa* induced by the copper chelator DDTC.

The infectious potential of *P. aeruginosa* cells in the culturable state, in the copper-induced VBNC condition and after resuscitation from 14-d-old suspensions (Fig. 4) was investigated by the determination of their cytotoxicity towards CHO-9 cells. Untreated culturable bacterial suspensions caused a cytotoxic effect with a

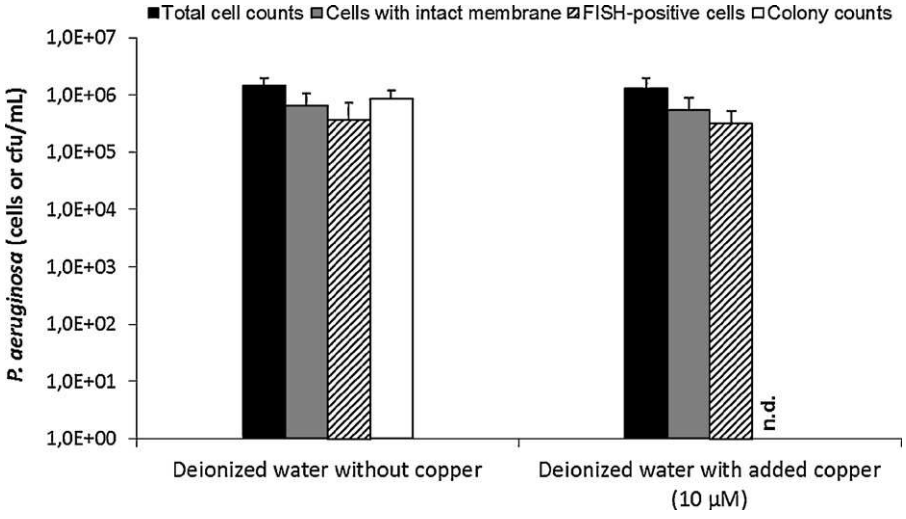

Fig. 3. Viability of *P. aeruginosa* after 24 h exposure to deionized water with or without 10 μM copper sulfate. Epifluorescence microscopy was used to determine total cell counts of DAPI-stained bacteria, the concentration of cells with intact membranes (LIVE/DEAD® *Bac*Light™ Bacterial Viability Kit) and the number of FISH-positive cells. Colony counts were determined on nutrient agar after incubation at 36 °C for 2 d. Error bars indicate standard deviation (n = 3). n.d., not detected.

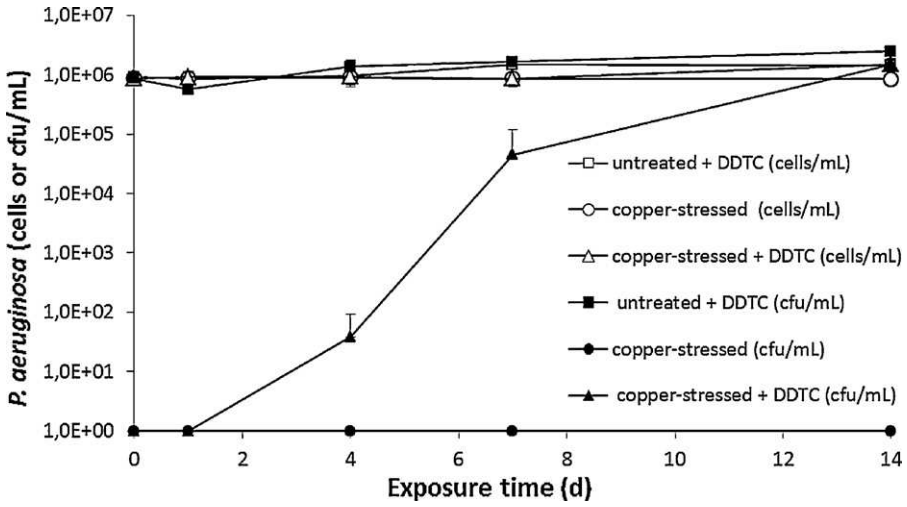

Fig. 4. Resuscitation of copper-stressed *P. aeruginosa* induced by the chelator DDTC. After 24-h pre-incubation of the bacteria in deionized water with copper sulfate (10 μM), DDTC (100 μM) was added at time zero to abolish copper stress. Controls were untreated bacteria with added DDTC and copper-stressed bacteria in the absence of DDTC. During subsequent incubation of the bacterial suspensions at 20 °C over a period of 14 d, total cell counts of DAPI-stained bacteria (open symbols) were determined by epifluorescence microscopy and colony counts (closed symbols) were determined on nutrient agar after incubation at 36 °C for 2 d. Error bars indicate standard deviation ($n = 3$). n.d., not detected.

complete loss of viability of CHO-9 cells after 24 h of incubation (Fig. 5). Copper-stressed bacteria did not affect viability of CHO-9 cells, while copper-stressed cells that had been resuscitated by DDTC treatment were cytotoxic again (Fig. 5). The chelator DDTC alone had no cytotoxic effect on CHO-9 cells.

Discussion

P. aeruginosa represents a water-related opportunistic pathogen of hygienic relevance in building plumbing systems where it can occur in water and persist in biofilms (Hambsch et al., 2004; Leoni et al., 2005; Moritz et al., 2010; Wagner et al., 1992; Wingender et al., 2009). Copper is a common material used to make pipes and fittings in building plumbing systems (WHO, 2006). Drinking water can corrode copper to some extent, resulting in the mobilization and release of copper ions (Turek et al., 2011). Thus, interactions between *P. aeruginosa* and copper ions can be expected to occur in water of copper plumbing systems. The bacteriostatic and bactericidal effect of copper on planktonic cells and biofilms of *P. aeruginosa* has been demonstrated in a number of studies, based solely on cultural methods (Harrison et al., 2005; Huang et al., 2008; Hwang et al., 2006; Leitão and Sá-Correia, 1997; Teitzel and Parsek, 2003; Teitzel et al., 2006). In these studies the impact of copper ions on bacterial survival was investigated in growth media, mineral salt solutions or deionized water, but not in real drinking water.

The basis of the present study was the observation that exposure of the environmental isolate *P. aeruginosa* AdS to tap water from buildings with copper plumbing resulted in a decrease of colony counts on nutrient agar by several log units over 24 h, while the number of total cells remained nearly unchanged. The loss of culturability was only observed in copper-containing tap water from two buildings with copper plumbing systems, while no change of culturability occurred in water from two drinking water distribution systems that served the buildings with drinking water and whose copper contents were near or below the detection limit. The elevated copper concentrations in tap water from the buildings probably originated from the copper plumbing. One of the main mechanisms by which copper is released into drinking water is

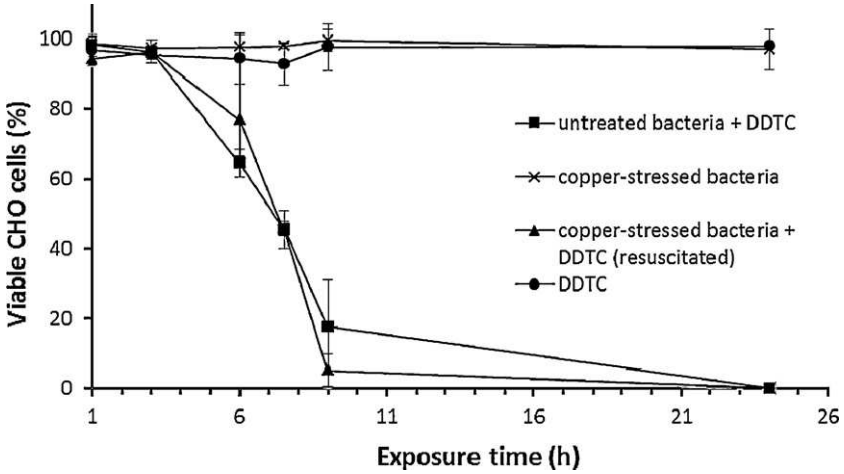

Fig. 5. Cytotoxicity of *P. aeruginosa* AdS towards CHO-9 cells. The bacteria had been incubated for 14 d in deionized water in the absence or presence of copper sulfate (10 μM), or resuscitated in the presence of the chelator DDTC (100 μM) (see Fig. 4), before they were added to CHO-9 cells. The CHO-9 cell cultures were incubated at 36 °C for 24 h, before the viability of CHO-9 cells was determined by trypan blue staining. Viability of bacteria-exposed CHO-9 cells was calculated as the percentage of intact cells in relation to control cell cultures that were incubated in the absence of bacteria (100% viable CHO cells). Error bars indicate standard deviation ($n = 3$).

through dissolution of copper scales on the inside surface of water distribution pipes (Turek et al., 2011). There was no decrease of culturability of *P. aeruginosa* in drinking water in the presence of the chelator DDTC. This chelator has previously been found suitable to complex Cu^{2+} and Ni^{2+} in biofilms of *P. aeruginosa* (Harrison et al., 2005). It could not be excluded that other metal ions or additional compounds also contributed to the inactivation of *P. aeruginosa* in real drinking water. To confirm that the decrease of colony counts was actually related to the presence of copper, comparative experiments were performed using deionized water with or without added copper (as $CuSO_4$), representing water without any substances which might possibly interfere with the biological effect of copper. In this environment, a decrease of colony counts was observed at copper concentrations that can be found in real drinking waters (Domek et al., 1984, this study), while cells which were not exposed to copper remained completely culturable. This observation indicated that copper alone could be the causative factor affecting *P. aeruginosa* culturability at concentrations relevant to drinking water in plumbing systems. This observation is supported by reports demonstrating that the survival of bacteria measured as colony counts was reduced by exposure to copper-containing well water and drinking water. Thus, copper was identified as the cause for injury of coliform bacteria in drinking water distribution systems (Domek et al., 1984) and was supposed to be responsible for the stronger decline of colony counts of pathogenic variant *E. coli* O157:H7 inoculated into well water with enhanced concentrations of copper compared to well water with copper levels below the detection limit (Artz and Killham, 2002).

Complete loss of culturability (>6 log units) was determined after 10 h exposure of *P. aeruginosa* AdS to 10 μM (0.6 mg/L) copper. This observation is in accord with data from the literature indicating a copper-induced reduction of colony counts by several log units at similar levels of copper ions as in the present study, when the exposure of other strains of *P. aeruginosa* was conducted in synthetic drinking water (Hwang et al., 2006) or deionized water (Huang et al., 2008). Using strain *P. aeruginosa* ATCC 10145 a complete inactivation (>7 log units) was observed after 3 h and 8 h at copper concentrations of 0.4 mg/L and 0.15 mg/L, respectively (Hwang et al., 2006). At copper concentrations in the range of 0.1–0.8 mg/L a reduction of the colony counts of an environmental isolate of *P. aeruginosa* by more than 5 log units was observed after 1.5 h in deionized water (Huang et al., 2008). In summary, these results indicate that copper ions concentrations as they can be found in drinking water environments cause a significant loss of culturability of *P. aeruginosa* within hours.

Total cell counts of copper-exposed *P. aeruginosa* were quantified microscopically by enumeration of bacteria fluorescing brightly blue after staining with the fluorochrome DAPI that binds to double-stranded DNA. Total cell counts remained unchanged over the whole range of copper concentrations tested, also at copper levels where *P. aeruginosa* could not be detected culturally. To discriminate between living and killed bacteria, copper-treated cells were assayed for viability using the LIVE/DEAD *Bac*Light bacterial viability kit. This method allows the differentiation of cells with an intact cytoplasmic membrane (viable cells) from those with a compromised cytoplasmic membrane (dead cells) (Haugland, 2005). The results obtained with the LIVE/DEAD *Bac*Light kit showed that under the conditions of the current study copper ions did not cause damage to the cytoplasmic membrane. In addition, the FISH technique was applied using a *P. aeruginosa*-specific oligonucleotide probe targeted at the 16S rRNA as an indicator of the presence of rRNA as constituents of ribosomes in viable cells. Copper treatment of *P. aeruginosa* did not change the concentration and fluorescence intensity of FISH-positive cells, indicating that no damage to the ribosomes or decline in the number of ribosomes had occurred by the action of copper ions. Taken together these experiments demonstrated that, although inhibiting culturability, copper did not damage the bacterial cell membrane and did not disrupt ribosomal RNA. Thus, it can be concluded that copper ions apparently induced a VBNC state in planktonic cells of *P. aeruginosa* at concentrations that are relevant in drinking water systems.

Although many studies have demonstrated the inhibitory effect of copper ions on *P. aeruginosa* by cultural methods monitoring the decrease of colony counts on agar media, the possible copper-induced VBNC state has not been considered in these investigations. However, copper ions have been reported to induce the VBNC state in a number of other gram-negative bacteria, including soil and phytopathogenic species such as *Agrobacterium tumefaciens* and *Rhizobium leguminosarum* (Alexander et al., 1999), *Xanthomonas campestris* pv. *campestris* (Ghezzi and Steck, 1999), *Ralstonia solanacearum* (Grey and Steck, 2001a), *Erwinia amylovora* (Ordax et al., 2006) and *Xanthomonas axonopodis* pv. *citri* (Del Campo et al., 2009), and the faecal bacterium *Escherichia coli* (Grey and Steck, 2001b). For all species, the transition into the VBNC state was observed on planktonic cells suspended in distilled water or mineral salts media. As reported here for *P. aeruginosa*, cell populations of *A. tumefaciens*, *R. leguminosarum*, *E. amylovora* and *X. axonopodis* entered the VBNC state over periods of ≤24 h at copper concentrations varying between 5 μM and 500 μM (Alexander et al., 1999; Del Campo et al., 2009; Ordax et al., 2006), while populations of *R. solanacearum*, *X. campestris* and *E. coli* needed more than two days to convert to the VBNC state (Ghezzi and Steck, 1999; Grey and Steck, 2001a,b). These observations indicate that the adoption of the VBNC state as one of several possible strategies to adapt to copper stress is not a rapid process, but usually takes at least several hours, as observed for *P. aeruginosa*.

In most of the studies mentioned above, membrane integrity, determined with the LIVE/DEAD *Bac*Light kit, was used as an indicator of viability in copper-treated cells that had lost their culturability. This approach also proved suitable to demonstrate maintenance of membrane integrity in *P. aeruginosa*. The FISH method used successfully for the detection of rRNA in viable cells of *P. aeruginosa* has not been described before for the analysis of other bacterial species in the copper-induced VBNC state. However, Steinert et al. (1997) employed a 16S rRNA-targeted oligonucleotide probe to detect *Legionella pneumophila* cells in as VBNC state induced by starvation under low-nutrient conditions in tap water. Thus, FISH may be a complimentary method suitable to indicate the copper-induced VBNC state.

A characteristic of the VBNC condition is the ability of bacteria to become culturable again upon resuscitation (Oliver, 2005). In this study, the presence of the chelator DDTC resulted in an increase in the culturability of copper-stressed and thus previously unculturable bacteria over 14 d to the concentration that was equal to the initial number of culturable cells (approximately 10^6 cfu/mL) before addition of copper, while total cell counts remained nearly constant. This effect was probably due to the complexing of copper ions by DDTC, so that copper stress was abolished and the bacteria regained their ability to grow, forming visible colonies on nutrient agar again. The fact that the total cell count did not increase showed that a regrowth of culturable cells probably did not occur, which was expected due to the absence of any nutrients in deionized water. Thus, it can be assumed that VBNC cells were truly resuscitated.

Resuscitation of copper-stressed bacteria has been studied for a number of other bacterial species with variable results. Removal of copper stress did not restore culturability of *R. leguminosarum* (Alexander et al., 1999) and *X. campestris* (Ghezzi and Steck, 1999). In contrast, addition of copper-complexing agents such as EDTA employed to abolish copper stress resulted in the restoration of the culturable state in *E. amylovora* (Ordax et al., 2006) and *E. coli*

(Grey and Steck, 2001b). In the case of *E. coli* complete resuscitation of copper-induced VBNC cells after abolishing copper stress was achieved after several days (Grey and Steck, 2001b), confirming that full resuscitation can be a slow process as was also observed for *P. aeruginosa* in the current study. In the present study DTTC was found to be an alternative copper chelator that allowed the resuscitation of *P. aeruginosa* from the VBNC state.

An important aspect of pathogens in the VBNC state is their potential to initiate infection (Oliver, 2005). In the current study, the interaction between *P. aeruginosa* and CHO-9 cell cultures was used as an infection model system to assess the cytotoxicity of untreated, copper-stressed (VBNC) and resuscitated bacteria towards mammalian cells. Previously, CHO cells were shown to be suitable to recognize biological effects, including cytotoxicity, of toxins secreted by *P. aeruginosa* (Vallis et al., 1999). *P. aeruginosa* bacteria in their different states were inoculated into cultures of CHO-9 cells and cell damage (permeability to trypan blue) was monitored over 24 h. The copper-stressed bacteria in the VBNC state did not affect the viability of the CHO-9 cells, while the untreated and the resuscitated bacteria caused an extensive lysis of the CHO-9 cells. Thus, the VBNC cells were not cytotoxic, but retained their potential to destroy the mammalian cells. It was reported for some phytopathogenic species (*R. solanacearum*, and *E. amylovora*) that resuscitated copper-induced VBNC cells regained their pathogenicity towards their host plants (Grey and Steck, 2001a,b; Ordax et al., 2006). These observations with copper as the inducer of the VBNC state is in accord with the general view that pathogens are mostly not able to start infection in the VBNC state, but that virulence is retained and infectivity is regained following resuscitation to the original actively metabolizing and culturable state (Oliver, 2010).

In conclusion, this study provides evidence that *P. aeruginosa* can be induced to enter the VBNC state by the presence of copper ions at concentrations relevant to drinking water environments. Thus, *P. aeruginosa* represents another gram-negative species and a human pathogen for which the adoption a copper-induced VBNC state was shown. The VBNC condition was reversible by abolishing copper stress, resulting in the restoration of culturability and cytotoxicity towards mammalian cells. The hygienic relevance of VBNC bacteria of *P. aeruginosa* in water and biofilms of plumbing systems has still to be established. It can be expected that *P. aeruginosa* may occur in the VBNC state in copper plumbing systems and thus not detectable by culture-based methods routinely employed for monitoring drinking water quality. Studies are under way to investigate the basic mechanisms underlying the copper-induced VBNC state in *P. aeruginosa* and to identify factors that can lead to resuscitation of VBNC bacteria under conditions relevant to building plumbing systems.

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