Recovery of *Pseudomonas aeruginosa* culturability following copper- and chlorine-induced stress

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NonTechnical Summary: The authors investigated the loss of culturability of *P. aeruginosa* in presence of copper and chlorine concentrations typically found in drinking water and demonstrated culturability recovery once stressors were removed.

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Abstract

This study investigated how quickly cells of the opportunistic pathogen *Pseudomonas aeruginosa* recover culturability after exposure to two of the most common environmental stressors present in drinking water, free chlorine and copper ions. Viable but non-culturable (VBNC) *P. aeruginosa* undetected by direct culturing following exposure to free chlorine or copper ions can survive in drinking water systems, with potential to recover, multiply and regain infectivity. Cells were exposed to copper sulphate (0.25 mg Cu^{2+} L^{-1}) or free chlorine (initial dose of 2 mg Cl_2 L^{-1}) for 24h. Despite total loss of culturability and a reduction in viability from $1.2x10^7$ to $4x10^3$ cells mL^{-1} (3.5 log), cells exposed to chlorine recovered viability quickly after the depletion of free chlorine, while culturability was recovered within 24 hours. Copper ions did not depress viability, but reduced culturability from $3x10^7$ to $2.3x10^2$ cells mL^{-1} (5.1 log); VBNC cells regained culturability immediately after copper ion chelation. A comparison between direct culturing and Pseudalert, a specific enzyme-based assay, was performed. Both detection methods were well correlated in the range of 10^2-10^{10} cells L^{-1}. However, correlations between the methods declined after exposure to copper ions.

Introduction

Pseudomonas aeruginosa is an important source of nosocomial infections and the most frequently recovered Gram-negative bacterium from patients with nosocomial pneumonia in the USA (Weinstein *et al.*, 2005). For cystic fibrosis patients, it is the primary cause of morbidity (Pier, 2012) and a major predictor of mortality (Emerson *et al.*, 2002). It can also cause bacteraemia, urosepsis and secondary wound infections, including burns infection (Kerr & Snelling, 2009). Sources of transmission are multiple, but water is believed to be an important contributor for the transmission of

P. aeruginosa in hospitals (Trautmann *et al.*, 2005). In most intensive care unit outbreaks investigated, water has been implicated either directly or indirectly (Ferroni *et al.*, 1998, Ehrhardt *et al.*, 2006, Vianelli *et al.*, 2006, Hota *et al.*, 2009, Durojaiye *et al.*, 2011, Yapicioglu *et al.*, 2011, Schneider *et al.*, 2012). According to recent information, 30 to 50% of *P. aeruginosa* infections in intensive care units are associated with water (Exner, 2012).

The detection of *P. aeruginosa* in drinking water presents several challenges, including its preference for biofilm vs planktonic state as well as its presence in low and variable concentrations. Moreover, environmental factors such as nutrient-poor conditions and the presence of inhibitors and stressors can lead to a dominance of the viable but non-culturable (VBNC) state. Cells in the VBNC state are still alive and capable of metabolic activity but fail to multiply and grow on routine media on which they would normally grow (Oliver, 2005). A VBNC state has not been typically associated with *P. aeruginosa* and its presence in environmental samples has not fully been assessed. When monitoring for *P. aeruginosa* at drinking water taps, two main environmental stresses can be present: disinfectant residual and metals ions from plumbing (copper, zinc and lead). Depending on prior stagnation and residual chlorine concentration at the time of sampling, traditional culture methods may fail to reveal the presence of VBNC *P. aeruginosa* because of the impact of these factors on culturability. This is a well-known phenomenon for *Vibrio cholerae* in aquatic environments (Xu *et al.*, 1982). The toxicity of copper ions to culturable *P. aeruginosa* cells has been documented at relatively high concentrations (Teitzel & Parsek, 2003, Harrison *et al.*, 2005, Teitzel *et al.*, 2006, Elguindi *et al.*, 2009). However, the direct relevance of these results to conditions prevailing in drinking water systems is limited because of: (1) the use of growth media or mineral salt solutions in which the availability of toxic free copper ions varies with the level of chelation and binding; and (2) the systematic quenching of copper ions before enumeration. The impact of copper ions on

culturable and VBNC *P. aeruginosa* was verified in drinking water containing realistic copper concentrations (63.5 µg Cu^{2+} L^{-1}) showing a greater sensitivity to copper ions and an overwhelming effect of added chelators (Moritz *et al.*, 2010). VBNC *P. aeruginosa* cells undetected by culture can survive in the system, and eventually recover and multiply when stressors are removed, e.g. free chlorine depletion during stagnation periods. Furthermore, VBNC cells can also recover cytotoxicity as shown by the reversible VBNC state of suspended *P. aeruginosa* induced by copper ions (Dwidjosiswojo *et al.*, 2011).

The objectives of this study were 1) to estimate the impact of free chlorine and copper ions stresses on culturable and VBNC suspended *P. aeruginosa* cells under conditions representative of internal plumbing, 2) to test a new specific enzymatic activity-based assay for the increased recovery of stressed bacteria and 3) to quantify the recovery of *P. aeruginosa* after stress interruption for both free chlorine and copper ions.

Materials and Methods

Bacterial strain and culture conditions

Experiments were performed with *P. aeruginosa* strain PA14 (Lee *et al.*, 2006) grown in Tryptic Soy Broth (Difco, Detroit, USA) overnight at 37 °C. Cells were harvested by centrifugation (3000 x *g* for 30 min), washed twice in sterile 2 mM phosphate buffer, and suspended at a final estimated cell density of 5 x 10^9 cells mL^{-1}.

Experimental conditions

Sterile polypropylene bottles containing 500 mL of 2 mM phosphate buffer (pH 7.3±0.1) were inoculated (final concentration 10^7 cells mL^{-1}). A control cell suspension was not exposed to free

chlorine or copper ions. A chlorine solution was added to inoculated water to obtain an initial free chlorine concentration of 2 mg Cl_2 L^{-1}. Free chlorine concentrations were measured by the *N*,*N*-diethyl-*p*-phenylenediamine method using a DR5000 spectrophotometer (HACH, USA). Sodium thiosulfate (1%) was added to neutralize chlorine prior to conducting microbiological analysis. Copper sulphate was used as the source of copper ions and was added to the water prior to inoculation ($CuSO_4$, final concentration 0.25 mg Cu^{2+} L^{-1}). Copper ions were chelated after 24 hours by the addition of diethyldithiocarbamate (Moritz, 2011). All samples were mixed thoroughly and divided to perform selected analytical methods.

For the enzyme-based assay and culture methods comparison in absence of free chlorine and copper ions, serial dilutions of an early exponential phase bacterial suspension were prepared (1 to 10^6 cells mL^{-1}).

Microbiological analysis

Culture was performed according to International Organization for Standardization method 16266:2006 for *P. aeruginosa* detection and enumeration in water (International Organization for Standardization, 2006). Briefly, three dilutions were done in duplicate for each sample, filtered on a 0.45 µm pore size, 47 mm diameter mixed cellulose ester membrane. The filters were deposited on cetrimide-nalidixic acid agar plates (45.3 g L^{-1} of Cetrimide Selective Agar (Remel, Lenexa, USA), 10 mL L^{-1} glycerol (Fisher, Fair Lawn, USA), 0.015 g L^{-1} nalidixic acid (Sigma-Aldrich, Steinheim, Germany) and incubated at 37.5°C for 24 h before enumeration. Detection was also performed on two sample dilutions using the Pseudalert/QuantiTray2000 (IDEXX, Chicago, USA) according to the manufacturer's instructions. Viable and total cell counts were determined using LIVE/DEAD BacLight Bacterial Viability Kit (Molecular Probes, Eugene, USA) (Boulos *et al.*, 1996). This kit differentiates viable from dead cells using membrane integrity criteria. Total cells are defined as the

sum of viable (green) and dead (red) cells (Fig. 1). Briefly, 1 mL of sample or dilution in 0.85% sterile saline solution was mixed with 3 µl of stain (propidium iodide and SYTO9), incubated in the dark for 15 min and filtered on black 0.2 µm pore diameter, 25 mm diameter polycarbonate filter (Millipore, Bedford, USA). Enumeration was done at 1000-fold magnification, with an epifluorescence microscope (Olympus, Tokyo, Japan). VBNC cells were estimated as the difference between the viable and culturable cells.

Real-time qPCR amplification

A volume of 25 mL was filtered through a 0.45 µm pore diameter, 47 mm diameter mixed cellulose ester membrane for DNA extraction performed directly on filters using the bead beating method adapted from Yu & Mohn, 1999. Briefly, the filter was inserted into an extraction tube (Lysing Matrix A, MP Biomedicals, Solon, USA). Extraction buffer containing 50 mM Tris-HCl (pH8), 5 mM EDTA (pH8), 3% sodium dodecyl sulphate and RNase (20 µg mL^{-1}, Invitrogen, Carlsbad, USA) was added to each tube prior to the bead beating step performed on a FastPrep-24 (MP Biomedicals, Solon, USA), followed by ammonium acetate precipitation (2M, Sigma-Aldrich, St.Louis, USA) and successive cold 70% ethanol washes. Quantification by quantitative polymerase chain reaction (qPCR) was performed on *gyrB* gene, using primers and probes previously described (Lee *et al.*, 2011). The *gyrB* gene was chosen for its specificity and sensitivity for *P. aeruginosa*. The following protocol was applied: 10 min initial denaturation at 95 °C followed by 50 cycles with denaturation at 95°C for 30s, annealing and elongation at 60°C for 90 s (Corbett Rotor-Gene 6000, San Francisco, USA). Final reaction volume (20 µl) included 5 µl of DNA, 2X Universal MasterMix (Life Technologies, Foster City, USA) and TaqMan Exogenous Internal Positive Control Reagent (Applied Biosystems, Austin, USA).

Results and Discussion

The objective was to measure the impact of chlorine-based disinfectant and copper-containing water on the detection of *P. aeruginosa* under environmental conditions and to document its culturability recovery after those stressors were removed. Suspensions of *P. aeruginosa* cells were monitored for up to 8 days, with free chlorine and copper ions present only during the first 24h.

Effect of chlorine stress on *P. aeruginosa* culturability

A representative concentration of free chlorine found in drinking water distribution systems was selected to reach a target concentration of < 0.1 mg Cl_2 L^{-1} after 24 h. Figure 2 shows the loss of culturability (final to initial CFU mL^{-1}, N/N_0) of *P. aeruginosa* as a function of exposure to free chlorine expressed as the product of concentration and contact time (also expressed as Ct in the literature) observed during the first 24 hours. Similar data at lower exposure to chlorine values reported by Xue *et al.*, 2013 are also presented. Elevated initial rates of apparent inactivation of culturable *P. aeruginosa* by chlorine have been documented in batch and chemostat experiments (Behnke *et al.*, 2011, Xue *et al.*, 2013) showing large reductions (3.5 to 6 log) at initial free chlorine dosage of > 1 mg Cl_2 L^{-1}. Results presented in Fig. 2 show a trend of inactivation similar to the one obtained by Xue *et al*. The loss of culturability observed in this study increased with exposure to free chlorine (product of concentration and contact time) resulting from low chlorine concentrations maintained over long periods of time (24h). These chlorination conditions are representative of those found in the internal plumbing of buildings of drinking water systems.

Cell inactivation (final to initial measured cell count ratio, N/N_0) as a function of time is shown for the various detection methods in Figure 3a. Despite complete loss of culturability during the exposure to free chlorine and a poor nutrient environment (phosphate-buffered water), recovery of culturability after an additional delay of 24 hours following stress interruption was observed with

135 both CFU-based and enzyme-based detection methods. A decrease in viable counts was also
136 observed during exposure to chlorine (Fig 3a), indicating membrane injury for a large portion of
137 cells. After the onset of chlorination, the majority of cells labelled with LIVE/DEAD stain (total and
138 viable) showed poor fluorescence in the presence of free chlorine, an observation in agreement with
139 the documented loss of DAPI fluorescence after chlorination at 5 mg Cl_2 L^{-1} (Saby *et al.*, 1997).
140 Poor fluorescence was attributed to the inability of the dye to bind to chlorine-modified DNA or to
141 physical damage to the DNA. However, as chlorine concentrations declined over time, a transition in
142 cell marking was observed, from typical red-stained associated with membrane permeabilization to
143 orange and light yellow before reverting to light green (Fig. 1). The dye combination of SYTO9 and
144 PI can detect both inner and outer membrane permeabilization in Gram negative bacteria as detected
145 by flow cytometry, and outer membrane damage can be reversible (Berney et al., 2007). According
146 to Xue *et al.* 2013, similar intermediate states of binding after chlorination are an indication of
147 reversible cell injury (i.e. enzyme activity or functional group deformation) as opposed to lethal
148 membrane damage. Although it is not possible to confirm the state of the cells showing intermediate
149 staining, the observed trends in viable and cultivable cells provide evidence that most cells can
150 survive and regrow rapidly after the dissipation of free chlorine. This is clearly observed for VBNC
151 cells after 24 hours, corresponding to the loss of significant concentration in free chlorine (Fig. 3a).
152 qPCR results show a decrease within the first hours of contact time with free chlorine, but revert to
153 levels comparable to total cell counts obtained by LIVE/DEAD staining after 24 h.

154 These results are highly relevant to conditions prevailing in drinking water distribution systems. The
155 levelling off of the inactivation curves observed in disinfection studies show that a significant
156 fraction of suspended bacteria can breakthrough primary disinfection and enter the distribution
157 system (Behnke *et al.*, 2011, Xue *et al.*, 2013). Secondary disinfection conditions are not sufficient

158 to completely inactivate suspended or clustered cells as free chlorine concentrations decline
159 progressively with water age (Rossman *et al.*, 1994). Internal plumbing in large buildings offer
160 highly favorable conditions for biofilm development because of the large surface area provided by
161 small diameter pipes and corrosion, long residence times including dead-end sections and
162 disinfectant consumption (Prévost *et al.*, 1997). A significant fraction of chlorine-injured cells in the
163 present study are still viable but unlikely to be detected by standard culture methods.

164 **Effect of copper ions stress on *P. aeruginosa* culturability**

165 The current World Health Organization guideline value for copper concentrations in drinking water
166 is set at 2 mg Cu^{2+} L^{-1} (World Health Organization, 2008). In the United States, the Environmental
167 Protection Agency fixed the maximum contaminant level goal for copper at 1.3 mg Cu^{2+} L^{-1} (United
168 States Environmental Protection Agency, 2009). California has the lowest recommended level with
169 an established public health goal of 0.3 mg L^{-1} (California Environmental Protection Agency *et al.*,
170 2008). In the present study, copper stress was evaluated by adding 0.25 mg Cu^{2+} L^{-1}, representative
171 of concentrations found in internal plumbing of large buildings in the study area (data not shown)
172 and meeting recommended levels in drinking water. *P. aeruginosa* counts dropped from $3x10^7$ to
173 $2.3x10^1$ CFU mL^{-1} (6.1 log) in culture and from $2.9x10^7$ to $1.6x10^2$ MPN mL^{-1} (4.7 log) as measured
174 by the enzyme-based method within 2 hours of exposure to copper ions (Fig. 3b). These results agree
175 with prior reports of steep decline in culturable *P. aeruginosa* and persistence of a resistant sub-
176 population at lower copper ions levels (Teitzel & Parsek, 2003, Huang *et al.*, 2008, Dwidjosiswojo *et*
177 *al.*, 2011). Unlike free chlorine, ionic copper did not affect viability and no transition period was
178 observed with fluorescent staining (suggesting the absence of significant membrane
179 permeabilization, data not shown). Results obtained by qPCR were constant throughout the
180 experiment and comparable with total cell count. Copper ions were neutralized after 24 hours of

contact time by adding a chelating agent (100 µM diethyldithiocarbamate) that was demonstrated to have no inhibitory impact (data not shown). The quenching resulted in recovery of culturability within 3 days, with cell densities approaching initial concentrations. No measurable changes in total or viable cell counts were noted, minimizing the importance of cell multiplication in the apparent recovery of culturability of *P. aeruginosa* although some contribution from surviving cells is possible. These results are in agreement with a previous study documenting the reversible inhibition of *P. aeruginosa* culturability by copper ions (Dwidjosiswojo *et al.*, 2011). Slightly higher losses of culturable cells (from 10^6 CFU mL^{-1} to below detection limit) than those observed here (were reported following exposure of an environmental strain of *P. aeruginosa* to 0.635 mg Cu^{2+} L^{-1} copper, with complete recovery in 14 days (Dwidjosiswojo *et al.*, 2011).

These results have significant implications for monitoring *P. aeruginosa* in drinking water of health care establishments because of the presence of copper in internal plumbing. While newer copper piping might inhibit *P. aeruginosa* attachment initially, passivation of the material results in biofilm colonization after 1 or 2 years of utilization (Critchley *et al.*, 2001, van der Kooij *et al.*, 2005). Although the main source of copper in water is piping, brass present in valves, faucets, meters, and fittings can also leach copper in water during stagnation. In spite of the impact of brass elements, environmental conditions in the volume of water contained in the tap and its connecting pipe (< 50 mL) are most favorable to culturable state. Indeed, copper concentrations will be lower than in the connecting copper piping and free chlorine will be depleted. Therefore, the selection of sampling volumes carries direct implications for culturability and the interpretation of monitoring results. Recommended and typical sampling volumes vary between 50 mL and 500 mL (Ferroni *et al.*, 1998, Trautmann *et al.*, 2001, Chaberny & Gastmeier, 2004, Department of Health (DH), 2013), large enough to include water from the connecting pipe containing potentially high copper concentration.

Cells from the initial stagnant volume would then be exposed to enough copper to enter a VBNC state, potentially leading to cell count underestimation when using standard culture methods. Bacteria collected in copper-rich water continue to be exposed to this inhibitor until processing for detection (up to 24 h), unless sufficient quencher or chelating agent are added.

Impact of free chlorine and copper ions stresses on enzyme-based detection compared to culture method

Pseudalert is a new enzyme-based assay used to provide a quick response detection of *P. aeruginosa* currently applied to pools, spas and bottled water. It is based on the same platform as Colilert, a US Environmental Protection Agency-approved kit used for the detection of *Escherichia coli*. The enzyme-based assay for *P. aeruginosa* offers an attractive alternative to culture and is currently undergoing validation for monitoring of *P. aeruginosa* at water points of use in healthcare establishments. Technical data from the manufacturer and recent publications provide information on the positive correlation with culture enumerations in pool samples and bottled water (Idexx Laboratories, 2010 a, b, Mannisto 2012, Semproni *et al*. 2014). However, the influence of direct exposure to free chlorine or copper ions stress on the performance of the test has not been documented in drinking water on a wide range of cell concentrations.

Figure 4 shows that paired measurements for a control culture in early exponential phase using direct culturing and the enzyme-based methods are highly correlated ($R^2 = 0.99$, n = 24) as confirmed by the 95% prediction interval (Fig. S1a). This correlation is observed over a wide range of concentrations and remains in agreement with the previously published dataset on pool and spa water samples ($R^2 = 0.95$, n = 14) established with lower detected concentrations of *P. aeruginosa* (Idexx Laboratories, 2010 a). Overall, results obtained by the enzyme-based assay led to slightly higher estimates than direct culturing with a positive bias of 7% (Fig. 4).

Figure 5 presents paired measurements by enzyme-based assay and culture methods on stressed cells. Exposure to free chlorine did not modify the correlation observed in the absence of stress (R^2 = 0.99) (Fig 5). The 95% prediction intervals on the log transformed data without a forced intercept confirms that chlorine exposure does not modify the correlation between the enzyme-based and the culture results (Fig . S1a and b). However, for copper ion induced stress conditions, an apparent scatter from the regression line is noted (Fig. 5) and the 95% prediction interval on the log transformed data without a forced intercept clearly shows the poor correlation in the presence of 0.25 mg Cu^{2+} mL^{-1} (Fig. S1c). As no interference with the reagent was observed (data not shown), the increased response of the enzymatic assay may be attributed to the interference of copper ions with the enzymatic hydrolysis of the substrate, possibly because of the enhanced production of the targeted enzyme (Teitzel *et al.*, 2006) .

Implications for environmental monitoring

Culture-based methods are most commonly prescribed to monitor *P. aeruginosa*, and standards and guidelines in drinking water and clinical settings are still almost entirely expressed in CFU mL^{-1}. Results obtained in this study demonstrate the presence of viable *P. aeruginosa* cells in some water samples that would not be detected by standard plate count culturing or by an enzyme-based assay due to prior exposure to free chlorine or copper ion stress, a common situation within internal plumbing. Residual chlorine is often detected in municipal cold water and standard sampling protocols recommend the addition of sodium thiosulfate to neutralize residual chlorine upon sample collection (American Public Health Association (APHA) *et al.*, 2012). Even with neutralization, bacteria previously exposed to free chlorine may not fully recover culturability before the start of culture, as samples are processed within 24h of sampling. Resulting counts may then be an underestimation, providing a false sense of security. The comparison of results from culture and the

250 enzymatic assay suggest a greater sensitivity of the enzymatic assay that, coupled with the quicker
251 response time (< 26 hours), may provide additional surveillance value and contribute to prevention.

252 Results of this study reveal the presence of a large proportion of VBNC cells in the presence of
253 common environmental stressors such as free chlorine and copper ions. It was shown that *P.*
254 *aeruginosa* exposed to chlorine and copper ions are unlikely to be measured by standard culture
255 methods, or even newer quicker response methods based on enzymatic reactions. This raises the
256 question of the sanitary significance of the presence of *P. aeruginosa* in a VBNC state. Although
257 some cells in the VBNC state are avirulent, the potential for VBNC cells to become infectious once
258 resuscitated has been clearly documented (Oliver, 2010, Dwidjosiswojo *et al.*, 2011). Conditions and
259 time lag for such resuscitation vary greatly, and the time of recovery in nutrient-rich environments
260 such as a sink drain or susceptible host is poorly documented. As disinfectant residuals are not stable
261 or are often absent in internal plumbing, VBNC cells could regain culturability and infectivity
262 between sampling events. Copper ion concentrations in large building drinking water systems can
263 reach and surpass 0.25 mg L^{-1}. This is especially the case in large buildings such as hospitals, where
264 copper is a commonly used pipe material and stagnation between uses can lead to elevated copper
265 concentrations. These results also highlight the need to revisit the evaluation of Cu-Ag based-
266 disinfection methods efficacy. Moreover, they highlight the importance of using an appropriate
267 monitoring protocol, including sampling volume, sample treatment and analytical method to assess
268 the risks to which patients are directly or indirectly exposed.

269 On the other hand, the use of qPCR as routine monitoring in hospitals is still infrequent. The main
270 drawback cited against its wide use is its inability to distinguish between viable and dead cells, as all
271 intact DNA can be amplified. Nevertheless, qPCR can still be used as a monitoring tool, especially
272 to interpret changes to the baseline values in a drinking water system. Clearly, an increase in the

qPCR signal indicates cell multiplication and proactive action could be taken to resolve the issue before it is detected by culture methods. In critical situation, this approach could complement culture and enzymatic methods and help reduce the risk associated with the presence of *P. aeruginosa* in water. Viability PCR is being developed, where an intercalating dye prevents the amplification of DNA in membrane compromised dead cells, but it still suffers from practical limitations. Given the demonstration of the potential of VBNC cells to regain virulence, viability PCR holds great promise for future monitoring improvements.

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403

404 **Figure legends**

405 **Fig. 1.** LIVE/DEAD stained *P. aeruginosa* PA14 in buffered sterile water (pH = 7.3) for various
406 contact times after chlorination illustrating the transient cell marking observed: green prior to

chlorination (a), red at t = 0.5 h (b), faded yellow at t = 48 h (c), light green t = 96 h (d) and green at t = 192 h (e). Green cells represent viable cells and red cells represent dead cells.

Fig. 2. Loss of culturability for *P. aeruginosa* PAO1 (Xue *et al*. 2013) and PA14 (this study) assessed by culture method (final to initial CFU mL^{-1} ratio, N/N_0) as a function of free chlorine concentration (mg $Cl_2 \cdot L^{-1}$) and contact time (min) product expressed as Ct. Error bars for this study indicate standard deviation.

Fig. 3. Cell survival of *P. aeruginosa* PA14 in buffered sterile water (pH = 7.3) as a function of time elapsed after application of 2.0 mg $Cl_2\,L^{-1}$ of free chlorine (a) and 4 µM copper ions (b). Cell survival is expressed as the logarithm of N/N_0, where N_0 is the initial cell count and N is the cell count at sampling time. Enumeration was done by culture (▲, $CFU \cdot ml^{-1)}$, enzyme-based assay (∆, $MPN \cdot ml^{-1}$), qPCR (X, genomic units$\cdot L^{-1}$) and LIVE/DEAD staining (◊, viable cells and ♦, total cells). Viable cells are defined as cells with membrane integrity and total cells as the sum of viable and dead cells. Error bars indicate standard deviation (*n* = 3). Free chlorine concentration decay (●) and copper ion concentration (○) are presented on the secondary y axis. Copper ions were chelated with diethyldithiocarbamate at t = 24 h (b).

Fig. 4. *P. aeruginosa* PA14 cells concentrations suspended in buffered sterile water measured by enzyme-based assay in Most Probable Number (MPN) $\cdot mL^{-1}$ and standard culture methods in $CFU \cdot mL^{-1}$ (*n* = 24; y = 1.07 x; R^2 = 0.995). Error bars indicate 95% confidence range for MPN and standard deviation for culture.

Fig. 5. *P. aeruginosa* PA14 cells concentrations measured by the enzyme-based assay in Most Probable Number (MPN) $\cdot mL^{-1}$ and standard culture methods in $CFU \cdot mL^{-1}$ in presence of 2 mg L^{-1} initial chlorine concentration (•, *n* = 7; y = 0.99 x; R^2 = 0.99) and 4 µM copper (○, *n* = 7).