Plasma Activated Water is capable to inactivate dormant bacteria in drinking water

Hummert M.[1], Leenders P.H.M. [2], van Ooij P. [2], Kuczius T. [1]

[1]Institute of Hygiene, University of Münster and University Hospital of Münster, Münster, Germany
[2]VitalFluid B.V., Nijmegen, Netherlands

The contamination of water sources with pathogenic and drug-resistant microorganisms is considered a major public health problem. Drinking water quality is controlled by cultivation on selective nutrients. Planktonic and viable bacteria are verifiable; however, most microorganisms live in biofilms or survive in an dormant or viable-but-non-cultivable (VBNC) state.
Bacteria living in and adapted to water (e.g. in tap water pipes) develop an increased tolerance to disinfections while cultivated microorganisms are highly susceptible. We analyzed the influence of plasma activated water (PAW) as a novel approach to efficiently inactivate such tolerant bacteria. During plasma activation many active and reactive substances and radicals arise associated with a radical decrease in pH.

Methods and Results:

Adaption of bacteria to drinking water

Single colonies of *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus* and *Bacillus subtilis* were cultivated in Luria broth (LB) for extensive growth at 36°C over night. A nine-fold volume of sterile tap water (Institute of Hygiene, Münster) was added, followed by incubation at 25°C with constant, moderate shaking (89 rpm) for 72 h in order to minimize biofilm formation. Residual nutrients were removed by harvesting the cells using a centrifugation step (20 min, 2000 xg) and resolving in half of the original volume of sterile tap water. The bacterial suspension was incubated under shaking (89 rpm) at 25°C for a prolonged time until respective use.
Viability was verified by plating in dilutions on LB agar plates after distinct time periods (Fig. 1).

Flow cytometry:

Changes of the physiological states of the microorganisms during water adaption were followed by standard flow cytometry using NovoCyte Flow Cytometer 2000R (ACEA Biosciences Inc., USA) configured with a 488 nm laser. Bacterial labeling was achieved with SYBR Green I (Invitrogen, S7563; final concentration 1 x) and propidiumiodide (PI, Roth, CN74.2; final concentration 0.6 µM). Data collection and analysis was performed with the NovoExpress 1.3.0 software (Fig. 2).

Disinfection assay:

Bacteria grown in rich nutrient media (LB-medium) and bacteria, adapted to water for distinct time periods, were exposed to plasma activated water (PAW) and disinfectants. The plasma activation was performed with the use of the VitalFluid LabUnit with 120 W for 10 and 30 minutes as indicated. The bacterial inactivation with PAW was followed in comparison to standard procedures as chlorine and hydrogen peroxide treatments for 120 min at room temperature. After neutralization for stopping the reaction, viable cells were verified by plating in dilutions on LB-agar plates as colony forming units. The percentage of survival was normalized to the survival after incubation without disinfection (Fig. 3).

Fig. 1: Bacterial viability in tap water. Microorganisms grown overnight were diluted in tap water (1:10) and incubated for 72 h (3d), were finally harvested and resuspended in sterile tap water (100%dw) followed by long term incubation. After defined time intervals (weeks) aliquots were plated on LB-agar for determination of the viable cell counts in CFU. Results display the mean of two experiments conducted independently of one another with each duplicate samples.

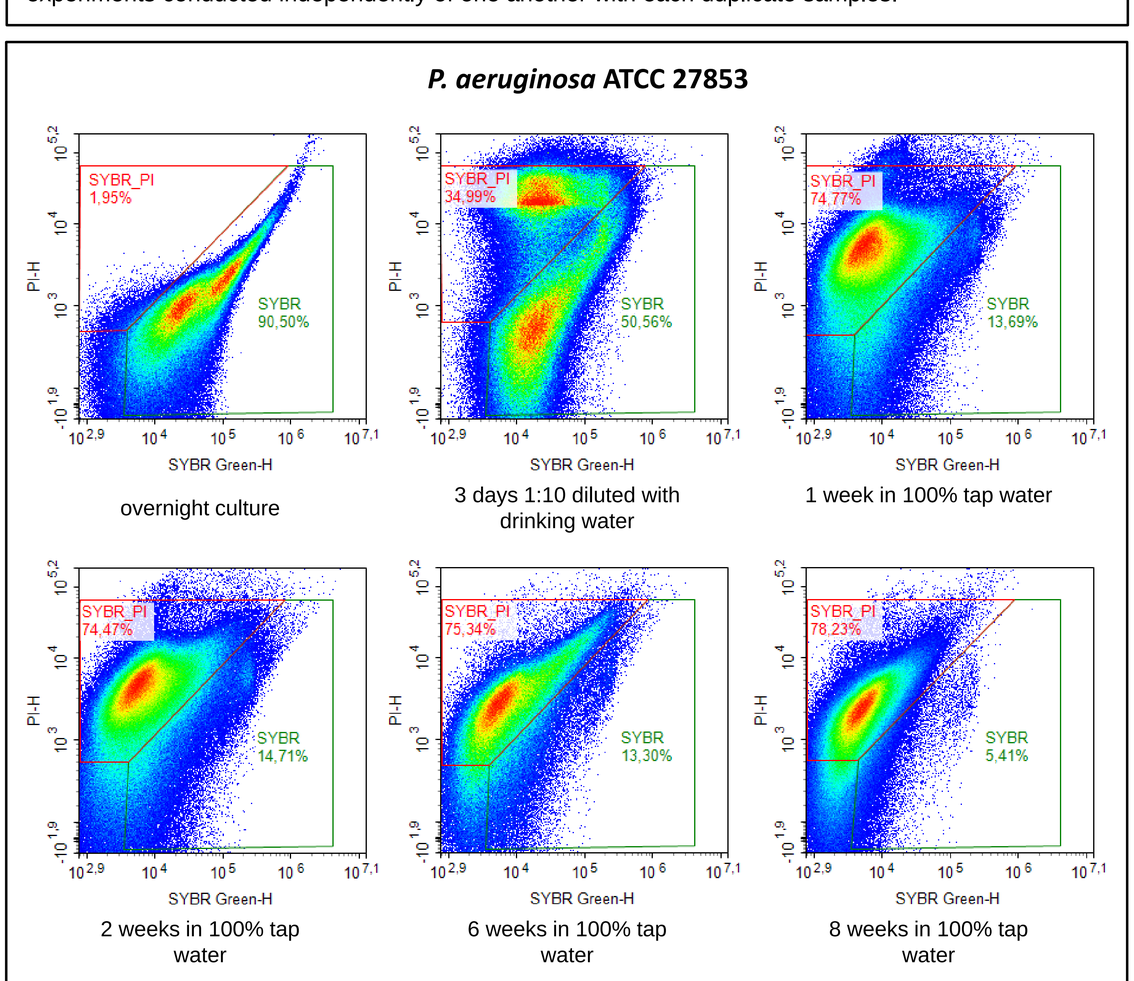

Fig. 2: Scattered plot of the population of *P. aeruginosa* ATCC 27853 stained with SYBR Green and PI. Bacteria cell population were subjected to Live/Dead-staining and differentiated via flow cytometry. The gate labeled "SYBR" refers to bacteria stained only with SYBR Green, with are generally considered to be viable. The gate labeled "SYBR_PI" refers to bacterial cells that are stained both with SYBR Green and PI, which are more likely considered as dead or membrane compromised bacteria. Each panel represents the events of 50 µL bacterial tap water culture.

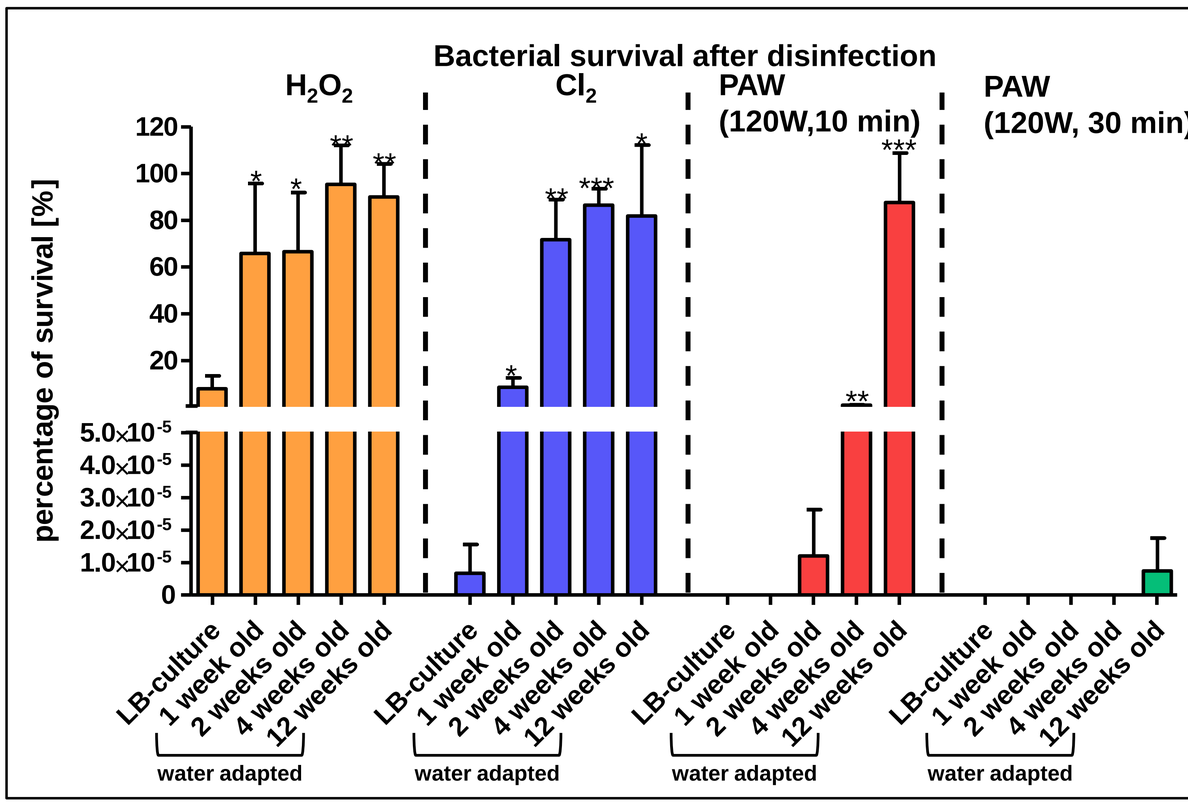

Fig. 3: Percentage of *P. aeruginosa* ATCC 27853 survival after exposure to disinfections. The colony forming units were determined by plating on LB-agar after 120 min-exposure to plasma activated water (PAW; activated for 10 and 30 min at 120W), H_2O_2 (50 mg/L) and Cl_2 (1.2 mg/L) . The survival percentage was normalized to the survival after incubation without disinfection. Results represent the mean values of at least four experiments performed independently with errors corresponding to the standard deviation. The statistical analysis was conducted between bacteria, grown in rich nutrient medium, and water-adapted bacteria using two-sided, unpaired t-tests without equal variances.

Conclusion:

- **Microorganisms change their physiology to dormancy after prolonged incubation in tap water and develop an increased tolerance to disinfection**
- **Plasma activated water (PAW) is able to inactivate dormant bacteria in water more efficiently than common disinfections**
- **Flow cytometry technique indicates at least a change in bacterial cell integrity during water adaption**