Journal of Water & Health

IWA PUBLISHING

Journal of Water and Health Vol 19 No 5, 808 doi: 10.2166/wh.2021.075

Thermal and chemical disinfection of water and biofilms: only a temporary effect in regard to the autochthonous bacteria

Andreas Nocker [a,*], Elisa Lindfeld [a,b], Jost Wingender [a,c], Simone Schulte [d], Matthias Dumm[e] and Bernd Bendinger[a]

[a] Applied Microbiology, IWW Water Centre, Moritzstraße 26, 45476 Mülheim an der Ruhr, Germany
[b] Department of Chemical Engineering, University of Applied Sciences Münster, Stegerwaldstraße 39, 48565 Steinfurt, Germany
[c] Faculty of Chemistry, Environmental Microbiology and Biotechnology, Aquatic Microbiology, University of Duisburg-Essen, Universitätsstraße 5, 45141 Essen, Germany
[d] Evonik Operations GmbH, Goldschmidtstraße 100, 45127 Essen, Germany
[e] THYSSENKRUPP Steel Europe AG, Kaiser-Wilhelm-Straße 100, 47166 Duisburg, Germany
*Corresponding author. E-mail: a.nocker@iww-online.de; andreas.nocker@gmail.com

AN, 0000-0002-5343-9418; EL, 0000-0003-3519-7694; JW, 0000-0003-3143-4760; SS, 0000-0003-2336-9313

ABSTRACT

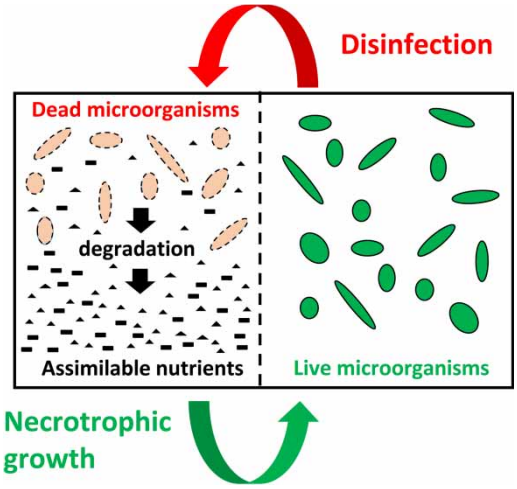
Thermal and chemical disinfection of technical water systems not only aim at minimizing the level of undesired microorganisms, but also at preventing excessive biofouling, clogging and interference with diverse technical processes. Typically, treatment has to be repeated in certain time intervals, as the duration of the effect is limited. The transient effect of disinfection was demonstrated in this study applying different treatments to water and biofilms including heat, chlorination, a combination of hydrogen peroxide and peracetic acid and monochloramine. Despite the diverse treatments, the reduction in live bacteria was followed by regrowth in all cases, underlining the universal validity of this phenomenon. The study shows that autochthonous bacteria can reach the concentrations given prior to treatment. The reason is seen in the nutrient concentration that has not changed and that forms the basis for regrowth. Nutrients are released by disinfection from lysed cells or are still fixed in dead biomass that is subsequently scavenged by necrotrophic growth. Treatment cycles therefore only provide a transient reduction of water microbiology if nutrients are not removed. When aiming at greater sustainability of the effect, biocidal treatment has to be equally concerned about nutrient removal by subsequent cleaning procedures as about killing efficiency.

Key words: biocides, disinfection, heat, necrophilic growth, necrotrophy, regrowth

HIGHLIGHTS

- The reduction in live bacteria by disinfection is followed by renewed growth.
- The reason for post-disinfection growth is seen in nutrient availability.
- Nutrients may originate from killed biomass, allowing necrotrophic growth.
- The sustainability of disinfection relies on the removal of killed biomass.

GRAPHICAL ABSTRACT



INTRODUCTION

The majority of water treatment processes focus on minimizing the concentrations of undesired microorganisms. Apart from filtration and UV irradiation, this goal is typically achieved by disinfection applying heat or chemicals. In technical water systems, the term biocidal treatment is more common. Although a wide array of treatment options is available with different rates of success for different applications, all of them share the common problem that the duration of the effect is limited even if the treatment was initially highly efficient. In industrial applications, it is mainly bacteria whose levels are monitored. In contrast to fecally shed pathogens that typically cannot replicate in the environment outside of the host, autochthonous bacteria regrow annihilating the effect of treatment (Chatzigiannidou *et al.* 2018). This is especially problematic where excessive loads of the autochthonous flora interfere with production processes, e.g. by clogging of tubes or biofouling of filters or other surfaces (Drescher *et al.* 2013; Di Pippo *et al.* 2018). High concentrations of autochthonous bacteria can also provide the basis for hygienic problems as in the case of pathogenic *Legionella* whose presence is typically associated with biofilms that form the nutritional basis for amoebae (Barbeau & Buhler 2001; Abdel-Nour *et al.* 2013). *Legionella* that replicate intracellularly in amoebae profit from this buildup of biomass (van der Kooij *et al.* 2017).

An essential requirement for regrowth lies in the availability of live microorganisms and nutrients (Prest *et al.* 2016). The first is typically given as the disinfected compartment is not sterile. Even if the vast majority of planktonic cells are inactivated, surface-attached cells are notoriously hard to kill (Bridier *et al.* 2011). 'Regrowth' can, however, be caused not only by bacteria surviving treatment, but also by bacteria that are introduced into the disinfected water further downstream (e.g. from biofilm along pipe walls). As typically the source of regrowth is not known, the term 'regrowth' is used here in the sense of 'recovery of bacterial numbers', independent of whether it is caused by sublethally damaged bacteria or newly introduced bacteria. Also nutrients, the other essential requirement, are typically present. In the case of flow-through water systems, nutrients are constantly carried along with the water stream. The microorganisms that remain in the water utilize these nutrients to regrow. Nutrients can also originate from dead biomass that contains nutrients in the same composition as required for the formation of new biomass (Herrero *et al.* 2017; Whitton *et al.* 2018). Regrowth kinetics can be seen to be hugely dependent on available nutrient concentrations, the bioavailability of those nutrients, the composition and the vitality of the scavenging community and temperature (Temmerman *et al.* 2006; Chatzigiannidou *et al.* 2018).

As a consequence in practice, regrowth results in the obligation of reiterative disinfection or biocidal treatment to maintain microbial concentrations at the desired level. Typically, treatment leads to a time window where microbiological concentrations are in line with the desired level before the treatment needs to be repeated. Time cycles very much depend on the actual situation and can widely vary even for the same application. A typical example is water treatment in cooling towers where intervals of biocide dosage and required treatment strengths can greatly vary (García & Pelaz 2008; Iervolino *et al.* 2017).

Although the rapid recovery of the bacterial population after disinfection or biocidal treatment is an accepted phenomenon, little knowledge is given about its extent, the parameters that affect its kinetics and about means to mitigate it. Research to

understand these relationships including the effect of nutrient composition seems essential to minimize regrowth. Necrotrophic growth, defined as the phenomenon that certain bacteria can use dead microbial cells as a nutrient source, appears as an emerging area of research (Chatzigiannidou *et al.* 2018). On the other hand, there are open questions about the regrowing microbiology in such man-made environments where many ecological niches are opened up. In other words, the consequences of disinfection and biocidal treatment are under debate.

In this study, we examined the regrowth of bacteria after applying a diverse range of treatment procedures, matrices and monitoring techniques. Using the example of drinking water subjected to heat or chlorination, we studied which concentrations bacteria can reach after treatment and how quickly. Further experiments addressed the consequences of repeated hydrogen peroxide and peracetic acid treatment on a drinking water-derived biofilm and the effect of daily monochloramine dosages to an industrial cooling water system on intracellular ATP levels. Eventually, the capacity of *Escherichia coli* to necrotrophically feed on dead *Pseudomonas putida* cells (killed either by heat or chlorine) was examined.

MATERIALS AND METHODS

Preparation of assimilable organic carbon-free glassware

All glassware used for studying the regrowth of chlorine- or heat-treated water or bacteria were free of assimilable organic carbon (AOC). For this purpose, an alkaline solution of potassium permanganate was prepared by dissolving (A) 30 g of potassium permanganate (Cat. No. 105082; Merck KGaA, Germany) and (B) 100 g sodium hydroxide pellets (Cat. No. 106498; Merck KGaA, Germany) separately in each 500 mL deionized water. The latter is strongly exergonic and was allowed to cool down. The two solutions were subsequently combined in a 1 L bottle and stored in the dark prior to use. 50 mL glass vials (Cat. No. 7612150; Th. Geyer GmbH & Co. KG) used for flow cytometry samples and other glassware that should be AOC-free were filled approximately one-third of the total volume with this alkaline solution and sealed with appropriate caps. Glassware was subsequently shaken in regular intervals for at least 15 min to ensure contact of the entire inner surface area with the solution. The permanganate solution was decanted (for recycling purposes), and glassware and caps were rinsed three times with tap water and twice with deionized water. Dried glassware and caps were wrapped in aluminum foil. Glassware was heated in a muffle furnace at 280 °C for at least 8 h or overnight, and caps were heated at 180 °C during that time.

Bacteria and growth conditions

E. coli (DSM 1058) was grown on tryptic soy agar (TSA, Carl Roth GmbH & Co. KG) for 24 h at 37 °C. One colony was transferred into 20 mL of tryptic soy broth (TSB) and the culture was shaken for 16 h at 37 °C at 280 rpm. *P. putida* (DSM 6413) was streaked on TSA and grown for 24 h at 30 °C. A single colony was transferred into 20 mL of TSB and shaken for 24 h at 30 °C at 280 rpm. The optical densities (OD_{600}) of the cultures were adjusted to 1.0 ± 0.05 by addition of TSB, using a photometer (HACH LANGE, DR5000). Aliquots of 1 mL were spun down by centrifugation (5,000 g, 5 min) and pellets were resuspended in the same volume of membrane-filtered (0.2 μm) mineral water (Evian, France) to ensure osmotic stability. The washing step was repeated twice to remove disinfectant demand by broth residuals.

Killing conditions

Chlorine disinfection

A chlorine stock solution (1,000 ppm free chlorine) was prepared by dilution of a sodium hypochlorite stock solution (10–15% free chlorine, Acros Organics, Geel, Belgium) with ultrapure water. Aliquots of this stock solution were added to water samples (20 mL) or bacterial suspensions (10 mL) to obtain the desired chlorine concentrations. Chlorine exposure at room temperature was stopped after 30 min by the addition of 200 μL 0.1 N sodium thiosulfate (Acros Organics, Geel, Belgium).

Thermal disinfection

Alternatively to chlorine killing, bacteria were killed by heat exposure. 20 mL aliquots of water samples or 10 mL of *P. putida* suspensions were transferred into 40 mL glass vials free of AOC. Water or cell suspensions were placed for 45 min into a 70 °C or 90 °C pre-heated water bath and subsequently cooled down to room temperature.

Experiments with tap water

Tap water (approximately 5 L) was flushed until a constant temperature was reached before collecting 250 mL tap water for further processing. The corresponding building was supplied with unchlorinated drinking water by a waterworks distributing treated surface water. Aliquots of 20 mL tap water were transferred into 40 mL AOC-free glass vials and treated with either chlorine (0.1 and 1 ppm final concentrations) or heat (70 °C and 90 °C) as described before. After treatment and neutralization of chlorine by the addition of sodium thiosulfate, vials were shaken every 30 s over a time period of 5 min at room temperature to ensure sufficient oxygen content. Samples were spiked with 200 μL untreated tap water or not and incubated at 22 °C (±1 °C) for a total of 14 days. Water samples not treated with chlorine or heat served as controls.

Necrotrophic growth on dead *P. putida*

10 mL of washed *P. putida* suspensions (approximately 10^8 intact cells per mL filtered mineral water, determined by flow cytometry) were transferred into AOC-free 40 mL glass vials. Bacteria were either exposed to chlorine (20 or 80 ppm for 30 min, followed by chlorine neutralization) or to heat (70 °C or 90 °C for 45 min) as described above. The suspensions of killed *P. putida* were spiked with 10 μL (1:1,000th final volume) of untreated washed *E. coli* (10^8 *E. coli* per mL, $OD_{600} = 0.1$) to obtain a final concentration of approximately 10^5 *E. coli* cells per mL. Samples were subsequently incubated at 37 °C for 7 days without shaking. Samples with only killed *P. putida* or with only live *E. coli* served as controls.

Flow cytometry

Fluorescent dyes used in this study were SYBR Green I (SG I; 1,000× stock; Invitrogen™) and propidium iodide (PI; 1 mg mL^{-1}, Invitrogen, Thermo Fischer). Water samples were processed undiluted or 10× diluted with 0.2 μm membrane-filtered mineral water (Evian, Évian-les-Bains, France) in case the total signal exceeded approximately 5,000 signals. SG I was diluted to a working stock concentration of 100× using dimethylsulfoxide (DMSO; Sigma Aldrich) and stored at −20 °C until use. Aliquots (250 μL) of water samples were transferred into a 96-well microtiter plate (0.3 mL polypropylene microtiter plate, U-bottom, Cat. No. 601808, HJ-Bioanalytik GmbH, Erkelenz, Germany). To determine concentrations of intact cells, 200 μL sample aliquots from this plate were transferred into the wells of a second 96-well plate with a pre-aliquoted 2.4 μL mixture of a 100× SG I and PI in the volume ratio of 5:1 followed by thorough mixing by pipetting up and down several times using a multichannel pipette. Staining was performed statically at 37 °C for 13 min in an incubator. Data were collected using an ACEA NovoCyte® flow cytometer equipped with a 488 nm laser (OLS OMNI Life Science, Bremen, Germany) and a NovoSampler® Pro that allowed automatic sample loading from 96-well microtiter plates. Data were analyzed using the instruments-specific NovoExpress software and a gating procedure similar to the one described by Gatza *et al.* (2013).

Biofilm reactor experiments

Continuous flow annular reactors as described by Griebe & Flemming (2000) and modified according to Wingender *et al.* (2004) were employed for the biofilm experiments. The reactors consisted of a stationary outer cylinder made of polytetrafluoroethylene (Teflon) and a rotating inner cylinder made of stainless steel (Figure 1). The inner wall of the outer cylinder held 12 flush-mounted removable stainless steel coupons (V4A steel, 1.5 cm × 22.0 cm, thickness of 0.5 mm). Prior to use, the coupons were degreased with acetone, rinsed with water and sterilized by autoclaving at 180 °C for 4 h. The biofilm reactor and connected tubing were sterilized at 121 °C for 20 min.

Drinking water supplied from the local drinking water distribution system and collected from a laboratory tap in an aerated tank served as the inoculum for biofilm cultivation. Four annular reactors were operated in parallel. For 3 days, the reactors were continuously fed with drinking water supplemented with tryptic soy broth (TSB; final concentration of 0.3 g/L TSB granulate, Merck) in a volume ratio of 10:1 at a flow rate of 4 mL/min by means of a peristaltic pump with a mean flow velocity of 0.45 m/s. The inner cylinder was operated with a rotational speed of 200 rpm. The Reynolds number was 2,226 (laminar flow). On day 4, the dosage of TSB was stopped and the flow-through of drinking water was continued until day 7. TSB was initially added to simulate a temporary contamination of drinking water promoting biofilm development followed by a flow-through period of clean water to finally generate biofilms of drinking-water microorganisms with high cell numbers within a relatively short time period. The mean temperature of the drinking water inside the reactors as measured in the reactor effluent was 28 ± 2 °C. After 7 days of reactor operation, biocide experiments were started with intermittent treatments on four consecutive days using a commercial product consisting of a combination of H_2O_2 and peracetic acid. The product was

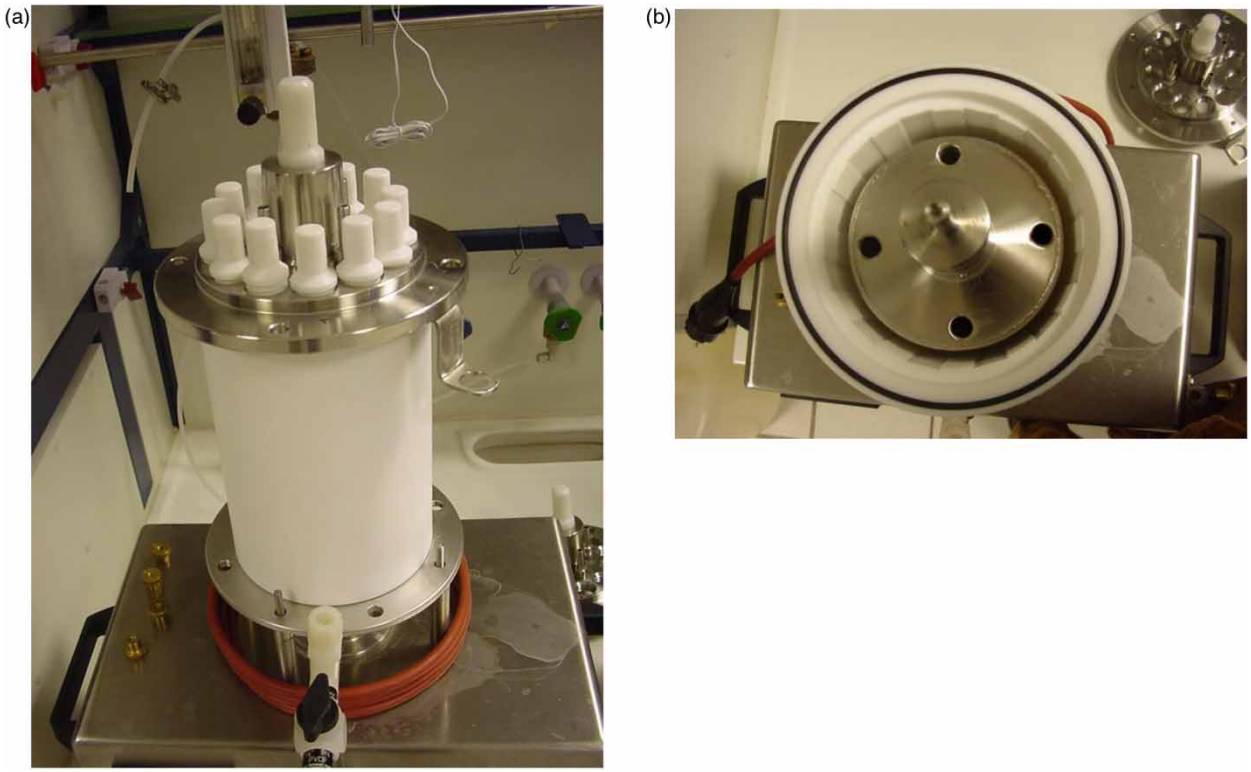


Figure 1 | Rotating annular reactor employed for cultivation and biocide treatment of drinking water-derived biofilms: (a) Assembled reactor and (b) assembled inner and outer cylinder (top view of opened reactor without lid). Drinking water and biocide solutions were supplied to reactor through an influent port at the top and discharged through an outlet at the bottom.

diluted in water of standardized hardness to final concentrations of 12,600 ppm H_2O_2 and 9,000 ppm peracetic acid. The reactors were emptied and three reactors were filled with 600 mL of the biocide solution. One reactor, filled with water of standardized hardness instead of biocide solution, served as a control. After a static incubation for 15 min at ambient temperature, the reactor was emptied and flow-through was resumed with drinking water under the conditions described above. Before and after the 15-min biocide exposure, coupons were aseptically removed from the reactors and replaced by fresh coupons in order to ensure constant hydraulic conditions throughout the complete reactor operation. Biofilms were removed from the coupon surfaces with a razor-blade surface scraper and suspended in 10 mL 10 mM phosphate buffer (0.31 g KH_2PO_4 and 1.37 g $Na_2HPO_4 \times 2H_2O$ per L, pH 7.54). With this procedure between 90 and 99.9% of all bacterial cells were removed from the coupon surfaces (data not shown). Homogenization of biofilms was conducted with a rotating (150 rpm) Teflon piston in a glass cylinder for 2 min (Griebe & Flemming 2000).

The heterotrophic plate count (HPC) in the biofilm suspensions was determined using the spread plate method on R2A medium (Difco™; Reasoner & Geldreich 1985). After incubation at 20 °C for 7 days, colonies were enumerated and the HPC was calculated as colony-forming units (CFU) per cm^2 of the steel coupons. For the determination of total cell counts, bacteria were stained with the DNA-binding fluorochrome 4′,6-diamidino-2-phenylindole (DAPI; Sigma). 1 mL of DAPI solution (5.5 μg/mL) was added to 4 mL of decimally diluted biofilm suspensions (dilution in 10 mM phosphate buffer). After incubation at room temperature for 20 min in the dark, the suspensions were filtered through black polycarbonate membrane filters (0.2 μm pore size; Millipore). The filters were rinsed with 10 mL of sterile-filtrated deionized water and subsequently air-dried. The cells on the membrane filters were enumerated under an epifluorescence microscope at 1,000-fold magnification in 20 randomly selected fields of view for each filter with a counting grid (100 μm × 100 μm), and total cell counts per cm^2 of the steel coupons were calculated.

Biofilm cells attached to the coupons were stained with the DNA-binding fluorochrome SYTO 9 (Molecular Probes, Live/Dead BacLight Bacterial Viability Kit, No. L-7012, component A; 1.5 μL SYTO 9 solution (3.34 mmol/L in DMSO) added to

1 mL of deionized water). 100 μL SYTO 9 solution was applied on top of the biofilms and the samples were incubated in the dark at room temperature for 30 min. The examination of SYTO 9-stained biofilms was performed using an LSM 510 confocal laser scanning microscope (Zeiss, Jena, Germany). Images were recorded at an excitation wavelength of 488 nm and an emission wavelength of 500 nm at 400× magnification. Digital image analysis of optical thin sections was performed with the Zeiss LSM software (version 2.5).

Cooling water: ATP measurements and colony counts

Cooling water at a steel manufacturing site was tested. The open cooling circuit had a total volume of 10,000 m^3 with a circulation rate of 10,000 m^3/h. Settling tanks and sand filters preceded the cooling tower to remove suspended solids, which enter the system due to the contact of cooling water with hot steel products (e.g. mill scale). Furthermore, oil contamination could enter the water through various sources. The exchange rate of cooling water was approximately 500 m^3/h corresponding to 1/20th of the total volume.

ATP measurements were carried out with a semi-continuous ATP-Analyzer (EZ7300, Hach, Loveland, CO, USA). The analyzer works according to the standard test method ASTM D4012-81 (ASTM D4012-81(2002) 1981) and uses sonication lysis to differentiate between free and total ATP. Calibration solutions and control standards (Cat. No. APPAZX000513, Hach), as well as the detection reagent (Cat. No. APPAZX000511, Hach), were ready to use. Due to high ATP concentrations in the cooling water, the linearity of the measuring range above 200 pg/mL was checked and verified by Hach. The analyzer was set to an average of 22 readings per day.

Colony counts from cooling water samples were determined at 36 °C following ISO 6222.

RESULTS

Thermal treatment of drinking water

Drinking water from a tap was subjected to either 70 or 90 °C for 45 min each and intact cell concentrations (ICC) were quantified by flow cytometry. Compared to an untreated control, ICC dropped from approximately 10^4 intact bacteria per mL by 1 and >2 log units (Figure 2(a)). In the case of the sample exposed to 90 °C, ICC were near the detection limit. These concentrations remained unchanged over 14 days, suggesting efficient killing of bacteria by the applied thermal treatments. Spiking of the thermally treated samples with 1:100th volume of untreated water, on the other hand, resulted in growth. The addition of low numbers of bacteria unaffected by disinfection resembles the situation in a drinking water system where new bacteria are introduced downstream of the treatment site when water has cooled down. Within 5 days, ICC of the sample treated at 90 °C reached the same level as the sample treated at 70 °C. After 7 days, ICC of both thermally treated samples were identical with the one of the untreated control whose cell concentrations also slightly increased. The latter effect is typical for stagnating water. Results suggest rapid necrotrophic growth by the transformation of thermally killed bacterial biomass into new one. After 14 days, bacterial concentrations in heat-treated samples after necrotrophic growth exceeded the ones in the untreated sample by 59% (70 °C) and 77% (90 °C). In other words, the bacterial concentrations not only fully recovered, but even exceeded the ones prior to disinfection.

Disinfection of drinking water by chlorine

Similar to thermal treatment, drinking water from a tap was subjected to chlorination for 30 min. Following disinfection, chlorine was neutralized by the addition of thiosulfate. Final concentrations of free chlorine of 0.1 and 1 ppm resulted in a drop of ICC from approximately 10^4 intact bacteria per mL by 1 and 2 log units, respectively (Figure 2(b)). Cell concentrations in the disinfected samples remained constant over 14 days, suggesting that all bacteria were killed. Spiking of the chlorine-treated samples with 1:100th volume of untreated water led, as in the case of thermally treated samples, to growth. Spiking of samples imitates the scenario in a drinking water distribution system or plumbing system, where chlorine has been rendered inactive by chlorine demand. Growth in the sample with a free chlorine concentration of 1 ppm was stronger than in the sample exposed to 0.1 ppm with identical ICC being reached after 5 days. After 7 days, ICC resembled the ones in the untreated control whose cell concentrations also increased slightly due to stagnation. The presence of thiosulfate did not cause significant differences in growth when comparing an untreated control with and without the addition of this neutralizing agent. As in the case of thermal disinfection, overall results suggest necrotrophic growth of newly introduced bacteria feeding on the dead biomass. Bacteria present in chlorine-disinfected water reach concentrations comparable to untreated

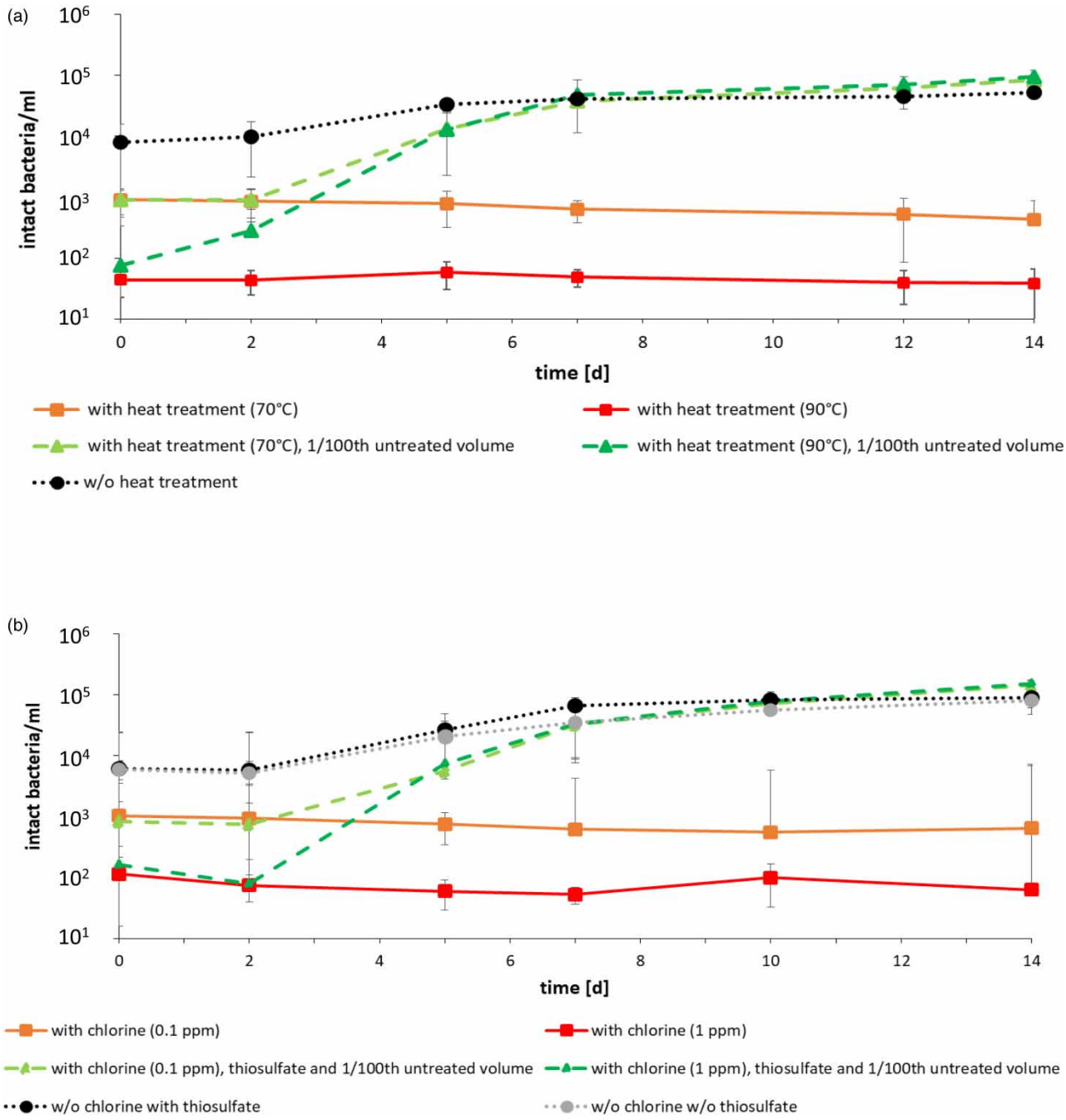


Figure 2 | Increase in intact cell numbers in drinking water samples after treatment with (a) heat and (b) chlorination in relation with untreated controls. (a) Samples were heated to 70 or 90 °C for 45 min and either supplemented with 1/100th volume of untreated water or not. (b) Samples were exposed to 0.1 or 1 ppm free chlorine for 30 min and either supplemented with 1/100th volume of untreated water or not. Untreated samples served as controls. In the case of the chlorine experiment, one control sample contained thiosulfate, the other not.

samples. On day 14, ICC in samples with necrotrophic growth even exceeded the ones of the untreated controls by 69% (0.1 ppm free chlorine) and 76% (1.0 ppm free chlorine).

Regrowth of biofilm after biocide treatment

Drinking water biofilms were grown on stainless steel coupons in four annular biofilm reactors for 7 days. Subsequently, starting on day 7, biofilms of three reactors were subjected in parallel to a once-daily 15-min treatment with a commercial product

containing a mixture of hydrogen peroxide and peracetic acid with a final concentration of 12,600 and 9,000 ppm, respectively, over a time period of 3 days (days 7–10). An identical biofilm without treatment served as a control. HPC (culturable bacteria) and total cell numbers (microscopic quantification after cell staining with DAPI) were determined in suspensions of biofilms removed from the coupons. The first biocide exposure resulted in a large drop in the HPC by up to more than 4 log units, whereas the HPC remained unchanged in the control reactor (Figure 3). HPCs in the treated biofilms remained essentially unchanged on day 8 and dropped further by up to 2 log units after a second biocide treatment. On day 9, numbers of culturable bacteria had recovered and increased by approximately 3 log units. A third biocide exposure resulted in a repeated drop in the HPC followed by recovery on day 10. A fourth biocide treatment again led to a decrease in the HPC. Total cell counts of the biocide-treated biofilms remained unchanged until the third biocide treatment where a moderate drop in total cell numbers was observed. The latter suggested a more severe treatment effect, which not only led to a loss of culturable cells, but also to a loss of overall cells of the biofilm biomass. Total cell counts recovered until day 10 concomitantly with culturable bacteria. Both parameters remained essentially unchanged in the case of the control biofilm over the experimental period. Results corroborate that biofilms can recover after biocide treatment showing a cyclic behavior of death and regrowth when applying repetitive biocidal treatments. Microscopic examination revealed that untreated biofilms were characterized by a heterogeneous distribution of biofilm cells and varying biofilm thickness, while hydrogen peroxide/peracetic acid exposure resulted in compact biofilms with a more homogeneous cell distribution and uniform biofilm thickness (Figure 4).

Regrowth in cooling water

In a 13-day field trial, the impact of monochloramine on bacterial growth in cooling water on a steel manufacturing site was tested. During the observation period of 13 days, daily biocide treatments were applied. With the exception of the initial dosage, where monochloramine was dosed for 5 h, each treatment lasted 3 h with a biocide peak concentration of 2.5–5 ppm total chlorine in the system. To assess the effect of the biocide treatment, a semi-continuous online ATP measurement was carried out. Total, free (extracellular) and intracellular ATP levels were determined. The initial addition of monochloramine led to a rapid decrease of intracellular ATP 400 to 5 ppm mL^{-1} (Figure 5). After the treatment was stopped, a rapid increase of ATP could be observed reaching ATP concentrations of up to 810 pg mL^{-1}. The next 12 dosages showed the same overall pattern in terms of ATP concentration. An immediate biocide-induced decrease of ATP was followed by increasing concentrations resulting in a steady-state phase with fluctuating ATP readings. The minimal intracellular ATP

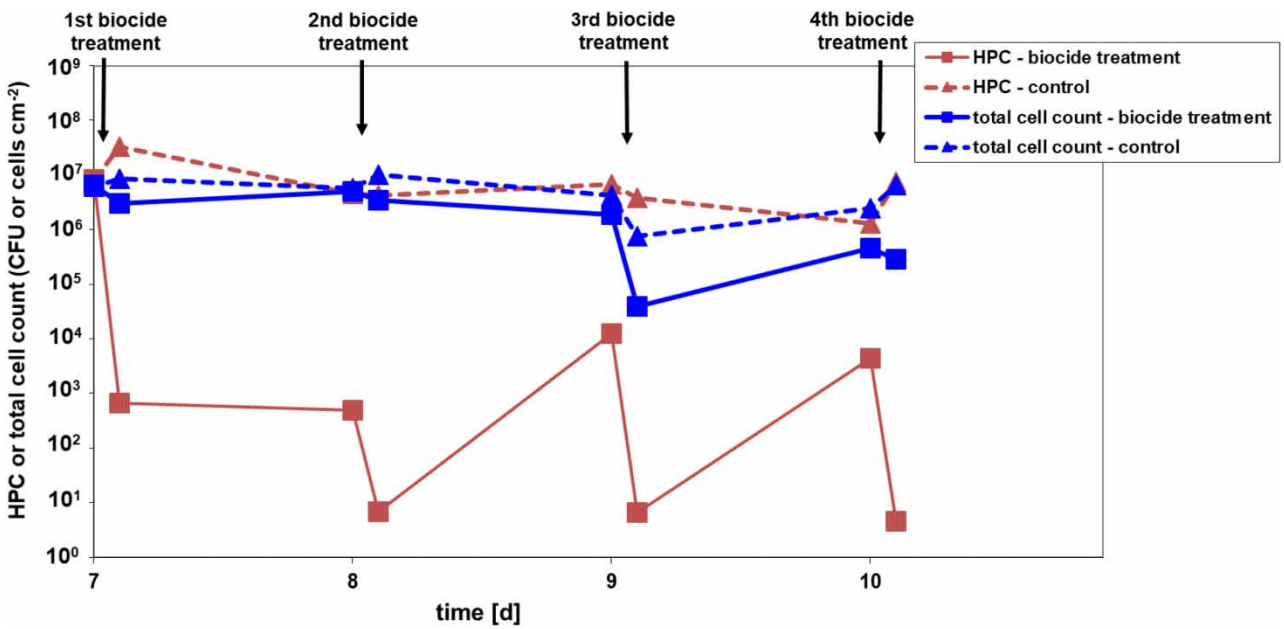


Figure 3 | Effect of repeated treatment of a mixed-population drinking water biofilm with H_2O_2 (12,600 ppm) and peracetic acid (9,000 ppm) for 15 min on HPC and total cell count of biofilms on steel coupons sampled from the biofilm reactors. Time points of treatment are indicated by arrows. Shown are representative results of one of the three biofilm reactors with biocidal treatment and of one reactor without biocidal treatment serving as a control.

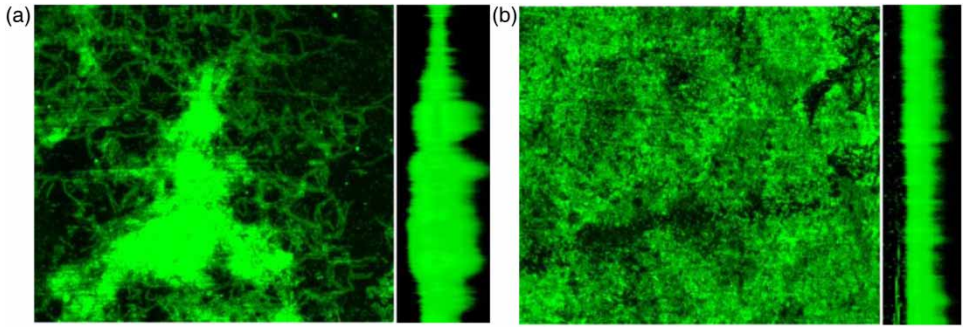


Figure 4 | Confocal laser scanning microscopic images of biofilms stained with SYTO 9 on stainless steel coupons (400× magnification): (a) untreated biofilm (control); (b) biofilm after biocidal treatment (12,600 ppm H_2O_2 and 9,000 ppm peracetic acid) for 15 min. Shown are top views (left) and side views (right) of the biofilms.

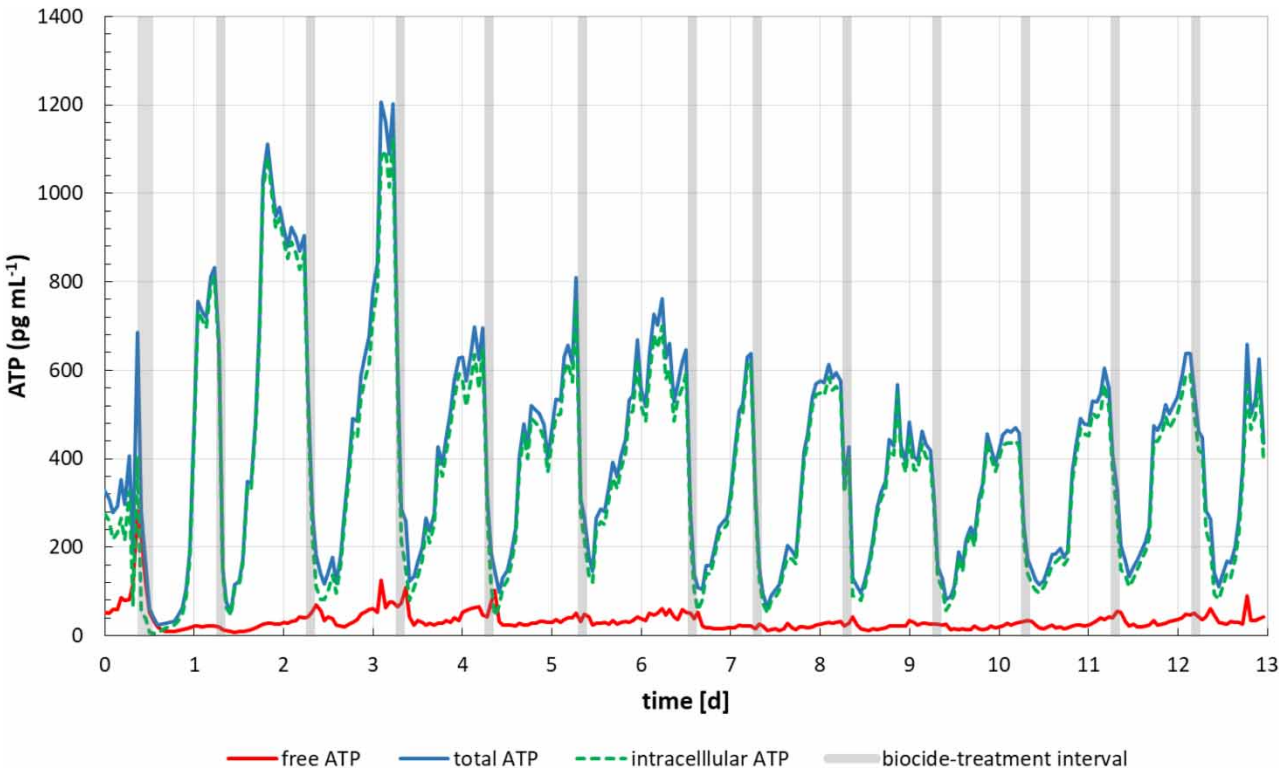


Figure 5 | Change in ATP concentration in cooling water subjected to daily dosage of monochloramine (range: 2.5–5 ppm) over 13 days. Biocide treatment intervals are indicated by gray bars.

concentrations after biocide treatment varied between 5 and 120 pg mL^{-1}, whereas the maximum concentrations reached 370–1,130 pg mL^{-1}. ATP measurements were supplemented by the assessment of colony counts (36 °C, according to ISO 6222) in the steady-state phase in the first 5 days of the experiment with samples taken shortly before the biocide dosage. Colony counts were between 10^4 and 10^6 CFU/mL at 36 °C corresponding to typical values for this system and did not show any overall decreasing tendency over time supporting the capacity for substantial microbial regrowth.

Necrotrophic growth of *E. coli* on dead *P. putida*

In the next step, the utilization of dead bacterial biomass by live bacteria of a different species by necrotrophic growth was demonstrated directly. A suspension of *P. putida* cells (approximately 10^8 total cells/mL) was killed by heat (90 °C for 45 min;

Figure 6(a)) or by chlorine (20 or 80 ppm for 30 min; Figure 6(b)). Dead cells were mixed with live *E. coli* (approximately 10^5 cells/mL) and numbers of intact bacteria were monitored over 7 days using flow cytometry in combination with cell integrity staining. Suspensions of only killed *P. putida* cells or only live *E. coli* cells served as controls. Neither of these controls showed increasing cell numbers, as in the first case dead *P. putida* cells could not replicate and in the second case live *E. coli* cells were deprived of nutrients. In the mixtures, on the other hand, increasing intact cell numbers were obtained. Growing bacteria were *E. coli* as confirmed by cultivation on membrane lactose glucuronide agar. Both heat-killed *P. putida* (Figure 6(a)) and chlorine-killed *P. putida* (Figure 6(b)) could thus serve as a nutritional basis for *E. coli* replication corroborating the necrotrophic capability of this bacterial species. Maximal numbers of *E. coli* were reached after 1 day in case chlorine-killed *P. putida* were administered (in case of treatment with 80 ppm chlorine), whereas 2 days were required in case heat-killed *P. putida* were added as a nutrient source. When comparing the two chlorine concentrations, the *E. coli*

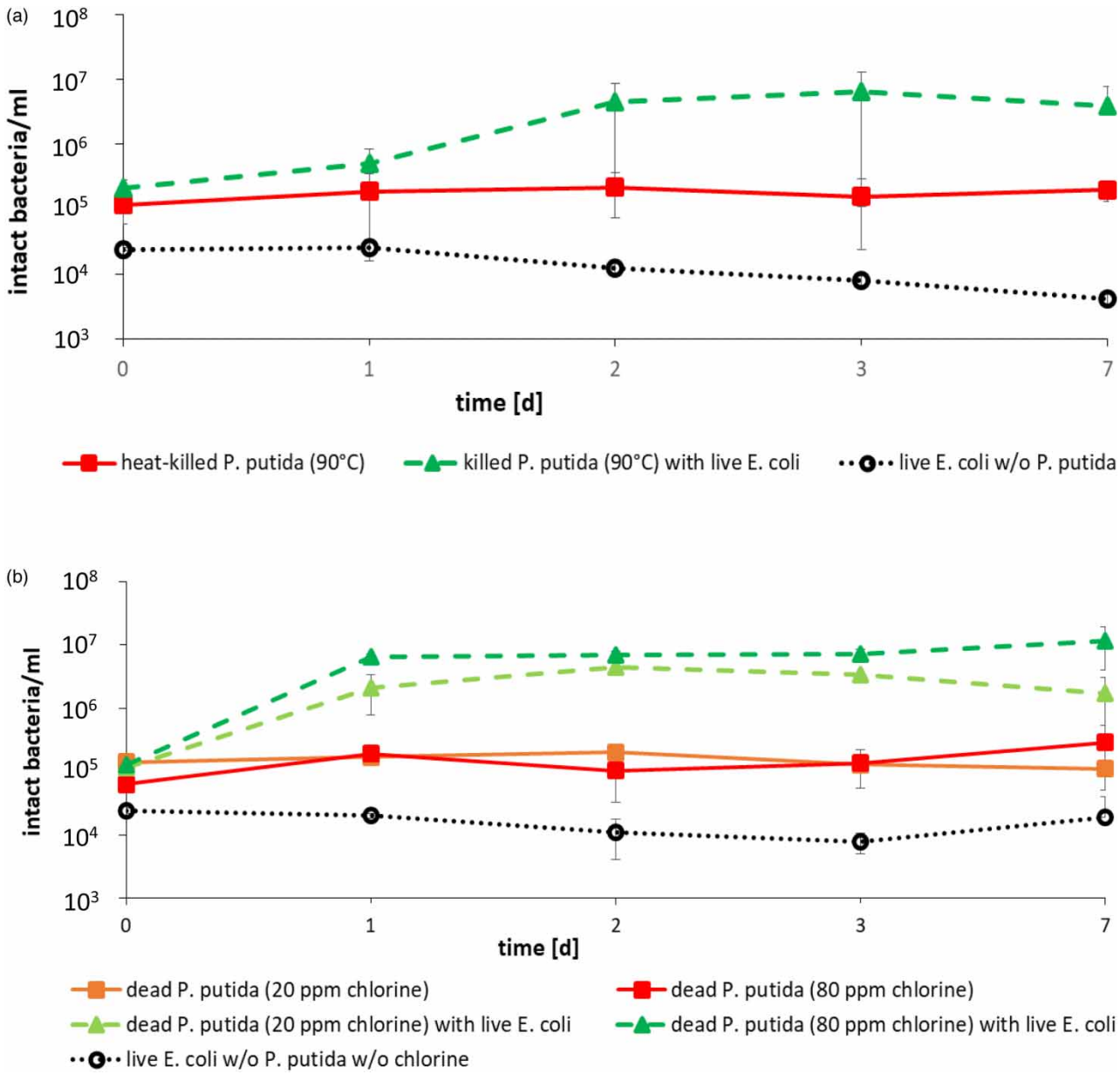


Figure 6 | Necrotrophic growth of *E. coli* on the basis of supplemented dead *P. putida* cells killed either by (a) heat or (b) chlorination. Samples with only killed *P. putida* cells or with only live *E. coli* cells served as controls.

fed with *P. putida* that were treated with 80 ppm chlorine, grew faster and reached higher concentrations than *E. coli* fed with *P. putida* treated with 20 ppm chlorine.

DISCUSSION

The study represents a compilation of examples where different waterborne bacterial populations were subjected to different types of disinfection or biocidal treatment. Regrowth behavior (in the sense of the recovery of the bacterial numbers) was monitored using flow cytometric quantification of intact cell numbers, total cell counts (DAPI), colony counts or ATP measurements. All examples show quick recoveries of bacterial numbers after thermal or biocidal treatment. Concentrations of bacteria can reach levels comparable with the ones of untreated control samples.

Parameters determining regrowth are diverse and include temperature, oxygen availability (in case of aerobic organisms), the spatial location of microorganisms and the availability of ecological niches (LeChevallier 2003; Nescerecka *et al.* 2014; Prest *et al.* 2016). Nutrient availability can be seen as another essential parameter for regrowth (van Nevel *et al.* 2013; Prest *et al.* 2016). With the exception of the cooling water and biofilm reactor systems, the experiments presented here can be considered closed systems in regard to the nutrients with no nutrients entering or leaving the systems. Whereas treatment did have a clear impact on bacterial viability, the effect was, as often seen in practice, not sustainable. The same patterns were seen both for planktonic cells as for biofilms.

In the case of drinking water, stronger treatment intensity led to more killing. If bacteria unaffected by treatment enter disinfected water, the recovery of cell numbers occurs within a few days as demonstrated with water subjected to different temperatures or to different chlorine concentrations. The stronger the thermal or biocidal treatment, the stronger subsequent regrowth. Concentrations of bacteria were the same after 5 days independent of the applied heat or the initial chlorine dose. Regrowth in the samples receiving more intense treatment tended to be stronger in the initial phase. After 14 days, ICC values of all treated samples exceeded bacterial numbers of the untreated controls. The reason for the latter can presumably be seen in a higher overall concentration of AOC generated by the application of heat or the administered chlorine. This effect has been reported previously for chlorine. The chlorination of drinking water had been shown to increase AOC concentrations (Ramseier *et al.* 2011). In certain cases, a correlation between chlorine concentrations and the extent of AOC was obtained with more chlorine generating more AOC (Liu *et al.* 2015). This effect was obtained in our study when feeding *E. coli* with *P. putida* killed either with 20 or 80 ppm chlorine. Higher *E. coli* numbers were obtained in the sample receiving biomass subjected to more disinfectant. The reason probably lies in the availability of higher concentrations of assimilable nutrients lending themselves to an altered substrate composition by oxidative disruption of chemical bonds (Okuda *et al.* 2009; Ramseier *et al.* 2011). More intense oxidation can be assumed to cause the stronger breakdown of organics and the more efficient conversion of large organic compounds into more bio-available smaller breakdown products.

Apart from the extent of regrowth, also its kinetics probably depends on the bioavailability of nutrients and their release. Depending on the mode of action of the biocide, intracellular contents are released by damage to the cell envelope (McDonnell & Russell 1999). The greater the damage, the faster the release and its extent can be assumed. The effect of different inactivation modes on the kinetics of necrotrophic assimilation still needs thorough analysis in this respect. When feeding *E. coli* with dead *P. putida*, chlorine-killed biomass resulted in stronger regrowth already after 1 day compared to heat-killed biomass. Although the conclusion that oxidized compounds might be better assimilable than denatured organic compounds was tempting, this did not hold true when subjecting drinking water to these different disinfection procedures. In this case, exposure to 90 °C heat resulted in the fastest regrowth compared to treatment at 70 °C or exposure to chlorine.

Post-treatment growth was not only observed in bulk water, but also in biofilms. Survival and regrowth of biofilm bacteria were demonstrated by repeated exposures to a combination of hydrogen peroxide and peracetic acid at high concentrations (12,600 ppm H_2O_2/9,000 ppm peracetic acid). Daily 15-min treatment of the biofilms resulted in a strong decline in the concentration of culturable HPC bacteria, while the number of total cells remained essentially unchanged, indicating that the biocides decreased bacterial viability, but did not result in the physical removal of the biofilms from the surface. In addition, within 24 h after the second and also after the following shock treatments, the HPC significantly increased again. Possible reasons may be a recovery of biofilm bacteria, becoming culturable again, multiplication of surviving bacteria or colonization and multiplication of autochthonous water bacteria entering the biofilm system via continuous biofilm reactor flow-through of drinking water between the biocide treatments, forming secondary biofilms on the residual surface-attached biomass and feeding on the dead biofilm biomass (necrotrophic growth). There are only few reports in the literature concerning the

discontinuous treatment of biofilms in technical water systems on the basis of peracetic acid-containing products. Mathieu *et al.* (1990) reported that repeated treatment of a 4 day-old wastewater biofilm with 30 ppm peracetic acid in a laboratory reactor for 10 min each day on four consecutive days resulted in an incomplete removal of the biofilms. After the first treatment, a removal of approximately 1 log unit of cell numbers occurred, but subsequent treatments did not cause any further reduction in attached biofilm cells. There was even a slight increase in biofilms (possibly regrowth) during the 24 h intervals between the treatments. In a laboratory-scale pipeline system, 4-week-old drinking water biofilms grown on polyvinyl chloride surfaces were continually perfused with peracetic acid (1 ppm) for 3 days (Kauppinen *et al.* 2012). The biofilms underwent an approximately 3.4 log decrease of HPC, while the total cell counts remained stable. The removal of the bulk biofilm biomass was not achieved. In the untreated pipeline control system, both HPC and total cell counts remained constant.

The effect of recolonization of peracetic acid-treated single-species biofilms was also demonstrated in a laboratory study (Akinbobola *et al.* 2021). After exposure of 96-h-old biofilms of *Pseudomonas aeruginosa* to 3,500 ppm peracetic acid for 5 min in 24-well plates, the complete killing of the biofilm cells, but only an approximately 40% reduction in the biofilm biomass compared to an untreated control was observed. Re-inoculation of the peracetic acid-killed biofilms with *P. aeruginosa* resulted in the formation of secondary biofilms, which displayed a significantly enhanced tolerance to peracetic acid compared to the original biofilms. Microscopic visualization of the distribution of living and dead cells in peracetic acid-killed and recolonized biofilms revealed that the living cells were distributed evenly throughout the biofilms, indicating that the embedding and multiplication of cells within the interior of biofilms may contribute to enhanced tolerance toward the biocide.

In the current study, microscopic analysis showed that the peracetic acid-exposed biofilms were still attached to the surface, but revealed a different architecture being more compact and of uniform thickness compared to the heterogeneous structure of the untreated biofilm. In a study of the effect of different disinfection protocols on microbial biofilms attached to tubes in real dental waterlines, scanning electron microscopy revealed that intermittent treatment with peracetic acid (2,600 ppm) throughout 3 months reduced only part of the biofilm, in particular bulk aggregates, compared to control biofilms, but significant surface coverage was still observed (Dallolio *et al.* 2014). This change of biofilm structure may reflect a protective adaptation and/or change in community composition to peracetic acid exposure. Overall results corroborate that the eradication of biofilms is extremely difficult and that microorganisms surviving in this protected environment can be the source of subsequent regrowth. This aspect is important to consider in the context of an integrated antifouling strategy (Flemming 2002, 2020). It is key to successful sanitation of biofouling in water systems, since biofilm killing alone is not sufficient, but has to be combined with cleaning procedures for the removal of dead biomass to prevent rapid regrowth of biofilms.

Biofilm might also explain the initial increase in ATP in water during the initial days of cooling water treatment. Cooling water has its unique water treatment challenges. The formation of biofilm caused by the organic load cannot only cause a number of problems including elevated *Legionella* concentrations, microbially enhanced corrosion and general fouling within the system, but can also be seen as a nutrient reservoir. Monitoring ATP levels has been shown to successfully reflect the efficacy of biocide impact (Pennings & Van de Velde 2019). ATP might have become increasingly released from biofilm deposits into bulk water as a consequence of monochloramine addition. In comparison with hypochlorite, which produces more biofilm sloughing, monochloramine was shown to better penetrate biofilms (Lee *et al.* 2018). The loss of cellular integrity was steady but slower in the case of monochloramine compared to hypochlorite, which might explain that the increase in ATP levels extended over 3 days. From days 3 to 10, a decrease in maximum ATP levels might have been the effect of slow replacement of cooling water with fresh feed water (and thus a decrease in nutrient content) with an exchange rate of approximately 500 m^3/h corresponding to 1/20th of the total volume. Nevertheless, despite nearly 2 weeks of daily biocide dosing, regrowth could not be eliminated. This can be explained by the fact that biofilm with its associated nutrient reservoir cannot be completely removed and nutrient influx by circulating cooling water continues.

It was finally demonstrated that *E. coli* can feed necrotrophically, in our example on *P. putida* killed either by heat or by chlorine. This is not surprising considering that *E. coli* live in the digestive tract of warm-blooded animals whose main purpose is to lyse cells contained in food and to release their nutrients to make them available for absorption by their hosts. The lifestyle in the intestines made it probably well adapted to utilizing nutrients released from dead biomass. The capability of *E. coli* for necrotrophic growth has also been supported by a recent study, where *E. coli* was found to thrive in sludge cakes after anaerobic digestion (Fane *et al.* 2019). The sludge underwent thermal treatment during anaerobic digestion and was subsequently subjected to mechanical dewatering. Thermal treatment and shear forces present during dewatering were hypothesized to be important factors causing lethal damage to bacteria contained in the sludge. Both treatments were

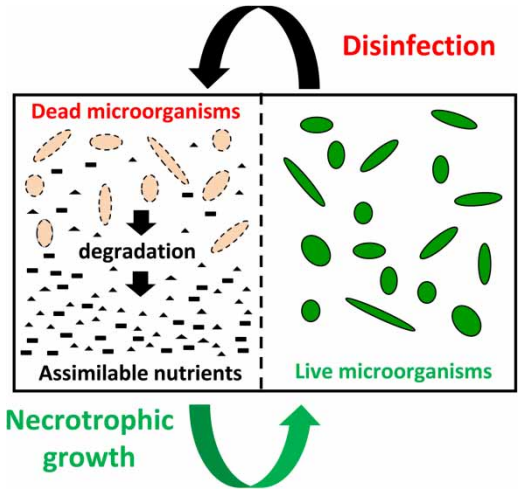


Figure 7 | Schematic representation of the transition between microorganisms and nutrients during necrotrophic growth and disinfection. Microorganisms are killed or damaged during disinfection. Cytosolic components are released into the surrounding environment. Dead microorganisms gradually degrade into assimilable nutrients which serve as building blocks for the formation of new biomass during growth by remaining live bacteria or bacteria newly introduced into the system. The nutrient pool and the pool of live microorganisms exist in equilibrium. The transition of dead biomass into live biomass is referred to as necrotrophic growth.

shown to increase the proportion of damaged cells compared to untreated sludge. The resulting release of cellular nutrients was seen as a significant factor contributing to subsequent necrotrophic *E. coli* growth in stored biosolids (Fane *et al.* 2019, 2020).

The cycle between disinfection/biocidal treatment and regrowth is schematically shown in Figure 7. Chemical disinfection results in a reduction of viable cells and often to damage of their cellular integrity. The intracellular contents are released and the remaining cell corpses degrade. The resulting organic compounds serve as building blocks for the assimilation of new biomass by surviving microorganisms or microorganisms newly introduced into the system. The nutrient pool (soluble nutrients) can be seen in equilibrium with microbially fixed nutrients. Necrotrophic regrowth can thus only be suppressed by removing dead biomass after treatment to withdraw nutrients from the system. In other words, to achieve sustainable disinfection, disinfection and biocidal treatment should therefore always be followed by thorough cleaning to minimize the nutrient basis.

A topic that deserves more attention in the future is the change of the bacterial microbiome during regrowth. Whereas the focus of this study was solely on the bacterial numbers reached by the innate waterborne community after treatment and how quickly this happened, the composition of the new microbial community is of hygienic relevance. Interestingly, it was recently reported that disinfection exerts a selective pressure on the drinking water microbiome toward microorganisms that can utilize decay products from inactivated biomass (Dai *et al.* 2020).

CONCLUSION

This study addressed regrowth behaviors of autochthonous bacteria contained in water subjected to different disinfection procedures. Biological stability was regained quickly in all cases. The stronger the thermal or biocidal treatment, the stronger the subsequent regrowth. The presented results are relevant for practical applications where disinfection is often considered finished after the first phase, where microorganisms are killed. In the second phase, the removal of dead biomass and released nutrients is often overlooked and interferes with a sustained effect of disinfection. Overall disinfection and biocidal treatment is not only about efficient killing, but should equally be concerned about the subsequent removal of the regrowth potential contained in the dead biomass. Minimizing this nutrient source together with the nutrient source that originally led to the buildup of biomass increases the likelihood to make the effect of disinfection and biocidal treatment more sustainable.

ACKNOWLEDGEMENTS

The study of biofilm regrowth after biocide treatment was financially supported by the German Federal Ministry of Education and Research (Grant No. 02WA9912). Otherwise, no financial support was received.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 2 March 2021; accepted in revised form 26 August 2021. Available online 7 September 2021