Influence of physicochemical surface properties on biofilm formation in drinking water distribution systems
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Chapter 3

Nanogel-based coating as an alternative strategy for biofilm control in drinking water distribution systems

Part of this chapter has been published in:
Abstract

Biofilm formation and detachment in drinking water distribution systems (DWDS) could lead to several operational issues. Here, an alternative biofilm control strategy of limiting bacterial adhesion by application of a poly(N-isopropylmethacrylamide)-based nanogel coating on DWDS pipe walls was investigated. The nanogel coatings were successfully deposited on surfaces of four polymeric pipe materials commonly applied in DWDS construction. Nanogel-coated and non-coated pipe materials were characterized in terms of their surface hydrophilicity and roughness. Four DWDS relevant bacterial strains, representing *Sphingomonas* and *Pseudomonas*, were used to evaluate the anti-adhesive performance of the coating in 4 h adhesion test. The presence of the nanogel coating resulted in adhesion reduction up to 97%, compared to a non-coated surface. These promising results motivate further investigation of nanogel coating as a strategy for biofilm prevention in DWDS.
Introduction

The purpose of drinking water distribution systems (DWDS) is to safeguard the transportation of potable water from a treatment plant to a consumer, and it is crucial that the water delivered to a tap is of high quality, both chemically and biologically. Nevertheless, this quality often deteriorates during the lengthy distribution process (Liu et al., 2017b). Next to pipe corrosion and scales formation, biofilm development on DWDS pipe walls is one of the main causes of quality degradation (Liu et al., 2013; Makris et al., 2014). Biofilms are microbial aggregates formed on a surface that consist of cells embedded in a matrix of extracellular polymeric substances (EPS) secreted by the microbial community itself (Flemming et al., 2016). Their presence in DWDS leads to several operational issues. For instance, a biofilm adhering on the pipe walls can impact distribution process efficiency in the long term by pipe damage through microbially induced corrosion of iron-based pipes (Wang et al., 2015) or by increasing the flow resistance (Cowle et al., 2014). Moreover, the detachment of biofilm clusters into the water flow can lead to downstream deterioration of water aesthetics (turbidity, colour, smell) (Husband and Boxall, 2011), and pose as a health hazard as biofilms were found to harbour pathogens (Wingender and Flemming, 2011).

Globally, the biofilm control approach in DWDS often involves disinfection strategies via chlorination and chloramination (Li et al., 2021). However, chlorine is incapable of complete inhibition of biofilm growth, affecting only the periphery of the EPS matrix, and does not target microorganisms within the biofilm (Liu et al., 2016). Chlorine has limited penetration within the biofilm matrix due to its reaction with organic and inorganic compounds (Schwering et al., 2013); this reaction with organics also produces harmful disinfection by-products, which are suspected human carcinogens (Le Roux et al., 2017). Water companies that resigned from chlorination, focus on limiting the organic matter and nutrients essential for bacterial growth through improved water treatment (Park et al., 2021). Nevertheless, it has been reported that oligotrophic bacteria are still found to accumulate and form biofilms even when little to no molecules, organic nor inorganic, are present in the water stream (Kulakov et al. 2002). Common cleaning practices, such as periodic pipe flushing with increased water velocity, also do not completely remove the biofilm since they were found to mobilize and remove only loose biofilm clusters (Douterelo et al., 2013).
In this study, we propose an alternative strategy to limit bacterial adhesion to pipe materials from DWDS with an anti-adhesive coating that targets biofilm formation in its onset. Antifouling solutions are already a recognized strategy in preventing biofilm development in numerous industrial applications, including membrane technologies (Zhao et al., 2021), the marine (Gu et al., 2020), and the biomedical field (Busscher et al., 2012). Such application offers an interesting alternative for DWDS biofilm control approaches, especially in DWDS biofilm hotspots, that are more prone to biofilm formation (higher temperatures, long stagnation periods, dead-ends) (Ling et al., 2018; Simunič et al., 2020).

Nanogels are nanosized hydrogel particles made of cross-linked swellable polymer networks with an ability to absorb and retain high volumes of water, without dissolving in aqueous media (Peppas and Hoffman, 2020). When deposited on a surface in a form of a coating, in an aqueous environment, nanogel particles create a hydration layer, which targets biofilm formation in its early stages by creating a physical and energetical barrier for initial adhesion of bacteria (Chen et al., 2010; Keskin et al., 2019). Nowadays, nanogel coatings are gaining great attention to kill bacteria and to limit bacterial adhesion on biomaterial surfaces in order to avoid biomaterial-associated infections (Zu et al., 2020; Keskin et al., 2021). Nanogel coatings have also been used in the marine industry to prevent biofouling and the costly consequences for marine equipment (Chen et al., 2016). Proposed nanogel coating deposition on various surfaces is a universal, very simple and time-efficient method based on the electrostatic interactions between the solid surface and the nanogels, which can also be applied to water distribution pipe materials (Keskin et al., 2019).

While the nanogel coatings have been tested against adhesion of proteins (Cross et al., 2016), mammalian cells (Schmidt et al., 2010), and clinical bacterial strains (Keskin et al., 2019), at this moment, this is the first study that investigates their influence on initial adhesion and biofilm formation of environmentally relevant bacterial strains. DWDS conditions vary considerably from those in clinical settings, including oligotrophic environment, exposure to high water flows, and spatial-temporal fluctuations in environmental and engineering parameters throughout the distribution process (Potgieter et al., 2018). Such conditions promote development of microbial communities equipped with different survival strategies and biofilm forming behaviours than those exhibited by the clinically relevant strains. Therefore, the effect of the coating on DWDS-
representative bacterial strains cannot be predicted from the results obtained by studies performed within biomedical settings. In this work, the poly(N-isopropylmethacrylamide)-based nanogel coating was deposited on a selection of polymeric pipe surfaces to validate the applicability of the coating on DWDS relevant materials. The nanogel coating was tested against the initial adhesion by four bacterial strains representing genera commonly identified in DWDS biofilms, *Sphingomonas* and *Pseudomonas*. Bacterial adhesion was assessed by flowing bacterial suspensions in a parallel-plate flow chamber and by enumerating attached bacteria after 4 h.

**Materials and methods**

**Nanogels synthesis, nanogel coating deposition and characterization**

**Reagents**

The monomer *N*-isopropylmethacrylamide (97%, NIPMAM), the cross-linker *N*,*N*-methylene bis(acrylamide) (99%, BIS), the surfactant sodium dodecyl sulfate (SDS), the initiator ammonium persulfate (98% APS) and polyethyleneimine (PEI, branched, M_w 25,000 g/mol) were purchased from Sigma-Aldrich, the Netherlands. *N*-Isopropylmethacrylamide was recrystallized from hexane; all other chemicals were used as received without purification. Ultrapure water (18.2 MΩ, arium 611 DI water purification system; Sartorius AG, Germany) was used in all experiments.

**Synthesis of P(NIPMAM) nanogel**

Synthesis of the poly(*N*-isopropylmethacrylamide) (P(NIPMAM)) nanogel was performed as described previously by Keskin et al. (2019), and the most optimal composition, µGel5, was selected for this study. Briefly, in a three-necked 250 mL flask equipped with a flat anchor-shaped mechanical stirrer, a reflux condenser, and a nitrogen in- and outlet, 1812 mg (4.8 mmol, 95 mol %) of NIPMAM (NIPMAM previously crys-}


purified by ultracentrifugation (three times at 179,200 g) followed by decantation and dispersion in water and the P(NIPMAM) nanogel was freeze-dried for the preparation of the coatings.

**Dynamic light scattering (DLS) and ζ-potential measurement**

The hydrodynamic diameter ($d_h$) and particle size distribution of the nanogels were measured using a Zetasizer Nano-ZS (Malvern Instruments, UK). Temperature-dependent measurements were recorded at a fixed scattering angle of 173 degrees and a wavelength $\lambda = 633$ nm of the laser beam while the temperature was varied in the range of 2 to 60 °C at 2 °C intervals and with a measurement time of 10 s and 11 runs, performed in triplicate. For data evaluation, the cumulant fit analysis was used and the hydrodynamic $d_h$ was calculated by use of the Stokes–Einstein equation. ζ-potential measurements were performed in disposable capillary cells (Malvern, DTS1070) in water at an angle of 17 degrees and a laser beam wavelength of $\lambda = 633$ nm. The ζ-potential was calculated from the electrophoretic mobility by use of the Smoluchowski equation.

**Transmission electron microscopy**

To evaluate the size and the morphology of the nanogel particles, transmission electron microscopy (TEM) analysis was performed using a Philips CM12 microscope operating at an accelerating voltage of 120 kV and coupled to a 4 k CCD camera. The nanogel particles were negatively stained with 2% uranyl acetate that were prepared by drop-casting of a nanogel suspension (0.2 mg/mL) in water onto a carbon coated copper grid.

**Surface preparation and nanogel coating deposition**

To test the nanogel coating applicability on pipe materials, the coating was deposited on coupons (8 mm x 8 mm) cut from polymeric pipes used in DWDS, such as high-density polyethylene PE100 (HDPE 1, HDPE 2) and unplasticized polyvinyl chloride (PVC 1, PVC 2). Additionally, nanogel coating was deposited on transparent polyvinyl chloride slides (transparent PVC; Eriks, the Netherlands) of 76 mm x 26 mm x 1 mm and used in a parallel-plate flow chamber for bacterial adhesion experiments. The coating procedure has been described previously (Keskin et al. 2019) and was implemented here with slight modifications. Briefly, prior to coating, transparent PVC and pipe materials were rinsed with ethanol (70%) and water, sonicated for 10 min (Transsonic TP690, Salm en Kipp...
B.V., the Netherlands) in water and air-dried at RT. Plasma oxidation was performed for 5 min (at 100 mTorr and 0.2 mbar, on Plasma Active Flecto 10 USB, Plasma Technology GmbH, Germany) after which the solid surfaces were immersed in PEI solution (1.5 mg/mL, 0.15 wt%, pH 7) for 20 min and afterward rinsed five times with water. After drying at RT, nanogel suspension (5 mg/mL, 0.5 wt%) was sprayed onto the PEI modified surfaces until the nanogel solution was uniformly distributed. The coated surfaces were dried first at room temperature followed by overnight drying in the oven at 50 °C. To remove the non-bound fraction of nanogel particles, the solid surfaces with the dried nanogel layer were washed three times by immersing in water for 2 h each time.

**Atomic force microscopy and average roughness measurements**

The surface topographies of nanogel coatings on transparent PVC, HDPE 1, HDPE 2, PVC 1, and PVC 2 were visualized using atomic force microscopy (AFM; Dimension Icon, Bruker, MA, USA) in contact mode with silicon nitride DNP-10 probes (spring constant $k = 0.06 \text{ N/m}$, frequency $f_0 = 18 \text{ kHz}$) in a dry and wet state. Average roughness ($R_A$) was calculated based on AFM images of non-coated and coated samples using NanoScope Analysis 2.0 image processing software (Bruker) on micrographs of 5 x 5 µm. $R_A$ measurements were performed on two different locations of three replicates per tested surface. To compare the roughness of uncoated and nanogel-coated pipe materials, Student’s $t$-test was used.

**Water contact angle measurements**

The wettability of non-coated and nanogel-coated surfaces was assessed by measuring advancing type water contact angles at room temperature using the sessile drop technique. Ultrapure water droplets of 1.5 µL were applied on the solid surfaces with and without a nanogel coating and measured with a contour analysis system (OCA35, DataPhysics Instruments, Germany). The measurements were performed on three different locations of three replicates per tested surface. All the samples were hydrated overnight before the measurement.

**Nanogel coating stability**

To test the stability of the nanogel coating, three coated and non-coated coupons (8 mm x 8 mm) of the four pipe materials were placed in a 24-well plate (Corning, NY, USA),
in 1.5 mL of sterilized tap water and agitated on an orbital shaker (KS 260 basic, IKA, Germany) at 240 rpm at room temperature for 30 days. The presence of the nanogel coating, prior and after the stability test, was measured using AFM. Water, in which the coupons were immersed during the stability test, was analysed for the concentration of organic carbon using Liquid Chromatography-Organic Carbon Detection (LC-OCD) method to evaluate potential effect of the nanogel coating on the water quality. LC-OCD was performed using Model 8 (DOC-LABOR, Germany) and data analysis was performed with DOC-LABOR software (ChromLog version). To compare the organic carbon concentrations in water in contact with uncoated and nanogel-coated pipe materials, Student’s t-test was used.

**Bacterial strains and growth conditions**

Four bacterial strains belonging to *Sphingomonas* and *Pseudomonas*, genera commonly identified in DWDS biofilms communities (Liu et al., 2014; Douterelo et al., 2014; Liu et al., 2017a), were selected for this study. *Sphingomonas* Sph5 and Sph10 were isolated from fouled spiral wound membranes and are part of the Wetsus institute collection (De Vries et al., 2019). *Pseudomonas aeruginosa* (DSM 50071) and *Pseudomonas extremorientalis* (DSM 15824) were purchased from the German Collection of Microorganisms and Cell Cultures GmbH (DSMZ, Germany). The bacteria were first grown on R2A agar (BD Difco, NJ, USA) plates at 30 °C over 72 h from frozen Viabank beads stock (Medical Wire, UK) that was stored at -80 °C. The plates were stored at 4 °C and were renewed every two weeks.

For preparation of bacterial cultures, one colony was used to inoculate 10 mL R2A liquid medium, grown overnight (18 h) at 30 °C under agitation (150 rpm). 0.5 mL of this pre-culture was used to inoculate 100 mL R2A broth (Dinkelberg Analytics GmbH, Germany), and the main culture was allowed to grow for 24 h. Bacteria were harvested by centrifugation (5000 g, 5 min, 10 °C) and washed three times with synthetic tap water (STW; 100 mg/L NaHCO₃, 13 mg/L MgSO₄·7 H₂O, 0.7 mg/L K₂HPO₄, 0.3 mg/L KH₂PO₄, 0.01 mg/L (NH₄)₂SO₄, 0.01 mg/L NaCl, 0.001 mg/L FeSO₄·7 H₂O, 1 mg/L NaNO₃, 27 mg/L CaSO₄, pH = 7.50 ± 0.05 (Gomes et al. 2019)) and sonicated on ice for 30 s at 30 W (Vibra Cell VCX130; Sonics and Materials Inc., CT, USA) to break bacterial aggregates. To prepare a bacterial suspension needed for the adhesion
experiments, bacteria were counted using a Bürker-Türk counting chamber (Marienfeld, Germany) and the bacterial suspension was diluted to $3 \times 10^8$ bacteria/mL in STW.

Parallel plate flow chamber experiments

Bacterial adhesion on nanogel-coated and non-coated transparent PVC was determined by triplicate experiments using a parallel plate flow chamber set-up (Figure S1) by flowing a bacterial suspension ($3 \times 10^8$ bacteria/mL) at room temperature for 4 h at a shear rate of 10 s$^{-1}$, as described previously (Busscher and Van der Mei, 2006). The parallel plate flow chamber was mounted on a phase-contrast microscope BH-2 (Olympus, Japan), equipped with a 40x long-distance objective (Olympus ULWD-CD Plan 40 PL) (Figure S2). Prior to the experiment, the system was filled with STW to remove air bubbles. Transparent PVC was used as an alternative for non-transparent pipe materials to allow real-time observation of bacterial adhesion. The transparent PVC slide with or without a nanogel coating was mounted as a bottom plate in the parallel-plate flow chamber from which live images were acquired. After 4 h, images were taken on ten different locations on each transparent PVC slide. To enhance the signal-to-noise ratio and to eradicate flowing bacteria from the analysis, live images were generated after summation of 15 consecutive images (1 s intervals). The adhered bacteria were counted and expressed per cm$^2$ using ImageJ Fiji software (https://imagej.net/software/fiji/) (Schindelin et al., 2012), automatically, or manually with the Cell Counter plugin, when the distinction of a single bacterium was not possible otherwise. As *Sphingomonas* Sph10 tends to adhere as aggregates, the number of aggregates was determined based on the images obtained after 4 h adhesion. A minimum of three bacterial cells clumped together was defined as an aggregate. Similar to single cell quantification, the number of Sph10 aggregates was evaluated after three independent assays, each time using images collected from ten different locations on a coated and non-coated surface. To compare the number of adhered bacteria on uncoated and nanogel-coated transparent PVC, Student’s $t$-test was used.

Results and discussion

Nanogels characterization

The synthesis of P(NIPMAM) nanogels was done by a precipitation polymerization method described earlier (Schmidt et al., 2010). To evaluate the nanogels stability in a
range of temperatures, their behaviour was investigated by DLS in water as presented in (Figure 1). Upon increasing the temperature, the nanogel hydrodynamic diameter ($d_h$) decreased, since the volume phase transition temperature of the P(NIPMAM) nanogels is around 44 °C. The measured $d_h$ of the nanogel at 30 °C was 572 ± 5 nm, which is comparable with previously reported values (Keskin et al., 2019). Synthesized nanogels exhibited spherical shapes with an average diameter of 340 ± 23 nm according to TEM results (Figure 1 B and C), which is lower than the diameter found by DLS analysis, since TEM is done in a dry state. Finally, the measured ζ-potential was -9.5 ± 0.1 mV, showing the net negative charge of the nanogel. Such negative charge of the nanogel particles potentially adds to the coating anti-adhesive performance, as negatively charged surfaces are known to repel bacteria through electrostatic repulsion, as bacterial cell walls are usually negatively charged themselves (Rzhepishevska et al., 2013).

Figure 1. (A) Hydrodynamic diameter ($d_h$) of nanogels as a function of the temperature. (B) and (C) Transmission electron microscopy (TEM) images of nanogels. Scale bars are 500 nm in (B) and 200 nm in (C).
Nanogel coating on polymeric pipe materials

Nanogel deposition was carried out on transparent PVC, and two brands of HDPE and PVC pipe materials, which were selected as surfaces of interest as they are a common selection for construction of new DWDS networks, due to their low price, light weight, and being non-corrosive (Wang et al., 2022). The polymeric surfaces were coated with P(NIPMAM) nanogel particles via spray-deposition and immobilization on a surface through electrostatic interactions with positively charged PEI as an anchoring polymer. Despite variations in topographies, AFM images confirmed that the nanogel coating was successful on all selected pipe materials, and nanogel particles were tightly packed on their surfaces (Figure 2), with only minor imperfections, where nanogel particles were missing (Figure S3).
Figure 2. Atomic force microscopy micrographs of transparent PVC and selected pipe materials non-coated (left column) and nanogel-coated (right column). Scale bars are 1 µm.
The thickness of the coating has been previously measured as the height of nanogel particles deposited on a surface by Keskin et al. (2019), with a result of 30 ± 4 nm in a dry state and 57 ± 8 nm in a hydrated state. Hydrophilicity of the coated and uncoated polymeric samples was assessed here by water contact angle measurement (Table 1). The water contact angles were similar on all uncoated samples and pointing to a hydrophobic surface (89 ± 6 degrees), while the presence of the nanogel coating increased the hydrophilicity of pipe materials, as evaluated after overnight hydration, resulting in an average decrease of 44 ± 5 degrees in the water contact angles. Hydrophilicity is directly connected to surface free energy of a substratum, which is an important indicator in predicting bacterial adhesion. The higher the surface free energy, the more hydrophilic the surface will be, which at the molecular level will increase energetical demand for an adhering bacterium to replace water molecules bound with that surface (Carniello et al., 2018). Therefore, theoretically, the more hydrophilic the surface, the more energetically unfavourable the conditions tend to be for a bacterium to adhere. Moreover, in aqueous solutions, the nanogel coating absorbs and immobilizes water molecules, creating a hydration layer that weakens the interactions between bacterial cell wall and substratum, limiting the transition from reversible to irreversible adhesion, and thereby hindering biofilm formation (Chen et al., 2010; Carniello et al., 2018; Liu et al., 2021). Generally, the presence of the nanogel coating creates not only an energetical, but also a physical barrier between bacterial cells and the surface. Another important surface feature in terms of supporting biofilm formation is surface roughness. Rougher surfaces provide increased surface area available for bacterial adhesion and bacteria on such surfaces are shielded from the shear stresses and turbulences of bulk flow, which makes them more resistant to detachment (Cowle et al., 2014). In this study, the average surface roughness $R_A$ was measured using AFM. The selected pipe materials varied significantly, with transparent PVC ($R_{A\,D\,Y} = 4 \pm 1$ nm), HDPE 1 ($16 \pm 13$ nm) and PVC 2 ($14 \pm 4$ nm) being smoother, and HDPE 2 ($32 \pm 10$ nm) and PVC 1 ($61 \pm 33$ nm) rougher (Table 2). The presence of the nanogel coating had no significant influence ($p > 0.05$) on pipe materials roughness, neither in a dry nor a wet state, potentially due to its thin film nature.

The long-term stability of the coatings on HDPE 1, HDPE 2, PVC 1 and PVC 2 was evaluated after 30 days immersion in sterilized tap water by AFM analysis, which confirmed that the coating remained on all the surfaces, as shown in Figure S4. Additionally, the water in which the coupons were immersed did not show any significant ($p >$
0.05) increase in organic carbon concentrations (Figure S5). While 30 days are not yet representative of the real operational periods of DWDS pipework, as water distribution pipework is installed with a purpose to serve for decades (Renaud et al., 2014), it provides an indication that the proposed protocol produces a coating, which is structurally and chemically stable, and remains on the surface for an extended period of time. Moreover, the conditions that have shown a negative effect on the nanogels stability, such as high temperature (above 45 °C), high salt concentration, and low pH (below 2.3) (Farooqi et al., 2017), are far from the average DWDS conditions. Temperatures in DWDS usually do not exceed 25 °C (Blokker and Pieterse-Quirijns, 2013), and drinking water is of neutral pH and low salt content, making nanogel coatings suitable for potential long-term applications.
Table 1. Water contact angles of pipe materials without and with the nanogel coating.

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<th>Transparent PVC</th>
<th>HDPE-1</th>
<th>HDPE-2</th>
<th>PVC-1</th>
<th>PVC-2</th>
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<tr>
<td>Non-coated</td>
<td>84±1°</td>
<td>97±1°</td>
<td>91±1°</td>
<td>83±2°</td>
<td>91±1°</td>
</tr>
<tr>
<td>Nanogel-coated</td>
<td>41±1°</td>
<td>55±5°</td>
<td>54±1°</td>
<td>38±1°</td>
<td>40±1°</td>
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Table 2. Average roughness ($R_A$) of pipe materials without and with the nanogel coating in a hydrated ($R_A\text{ WET}$) and a dry ($R_A\text{ DRY}$) state.

<table>
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<tr>
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<th>Transparent PVC</th>
<th>HDPE-1</th>
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<th>PVC-1</th>
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<td>$R_{A\text{ DRY}}$</td>
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<tr>
<td>Non-coated</td>
<td>4±1</td>
<td>4±1</td>
<td>16±13</td>
<td>22±8</td>
<td>41±5</td>
</tr>
<tr>
<td>Nanogel-coated</td>
<td>9±2</td>
<td>10±2</td>
<td>16±10</td>
<td>13±7</td>
<td>44±6</td>
</tr>
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</table>
Bacterial adhesion on nanogel-coated and non-coated transparent PVC

The effectiveness of nanogel coatings in reducing bacterial adhesion on non-coated and nanogel-coated transparent PVC in a parallel-plate flow chamber was evaluated after 4 h for *Sphingomonas* Sph5 and Sph10, *P. extremorientalis* and *P. aeruginosa*. The bacterial strains were selected as representative genera for the initial adhesion tests since they are commonly found in DWDS biofilm communities (Hong et al., 2010; Douterelo et al., 2014; Liu et al., 2017a; Chan et al., 2019). *Sphingomonas* spp. have been identified as primary surface colonizers and dominating species in many fouling phenomena, including those on spiral wound membranes (Bereschenko et al., 2010), and in membrane bioreactors (Huang et al., 2008). Moreover, *Sphingomonas* spp. are able to persist in oligotrophic environments (Vancanneyt et al., 2001; Ohta et al., 2004), and to resist chlorine (Sun et al., 2013), which make them suitable candidates to proliferate in DWDS conditions. Presence and potential growth of *P. aeruginosa*, an opportunistic pathogen, is of special concern in DWDS (Bressler et al., 2009). *P. extremorientalis* was selected as a representative *Pseudomonas* specie since it was first isolated from a water reservoir (Ivanova et al., 2002).

The number of bacteria adhering on the nanogel-coated transparent PVC decreased compared to non-coated PVC (Figure 3 A). The presence of the coating significantly (*p* < 0.005) reduced the number of adhered bacteria per unit area by approximately 1 to 1.5 log, namely by 88% ± 4%, 91% ± 3%, 97% ± 1% and 97% ±1%, for Sph5, Sph10, *P. extremorientalis* and *P. aeruginosa*, respectively (Figure 3 B), confirming the effectiveness of the coating in changing the surface properties. After 4 h, *Pseudomonas* strains adhered to the non-coated transparent PVC surface in significantly higher numbers than *Sphingomonas* strains (*p* < 0.05) (Figure 3 B).
Figure 3. (A) Phase contrast microscope images of bacteria adhered to the surface of non-coated transparent PVC (top row) and nanogel-coated transparent PVC (bottom row) in a parallel-plate flow chamber after 4 h. Scale bars are 20 µm. (B) Number of adhered bacteria in the parallel-plate flow chamber after 4 h on transparent PVC and nanogel-coated transparent PVC. The values are averages of experiments performed on three separately coated surfaces with separately prepared bacterial cultures indicated by the markers.
The same P(NIPMAM) nanogel coating also reduced adhesion of clinically relevant bacteria of *Staphylococcus aureus* by up to 99% in comparison to non-coated glass (Keskin et al., 2019). The lower efficiency against *Sphingomonas* and *Pseudomonas* adhesion assessed in this study could possibly be a result of morphological outer layer differences between the used strains and *S. aureus*. Both *Sphingomonas* and *Pseudomonas* possess surface appendages (as also showed by SEM analysis, Figure S6), such as flagella, which is important in DWDS context, since motility is essential for nutrients scavenging in oligotrophic conditions (Du et al., 2020). Flagellum and other cell appendages (e.g., pili) can also serve as tethers piercing the energy barrier (Wang et al., 2011) and facilitate adhesion to those limited adhesion sites between the deposited nanogel particles or spaces formed due to occasional coating imperfections (Figure S4). In comparison, *S. aureus*, as a non-flagellated bacterium (Pollitt et al., 2015), solely depends on adhesion sites that are big enough to support the cell body attachment.

It is worth to notice that, despite representing the same genus, *Sphingomonas* Sph5 and Sph10 exhibited different adhesion behaviours. On non-coated transparent PVC, Sph5 is adhering uniformly as single cells, and Sph10 as pre-formed aggregates that spontaneously formed in liquid phase (Figure 4 A). This behaviour is in agreement with the initial characterization of the isolate provided by De Vries and colleagues (2019), where Sph10 showed auto-aggregation of almost 100% of the cells in a liquid culture after 24 h. The nanogel coating significantly (\( p < 0.05 \)) reduced the number of adhered Sph10 aggregates by 97\% ± 2\%, compared to bare transparent PVC (Figure 4 B). Moreover, the aggregates detected on coated surfaces consisted of no more than four bacterial cells, while those attached on non-coated transparent PVC were multicellular clusters in sizes of up to 30 μm in diameter (Figure 4 A). This might indicate that while the coating might provide occasional adhesion sites to support (tethered) attachment of single cells, as observed for Sph5 and *Pseudomonas* strains, these sites are not big enough to hold big multicellular clusters under the applied flow conditions. It is important in the context of the discussed future coating application, as microbial aggregates play a considerable role in initial biofilm development stages in natural environments. Indeed, their adhesion can initiate biofilm formation in a more rapid way than adhesion of single cells (Kragh et al., 2016).
Aspects of nanogel coatings as an alternative biofilm control strategy for DWDS

The purpose of this study was to introduce the concept of P(NIPMAM) nanogel coatings application as an alternative control strategy for biofilm formation and regrowth in water distribution and storage systems via limiting the bacterial adhesion. As presented, the nanogel coating showed promising results in limiting bacterial adhesion and biofilm formation and appears to be a versatile solution to be applied on various surfaces utilized in
DWDS. Moreover, in line with the proposed application area, nanogel-based coatings present numerous advantages. As being soft deformable colloidal nanoparticles, nanogels can adhere to the surface and create a homogeneous thin coating. Compared to other chemical coating procedures that require grafting or covalent binding (Schmidt et al., 2010), nanogel coating deposition presented in this work depends on physical adsorption, and nanogels can be easily applied to a surface by spraying. Spray-deposition creates a considerable advantage in the manufacturing process, especially when applied on oddly shaped substrata, such as inner surface of a pipe. The polyacrylamides used to synthetize the nanogels are generally considered non-toxic/non-hazardous (Andersen, 2005), they are commonly selected for biomedical applications, due to their biocompatibility (Naha et al., 2010; Guo et al., 2017; Capella et al., 2019), and are also utilized as a coagulating agent in water treatment (Ma et al., 2019; Long et al., 2020). Also, if DWDS sections are considered to be the future coating target, despite significantly larger scale and surface area to coat, the nanogel coating shows potential to be effective as a thin monolayer, which volume-wise requires only little amounts of nanogel particles per surface unit. For instance, to coat 100 m of a 110 mm diameter pipe, only 1.1 g of P(NIPMAM) nanogels would be needed. Considering that residual concentrations of acrylamide in its polymerized form range from <0.01 to 0.1% (Andersen, 2005) and that big volumes of water are distributed daily, any potential sudden or gradual erosion of the coating components will not result in acrylamide concentration exceeding the guideline value of 0.1 µg/L (Directive 2020/2184). Together with high efficiency in limiting bacterial adhesion, P(NIPMAM) nanogel coatings present an interesting biofilm control strategy for DWDS, with a potential focus on biofilm hotspots characterized by low hydrodynamic stress, long stagnation periods and higher nutrients concentrations.
Conclusions

Until now, P(NIPMAM) nanogel coatings potential to limit bacterial adhesion has been investigated in the biomedical field, where they are proposed as an anti-biofilm strategy for in-body devices and have been tested against adhesion of medically relevant strains, such as *S. aureus*. This study showed that the nanogel coating has a much broader application, and it can be successfully applied to a variety of polymeric pipe materials, such as PVC or HDPE, that are commonly used for the construction of new DWDS pipelines. Obtained results show promise for the potential application of P(NIPMAM) nanogel coating against adhesion of drinking water bacteria. However, translation of the technology from biomedical application to DWDS circumstances will require further investigation, with a focus on long term stability, especially in DWDS hydraulic conditions, robustness, antifouling efficiency against complex, multi-species microbial communities, and any potential long term exposure health implications.
Figure S1. Parallel-plate flow chamber set-up.

Figure S2. Schematic representation of the parallel-plate flow chamber.
**Figure S3.** Atomic force microscopy images of occasional imperfections on the nanogel coated surface of transparent PVC, at different magnification. Bottom of the gradient bar is 0 nm.

**Figure S4.** Atomic force microscopy images of P(NIPMAM) nanogel coating on (A) HDPE 1 and (B) HDPE 2, (C) PVC 1 and (D) PVC 2 after 30 days exposure to drinking water under agitation. The scale bars are 1 µm.
**Figure S5.** Organic carbon concentrations measured in water after 30 days in contact with DWDS materials with and without nanogel coating in sterilized tap water.

**Figure S6.** Scanning electron microscopy images of (A) *Sphingomonas Sph5*, (B) *Sphingomonas Sph10*, (C) *Pseudomonas aeruginosa* and (D) *Pseudomonas extremorientalis* attached on the surface of HDPE 1 after 24 h incubation in R2A medium. The arrows indicate surface appendages involved in adhesion to the surface. Scale bars are 1 µm.