**Extracellular DNA in adhesion and biofilm formation of four environmental isolates: a quantitative study**

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**Abstract**

The importance of extracellular DNA (eDNA) in biofilm formation has become increasingly clear from research on clinically relevant bacteria. This study aimed to determine whether the quantity of eDNA produced can be linked to the ability to form biofilm. We systematically quantified eDNA over time during planktonic growth and biofilm formation in *Reinheimera* sp. F8 and three other environmental isolates belonging to the genera *Pseudomonas*, *Microbacterium* and *Serratia*. eDNA in biofilms was visualised by fluorescence microscopy and quantified by PicoGreen® labelling without further sample preparation, whereas eDNA in planktonic cultures was precipitated before labelling and quantification. The effect of eDNA removal was investigated by DNase treatment. eDNA appeared in the early exponential growth phase of planktonic batch cultures and the concentration peaked in the stationary phase. The concentration in biofilms differed substantially between strains and over time during biofilm development. eDNA was important for the initial attachment in all strains, and DNase treatment reduced biofilm formation in three of four strains. The extent to which eDNA accumulated in planktonic cultures or biofilms did not reflect its significance to biofilm formation, and even very low concentrations of eDNA affected biofilm formation strongly. The significance of eDNA for biofilm formation in nature may thus be more widespread than previously anticipated.

**Introduction**

Most bacteria in natural environments exist within biofilms where cells are encased in an extracellular matrix of biopolymers (Costerton et al., 1995). Extracellular DNA (eDNA) has been observed to be abundant in the biofilm matrix (Flemming et al., 2007) and was originally assumed to be a product of cell lysis with no apparent function besides carrying genetic information. Later studies showed that eDNA can have many functions. In 1998, it was discovered that eDNA promotes floc stability in the marine isolate *Rhodovulum* sp. (Watanabe et al., 1998), but this discovery did not receive much attention. However, because eDNA was reported to be important for the structural stability of young *Pseudomonas aeruginosa* biofilm in 2002 (Whitchurch et al., 2002), investigations into the role of eDNA has established that eDNA can stabilize biofilm structures (Izano et al., 2008; Thomas et al., 2008, 2009; Harmsen et al., 2010), promote bacterial attachment (Qin et al., 2007; Heijstra et al., 2009; Das et al., 2010) and have antimicrobial properties by chelating cations (Mulcahy et al., 2008).

Several studies have shown that eDNA accumulates in the late exponential growth phase of planktonic batch cultures (Petersen et al., 2005; Allesen-Holm et al., 2006). However, the timing and quantity of eDNA production during biofilm formation has not been systematically investigated despite its importance for the biofilm stability.

The role of eDNA in biofilm formation has mostly been studied in clinically relevant strains, while studies of eDNA in environmental isolates are sparse and count only a few examples from wastewater treatment plants (Heijstra et al., 2009; Dominiak et al., 2011), soil (Vilain...
et al., 2009) and freshwater (Böckelmann et al., 2006). In this study, we seek to determine whether there is a common pattern for eDNA accumulation during biofilm formation among different environmental isolates. The interaction of eDNA with abiotic surfaces is governed by electrostatic (Heijstra et al., 2009) or acid–base interactions (Das et al., 2010, 2011), and eDNA is expected to be most adhesive at low salinity. We therefore isolated bacteria from biofilms in a freshwater stream and used these as model organisms in comparison with the previously described environmental isolate ‘El’ (Böckelmann et al., 2006), a member of the genus Reinheimera (Merchant et al., 2007). We compared the four environmental isolates and quantified eDNA production and biofilm formation over time to investigate eDNA’s role through the process of biofilm formation from initial attachment to biofilm maturation. The aim was to address the following basic questions: (1) is there a common pattern for when in biofilm formation the presence of eDNA is most important? and (2) can the amount of eDNA produced be linked to a particular strain’s ability to form biofilm?

### Material and methods

#### Selection and identification of environmental isolates

Environmental isolates were obtained from a small iron-rich freshwater stream (Støvlebæk, Denmark) at water temperature of 9–11 °C, pH 5.5, and nitrate concentration of 90–108 μM. A biofilm sampler equipped with eight glass slides was submerged into the stream for 52 h. Bacteria attached to glass slides were scraped off with plastic or cotton sticks and streaked onto agar plates, or transferred to the agar plates by gently pressing the slide against the agar plate. The nutrient agar was composed of oligotrophic freshwater medium (FBM) (Böckelmann et al., 2000) (3.0 g L⁻¹ Na₂SO₄, 1.2 g L⁻¹ NaCl, 0.3 g L⁻¹ NH₄Cl, 0.1 g L⁻¹ yeast extract, 0.1 g L⁻¹ glucose, 0.15 g L⁻¹ CaCl₂·2H₂O, 0.4 g L⁻¹ MgCl₂·6H₂O, trace elements, vitamin solutions, 15 g L⁻¹ agar) or R2A medium (0.5 g L⁻¹ yeast extract, 0.5 g L⁻¹ trypticase® peptone, 0.5 g L⁻¹ casamino acids, 0.5 g L⁻¹ glucose, 0.5 g L⁻¹ soluble starch, 0.3 g L⁻¹ sodium pyruvate, 0.3 g L⁻¹ K₂HPO₄, 0.05 g L⁻¹ MgSO₄·7H₂O, 15 g L⁻¹ agar, pH 7.4).

Bacterial colonies were grown at room temperature and screened for eDNA production. Part of the colony was picked with a sterile toothpick and transferred to a glass slide, where cells were stained directly by addition of 5 μL diluted stain (1 μL SybrGreen I (Molecular Probes) in 1000 μL phosphate-buffered saline (PBS, 1.28 g L⁻¹ NaH₂PO₄·2 H₂O, 0.39 g L⁻¹ Na₂HPO₄·1 H₂O, 7.60 g L⁻¹ NaCl, pH 7.4) or 0.055 mM propidium iodide (3 μL mL⁻¹ component B from Live/dead BacLight L7007, Invitrogen), covered by a coverslip and incubated in the dark for 10 min before epifluorescence microscopy (Zeiss Axiocam 200M). Colonies where eDNA production could be observed were picked and streaked repeatedly onto new agar plates for isolation, and three of these were selected for further analyses based on their ability to form biofilm in microtitre plates (following the procedure described below).

The 16S rRNA genes of the selected isolates were amplified directly from bacterial colonies by PCR (15 min hot start at 96 °C, 23 cycles, 52 °C annealing temperature) using primers GM3F (5’AGA GTT TGA TCM TGG C 3’, positions 8–24) and GM4R (5’TAC CTT GTT ACG ACT T 3’, positions 1492–1507) (Muyzer et al., 1993), PCRs contained 2.5 units HotStarTaq polymerase (Qiagen), 1.5 mM MgCl₂, 0.2 mM each dNTP and 0.2 μM of each primer. Full-length sequences were analysed phylogenetically in ARB (Ludwig et al., 2004), while their closest relatives were found with the basic local alignment search tool (BLAST) on the National Center for Biotechnology Information (NCBI) homepage.

#### eDNA production in planktonic cultures

Bacterial strains were grown in 1-L conical bottles in 400 mL R2A medium at 28 °C with shaking (120 r.p.m.), and the biomass growth and eDNA production were followed over time. Cell density was estimated as the OD₅₀₀ₙₐₚₐ (Milton Roy Spectronic 601), and eDNA in the supernatant was quantified after precipitation: Bacterial cells were removed from 900 μL culture by centrifugation (4 min, 6800 g), and the supernatant (700 μL) was transferred to a sterile Eppendorf tube and mixed with 50 μL protein precipitation solution (Q-Biogene) by inverting (10 times) before centrifugation (10 min, 12 100 g, Minispin, Eppendorf). Then, 700 μL of the supernatant was mixed with 70 μL 2.5 M NaCl and 1400 μL 96% ethanol (62% final concentration) before storage at −20 °C for at least 24 h. DNA was precipitated by centrifugation (25 min, 4 °C, 23 500 g, Centrifuge 5417R, Eppendorf) and washed once in 70% ice-cold ethanol. The pellet was dried < 3 min at 43 °C before resuspension in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5) by vortexing for 25 s.

The eDNA concentration was determined by Pico-Green® fluorescent staining (Quant-it™ Invitrogen™ Molecular Probes®). A freshly prepared solution (1 μL PicoGreen® dye diluted in 199 μL TE buffer) was mixed with the precipitated DNA in the ratio 1 : 1 (2.5 μL dye mixture and 2.5 μL sample), and eDNA concentration in 2 μL was measured on a NanoDrop 3300 Fluorospectrometer (Thermo Scientific), using 470 nm excitation.
and 525 nm detection. The growth experiment was carried out with one culture for each organism, and the eDNA concentration was determined in three independent samples for each time point. To verify reproducibility between biological replicates, the experiment was also repeated with triplicate cultures for *Reinheimera* sp. F8 and *Microbacterium* sp. FW3. In the repeated experiments, the eDNA concentration was measured in clear LightCycler 480 96-well plates on a LightCycler® 480II (Roche), using 465 nm excitation and detecting emission at 510 nm.

eDNA production in the planktonic cultures was also inspected visually by fluorescence microscopy (Zeiss Axiovert 200M) after staining with propidium iodide (0.055 mM, Component B from BacLight Live/dead staining kit) during all experiments.

**Quantification of biofilm growth and eDNA production in biofilms**

Biofilm formation was quantified in 96-well microtitre plates designed for microscopy (Colstar®). Starter cultures were grown for 12 h (*Reinheimera* sp. F8, *Serratia* sp. FW2 and *Pseudomonas* sp. FW1) or 24 h (*Microbacterium* sp. FW3) in 5 mL R2A medium in test tubes incubated at 28 °C. Microtitre plates were inoculated with diluted starter cultures adjusted to a final OD600 nm of 0.01 in 200 μL R2A medium, and incubated at room temperature with shaking (80 r.p.m.). OD600 nm was measured at regular intervals with a Powerwave XS2 plate reader (BioTek). The amount of biofilm and the eDNA associated with the biofilm was quantified after 2, 4, 6, 12, 18 and 30 h. Each analysis was performed on three biological replicates for each strain, and at each time point, 5 (for eDNA) or 3 (for biomass) technical replicates were analysed for each of the three biological replicates. The experimental set-up thus included 15 wells for eDNA quantification, six wells for microscopy and nine wells for biomass quantification for each strain at each time point.

For all determinations, the supernatant was initially removed, and the biofilm was rinsed twice in PBS. The wells for quantification of biofilm biomass was left to dry for 1–2 h at room temperature (the biomass quantification was not significantly influenced by desiccation), while the remaining wells were used for immediate eDNA quantification. eDNA was quantified by addition of 100 μL TE buffer followed by 100 μL freshly made PicoGreen® solution (1 μL PicoGreen® dye in 199 μL TE buffer). The TE buffer and the PicoGreen® solution were mixed by pipetting 100 μL up and down 10 times. Wells with PicoGreen® were incubated for 2–4 min before measuring the fluorescence intensity (excitation 485 nm/emission 535 nm, 0.1 s) using a fluorescence plate reader (Wallace Victor 3, 1420 Multicolor, PerkinElmer). Lambda DNA (Invitrogen™ Molecular Probes®) was used to generate a standard curve for each run. For each time point, the biofilm was stained with PicoGreen® and observed by phase contrast and fluorescence microscopy (Zeiss Axiovert 200M). eDNA was observed as a cloud surrounding the cells or as strings in between microcolonies, and microscopy thus confirmed that PicoGreen® did not bind to intracellular DNA.

The biofilm biomass was quantified with a crystal violet staining assay modified from O’Toole & Kolter (1998). The supernatant was removed, and the biofilm was washed twice in 200 μL sterile PBS. A filter-sterilized (pore size, 0.2 μm) crystal violet solution (0.1 g mL⁻¹ in 96% ethanol mixed 1 : 4 with 1% (aq) ammonium oxalate) was added (200 μL to each well) and incubated for 12 min before rinsing the wells three times in 300 μL sterile distilled water. The remaining crystal violet was dissolved in 200 μL 96% ethanol, and the optical density at 585 nm was measured (Powerwave XS2 plate reader, BioTek).

**eDNA’s role in attachment and biofilm formation**

eDNA’s role in initial bacterial attachment and the subsequent biofilm formation was investigated by removing eDNA by DNase treatment and quantifying initial cell attachment or changes in biomass of the biofilms. Initial attachment of bacteria to glass surfaces was studied by circulating bacterial cultures in flowcells (DTU Biocentrum, Kgs. Lyngby, Denmark) followed by quantification of attached cells by microscopy. Glass coverslips were cleaned by sonication (30 min) in 95% acetone before mounting on the flowcells. After assembly, the flowcell set-up was sterilized with 0.5% hypochlorite for 2 h and rinsed with sterile distilled water overnight. The bacterial cultures were grown overnight in 30 mL R2A in 125-mL conical bottles at 25 °C with shaking (120 r.p.m.). The effect of DNase on attachment was studied both in R2A medium and PBS. The bacterial culture was diluted to OD600 nm = 0.05 in sterile R2A, or harvested by centrifugation [10 min at 5050 g (Sorvall® RC-5C plus)], washed twice in PBS, and resuspended to OD600 nm = 0.05 in PBS. Each suspension was divided into two 50-mL Falcon tubes (25 mL each), and DNase was added (90 Kunitz mL⁻¹, deoxyribonuclease I from bovine pancreas, Sigma) to one of the tubes. The samples were incubated at room temperature for 30 min to allow the DNase to degrade eDNA before starting the experiment. Each bacterial suspension was then circulated through three channels in a flowcell mounted on a Zeiss Axiovert 200M inverted microscope. Bacterial attachment was quantified after 30 and 90 min by phase contrast microscopy. More than 1000 cells were counted in most experiments, but...
some samples had so few cells attached that only 200 bacteria could be counted.

Biofilms were grown and quantified in microtitre plates as described above. The influence of eDNA on biofilm formation was investigated by adding DNase (90 kunitz mL\(^{-1}\)) at the same time as inoculating the wells. Biofilms were then quantified after 4, 6 or 12 h. The importance of eDNA for the stability of already formed biofilm was tested by 1-h DNase treatment after 4, 6 or 12 h of biofilm growth. At each time point, the bacterial culture above the biofilm was carefully removed from 11 wells by aspiration. Three wells were left to dry, while 200 µL sterile R2A medium was added to eight wells: Four wells with and four wells without DNase 1 (90 kunitz mL\(^{-1}\)). The dry wells were included to check whether the biofilm incubated in R2A medium grew during the experiment. After 1-h incubation, OD\textsubscript{600 nm} was measured and biofilm biomass was quantified by crystal violet staining as described above.

**Results**

**Selection and identification of bacterial isolates from freshwater**

A total of 110 colonies obtained from biofilms grown in a freshwater stream were screened for eDNA production by fluorescence microscopy, and 25 colonies contained large amounts of eDNA. These strains were isolated, and biofilm formation was observed in 19 of the 25 isolates. Three biofilm-forming, eDNA-producing isolates were selected for further experiments. Based on 16S rRNA gene sequence similarities of > 99% to their closest cultured relatives, they were provisionally termed *Pseudomonas* sp. FW1 (GenBank no. JN674082), *Serratia* sp. FW2 (GenBank no. JN674080) and *Microbacterium* sp. FW3 (GenBank no. JN674081). *Pseudomonas* sp. FW1 clustered within the group of *Pseudomonas fluureescens*, and the closest relative has been isolated from river water (GenBank no. DQ207731.2). *Serratia* sp. FW2 was related to *Serratia fonticula*, and the closest relatives were found in lake water (GenBank no. AY689057.1) and drinking water (GenBank no. AY236502.1). The closest relative of *Microbacterium* sp. FW3 has been isolated from a rice field (GenBank no. GQ369012.1). The three freshwater isolates were compared with the previously described environmental isolate F8, which produces large amounts of eDNA (Böckelmann et al., 2006, 2007). After F8 was reported, a new isolate, *Reinheimera texanensis* sp. nov. (Gammaproteobacteria) with 98% 16S rRNA gene sequence similarity to F8 has been characterized (Merchant et al., 2007), and F8 will therefore be referred as *Reinheimera* sp. F8 in the following.

**eDNA accumulates in the late exponential and death phase of batch cultures**

The concentration of eDNA in the supernatant of planktonic batch cultures was followed over time. eDNA was detected in all samples, starting at concentrations of 0.015 µg mL\(^{-1}\) in the beginning of the exponential growth phase. The concentration increased in the late exponential growth phase and peaked during the stationary and death phase (Fig. 1). The maximum concentration of eDNA varied substantially among the isolates; 33 µg mL\(^{-1}\) in *Reinheimera* sp. F8, 15 µg mL\(^{-1}\) in *Pseudomonas* sp. FW1, 1.5 µg mL\(^{-1}\) *Microbacterium* sp. FW3 and 0.07 µg mL\(^{-1}\) in *Serratia* sp. FW2 (Fig. 1). It should be noted that propidium iodide staining of the pellet after centrifugation showed that eDNA was present in the cell pellet of *Reinheimera* sp. F8, leading to underestimation of the total eDNA concentration in the culture. In the other cultures, eDNA could not be visualized in the pellet after centrifugation. Repetition of the experiment with biological triplicates of *Reinheimera* sp. F8 and *Microbacterium* sp. FW3 cultures demonstrated high reproducibility (see Supporting Information, Fig. S1).

Fluorescence microscopy with propidium iodide staining of eDNA in batch cultures showed that cells of *Pseudomonas* sp. FW1 (Fig. 2a), *Reinheimera* sp. F8 (Fig. 2b) and *Microbacterium* sp. FW3 were often surrounded by a cloud of eDNA, whereas hardly any eDNA was observed in *Serratia* sp. FW2. These observations correspond with the detection of eDNA in the supernatants. The filamentous network of eDNA previously described for *Reinheimera* sp. F8 (Böckelmann et al., 2006) was only transiently present in the late exponential growth stage (Fig. 2a). At later stages, eDNA comprised so much of the culture that it appeared as an intense background fluorescence without any visible structures. In *Pseudomonas* sp. FW1, filamentous eDNA structures appeared in the exponential growth phase (Fig. 2b) and became abundant after 50 h when the eDNA concentration in the supernatant increased dramatically. No filamentous structures were seen in *Microbacterium* sp. FW3 or *Serratia* sp. FW2 cultures.

**There is no consistent pattern of eDNA production during biofilm formation**

The quantity of eDNA in biofilms was studied with a novel procedure using PicoGreen®. When the fluorescence intensity was measured immediately after adding the stain, PicoGreen® had not yet penetrated the cells and only bound to eDNA. An example of the appearance of PicoGreen®-stained eDNA in the biofilms is shown in the matching phase contrast and fluorescence images of 12-h-old biofilms (Fig. 3). In most biofilms, eDNA...
appeared as a fuzzy cloud around some, but not all, cells in the biofilm. Only in *Serratia* sp. FW2, the stained cells were very bright, indicating that PicoGreen®/C226 had penetrated the cells. However, only very few cells in the biofilm were stained, and these were probably dead cells which allowed the stain to penetrate easily.

The timing and quantity of eDNA production varied greatly among the isolates, and the eDNA concentration in the biofilm did not correlate with the amount of biofilm formed. *Reinheimera* sp. F8 and *Pseudomonas* sp. FW1 both produced c. 100 ng eDNA per well, and while *Pseudomonas* sp. FW1 formed a thick biofilm, *Reinheimera* sp. F8 only formed little biofilm, which even dispersed after 12-h incubation (Fig. 4). Like *Pseudomonas* sp. FW1, *Serratia* sp. FW2 and *Microbacterium* sp. FW3 were both strong biofilm formers, although they accumulated much less eDNA. *Serratia* sp. FW2 produced c. 1/10 and *Microbacterium* sp. FW3 only 1/100 of the eDNA concentration measured in *Pseudomonas* sp. FW1 biofilms (Fig. 4). The concentration of eDNA increased over time as the biofilm developed in all isolates, except in *Serratia* sp. FW2, which showed a transient peak of eDNA after 6-h incubation. If the quantity of eDNA is normalized to the amount of biofilm, the amount of eDNA relative to the biofilm biomass increased...
with time in *Rheinheimera* sp. F8, decreased over time in *Pseudomonas* sp. FW biofilms, and showed a transient peak at 6-h incubation in *Serratia* sp. FW biofilms. No trend was observed for the amount of eDNA relative to the biomass of *Microbacterium* sp. FW3 biofilms (Fig. S2).

**eDNA removal affected initial attachment but had little impact on biofilm formation**

The influence of eDNA on initial attachment was investigated by running a bacterial suspension through flowcells in the presence or absence of DNase. The cells were either suspended in PBS or in R2A medium to test attachment under controlled conditions that did not permit cell growth (PBS), but also under nutrient-rich conditions (R2A medium) that allowed cell growth and facilitated formation of a conditioning layer on the glass surface prior to attachment.

eDNA played a highly significant role for initial attachment of all isolates in both PBS (t-test, $n = 3$, $P < 0.005$) and in R2A medium (t-test, $n = 3$, $P < 0.02$) (Fig. 5). For *Pseudomonas* sp. FW1, *Serratia* sp. FW2 and *Reinheimera* sp. F8, the effect of DNase treatment was not as strong in R2A medium as it was in PBS. Because the number of adhered cells increases over time during the incubation, we included two time points (30 and 90 min) in the experiment. However, microcolony formation after 90-

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**Fig. 3.** Biofilms visualised after 12-h incubation in microtitre plates. Cells are shown by phase contrast microscopy (left) and eDNA by PicoGreen® staining and fluorescence microscopy (right). The cultures are *Reinheimera* sp. F8 (a,b), *Pseudomonas* sp. FW1 (c,d), *Microbacterium* sp. FW3 (e,f) and *Serratia* sp. FW2 (g,h). (scale bar = 10 μm).
Fig. 4. Biomass (light grey bars) and eDNA (dark grey bars) quantified during biofilm formation in microtitre plates (n = 3). Biomass was quantified by crystal violet staining, and eDNA was quantified by PicoGreen® staining.

Fig. 5. Initial adhesion of bacteria to glass surfaces (n = 3) in the presence (light grey bars) and absence (dark grey bars) of eDNA. Cell suspensions were circulated in flowcells, and adhesion was quantified after 30 min in R2A media, or after 30 and 90 min in PBS. Asterisk indicates statistically significant effect of DNase treatment (t-test, P < 0.05).
eDNA has previously been shown to affect biofilm formation in numerous bacterial strains across several phyla, but can it be assumed that strains that produce large amounts of eDNA are also excellent biofilm formers? We investigated whether a general pattern of eDNA production and its role in biofilm formation exists by systematically quantifying eDNA production during planktonic growth and biofilm formation in four environmental isolates from freshwater.

We used a fast and simple method to quantify eDNA in intact biofilms by PicoGreen® staining followed by quantification of the fluorescence intensity. Previous studies have extracted eDNA from biofilms before quantification (Steinberger & Holden, 2004; Wu & Xi, 2009). The extraction efficiency is, however, strain dependent (Wu & Xi, 2009), and in situ quantification by fluorescence labeling is therefore better suited for comparing eDNA production by different strains. In situ eDNA quantification based on fluorescence labelling was previously carried out by Allesen-Holm et al. (2006) and Dominiak et al. (2011) using propidium iodide and DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one), respectively. DDAO and propidium iodide both penetrate and label intracellular DNA in dead cells, resulting in potential overestimation of eDNA within the biofilm. PicoGreen® only penetrated very few cells in the biofilms at the different development stages, and we therefore chose PicoGreen® as a good candidate for quantification of eDNA in biofilms directly in microtitre plates.

To determine whether the detected eDNA played a role for the formation and the stability of biofilms, DNase was added during biofilm formation in microtitre plates. DNase was either present from the beginning of the incubation or added after the biofilm had been allowed to establish. Due to the difference in growth rates, Pseudomonas sp. FW1 and Serratia sp. FW2 biofilms were analysed after 6-h incubation, while Rheinheimera sp. F8 and Microbacterium sp. FW3 were analysed after 12 h (Fig. 6). The presence of DNase did not prevent biofilm formation, but caused a modest but significant reduction in the amount of biofilm formed by Serratia sp. FW2, Microbacterium sp. FW3 and Rheinheimera sp. F8 (t-test, n = 3, P < 0.05). Despite the large amount of eDNA in Pseudomonas sp. FW1 biofilms, suggesting that eDNA had an important function in the extracellular matrix, these biofilms were unaffected by DNase treatment.

DNase had even less effect on already established biofilms. Only Microbacterium sp. FW3 was significantly affected by DNase treatment (t-test, n = 3, P < 0.05). When established biofilms were treated with DNase, we exchanged the liquid phase with fresh R2A medium to focus the DNase activity on eDNA in the biofilm and not in the overlying planktonic culture. The exchange of the liquid phase did induce some biofilm dispersal in Rheinheimera sp. F8, Pseudomonas sp. FW1 and Microbacterium sp. FW3 (data not shown).

**Discussion**

In situ eDNA quantification by fluorescence labelling is therefore better suited for comparing eDNA production by different strains. In situ eDNA quantification based on fluorescence labelling was previously carried out by Allesen-Holm et al. (2006) and Dominiak et al. (2011) using propidium iodide and DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one), respectively. DDAO and propidium iodide both penetrate and label intracellular DNA in dead cells, resulting in potential overestimation of eDNA within the biofilm. PicoGreen® only penetrated very few cells in the biofilms at the different development stages, and we therefore chose PicoGreen® as a good candidate for quantification of eDNA in biofilms directly in microtitre plates.
eDNA was present at all times during planktonic growth in batch cultures, but the concentration increased in the late exponential growth phase and peaked in the stationary and death phase (Fig. 1). Several other studies have also found a similar pattern of eDNA accumulation in batch cultures. For example, eDNA concentrations in the environmental isolates *Acidovorax temperans* and *Rhodovulum sulfidophilum* increased to a maximum of 5 and 1.4 μg mL$^{-1}$, respectively, during the late exponential growth phase (Heijstra et al., 2009; Suzuki et al., 2009). In the clinical strains *Pseudomonas aeruginosa* and *Streptococcus mutans*, the eDNA concentration peaked in the early stationary phase with 17 and 0.085 μg mL$^{-1}$, respectively, but subsequently decreased to below detection limit (Petersen et al., 2005; Allesen-Holm et al., 2006). The peak concentration of eDNA in our batch cultures is thus within the range of what is found in other studies of clinical and environmental isolates. Only *Reinheimera* sp. F8 stood out with up to 33 μg mL$^{-1}$ eDNA detected in the culture supernatant, and possibly even more attached to the cells. This value was much higher than eDNA concentrations reported for any other pure culture, but well within the range of 21–134 μg eDNA mL$^{-1}$ found in the mixed microbial communities of activated sludge (Dominiak et al., 2011).

The transient peak of eDNA found in batch cultures of several clinical strains indicates that extracellular DNases breakdown the eDNA to make the nucleotides available as nutrients (Petersen et al., 2005; Allesen-Holm et al., 2006). We found a transient peak of eDNA during biofilm formation by *Serratia* sp. FW2, suggesting that it can breakdown eDNA. The low concentration of eDNA in planktonic cultures of this isolate could thus be caused by degradation by extracellular DNases rather than lack of eDNA production. The relative importance of eDNA versus other extracellular matrix components may differ over time during biofilm development. Except for *Serratia* sp. FW2, we found that the concentration of eDNA in the biofilm increased as the biofilm grew. Despite the increasing amount of eDNA over time in the three isolates, only *Microbacterium* sp. FW3 biofilm was affected by DNase treatment, and the effect was rather modest (Fig. 6). It is possible that the DNase treatment was simply not sufficient to degrade the eDNA, but is more likely that other components in the extracellular matrix replace or complement the function of eDNA in securing the structural stability of the established biofilm, as previously suggested by Whitchurch et al. (2002).

It is tempting to look at the accumulation of eDNA in batch cultures when searching for a correlation between biofilm formation and eDNA production, and Yang et al. (2007) did find such a correlation for *Pseudomonas aeruginosa* under different growth conditions. However, we observed no such correlation, and hence, the amount of eDNA accumulating in batch cultures did not reflect its significance for biofilm formation. For example, *Serratia* sp. FW2 accumulated little eDNA in batch culture, but it was the most efficient biofilm producer (Fig. 1, Fig. 4). Conversely, *Reinheimera* sp. F8 accumulated the highest concentration of eDNA in batch cultures, but produced the least biofilm. When making the same comparison for the concentration of eDNA in biofilms, we also found no correlation. Again, *Reinheimera* sp. F8 accumulated the highest concentration of eDNA, but produced the least biofilm, while *Microbacterium* sp. FW3 produced substantial amounts of biofilm although the eDNA concentration was 1/10 of that observed in other biofilms (Fig. 4). A possible explanation is the fact that *Reinheimera* sp. F8 was isolated in a screening for bacteria from river aggregates using low-nutrient media. Thus for bacteria adapted to planktonic and aggregated growth under highly dynamic flow conditions, eDNA may have a different functionality (Neu & Lawrence, 2009) compared with the role of eDNA during biofilm growth at a solid–liquid interface. Another reason for these differences is probably that other adhesins are more important for biofilm formation in some strains. It is, however, interesting to note that eDNA did affect initial adhesion in all strains and that biofilm formation began in the exponential growth phase when eDNA concentrations were very low. Heijstra et al. (2009) reached a similar result when demonstrating that DNA-dependent attachment occurred in *Acidovorax temperans* during the early exponential growth phase, prior to eDNA being detectable in the culture supernatant. Together with our findings, these studies underline that eDNA can be important for biofilm formation even when it is not detected in high concentrations, or perhaps not detected at all. We found this to be the case despite strain-specific differences in eDNA production of three orders of magnitude, and eDNA is likely a universal principle for initial adhesion and biofilm formation in nature.

**Acknowledgement**

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**References**


### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Repetition of optical density measurements (triangles) and eDNA quantification (diamonds) in batch cultures in biological triplicates (separately grown batch cultures) of *Reinheimera* sp. F8 and *Microbacterium* sp. FW3 grown in R2A (error bars = SD, n = 3).

**Fig. S2.** Amount of eDNA relative to the bacterial biomass during biofilm growth.