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ORIGINAL PAPER

Quantitative contributions of bacteria and of *Deinococcus geothermalis* to deposits and slimes in paper industry

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Abstract *Deinococcus geothermalis* has frequently been isolated from pink colored deposits of paper industry processes. Laboratory studies have shown that *D. geothermalis* is capable of forming on nonliving surfaces patchy biofilms that are resistant to adverse agents such as extreme pH, desiccation, solubilising detergents and biocides. This study was done to quantitatively assess the role of *D. geothermalis* as a biofouler in paper industry. Colored deposits were collected from 24 European and North American paper and board machines and the densities of the bacterial 16S rRNA genes and those of the red slime producers *D. geothermalis* and *Meiothermus* spp. were measured by QPCR (quantitative real time PCR). *D. geothermalis* was found at nine machines, usually from splash area deposits, but its contribution was minor, 0.001–1%, to the total bacterial burden of 8.3 to log 10.5 log units per gram wet-weight of the deposits. When *D. geothermalis* was found in a measurable quantity, *Meiothermus* spp. also was found, often in bulk quantity (7–100% of the total bacteria). The data are in line with the properties of *D. geothermalis* known from laboratory biofilm studies, indicating this species is a pioneer coloniser of machine surfaces and may help other bacteria to adhere and grown into biofilms, rather than competing with them.

Keywords Biofilm · *Deinococcus geothermalis* · Paper machine · Biofouling · Quantitative RT-PCR

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Introduction

Deposits and slimes accumulating on the submerged and splash areas of paper machine process equipment are long time suspected of being caused, at least in part, by microorganisms [1, 28] but the quantitative role of the organisms is unclear. It is often stated that almost any species of bacteria is capable of forming biofilms on nonliving surfaces [3, 9]. However, some species of bacteria may generate more persistent biofilms than others in a given environment, for instance, *Staphylococcus epidermidis* on metal devices like pacemakers implanted in the human body [8, 17]. We recently found that *Meiothermus* spp. was present in high densities in colored deposits formed on paper machine process surfaces in the majority of paper machines studied from four countries [6].

In this paper, we present results obtained using quantitative real time PCR (Q-PCR) to assess the contribution of the well-known primary biofilm former *Deinococcus geothermalis* [14, 22, 26, 27] in the total bacterial burden of

colored biofilms, waters, deposits, raw materials and products of paper industry.

Materials and methods

Bacterial strains and culture media

Phylum *Deinococcus-Thermus* strains used in this study were *Deinococcus geothermalis* E50051 (AJ864721), *D. geothermalis* 11300^T, *D. geothermalis* E50053, *D. murrayi* ALT-1b^T (Y13041), *D. radiophilus* DSM20551^T (Y11333), *D. radiodurans* DSM20539^T (Y11332), *D. grandis* DSM3963^T (Y11329), *D. proteolyticus* DSM20540^T (Y11331), *D. radiopugnans* ATCC19172^T (Y11334) *Meiothermus ruber* DSM1279^T (Z150059), *M. silvanus* DSM9964^T (AY864723). Strains used from other phyla were *Pseudoxanthomonas taiwanensis* jk-M (AY864723), *Burkholderia cepacia* F28L1, *Roseomonas gilardii* ATCC499956^T (AY150045), *Bacillus stearothermophilus* DSM1550, *Escherichia coli* MT102 (Obtained from Otto Geisenberger). The strains were obtained from Prof Mirja Salkinoja-Salonen collection and HAMBI, the Culture collection of the Faculty of Agriculture and Forestry, University of Helsinki. The type strains of *Deinococcus* species probed for the primers DgeR866 and DgeF627a were *D. apachensis* KR-36^T (AY743264), *D. pimensis* KR-235^T (AY743277), *D. yavapaiensis* KR-236^T (AY743279), *D. maricopensis* LB-34^T (AY743274), *D. indicus* Wt/1a^T(AJ549111), *D. deserti* VCD115^T(AY876378), *D. ficus* CC-FR2-10^T(AY941086), *D. hohokamensis* KR-40^T (AY743256) *D. navajonensis* KR-114^T(AY743259), *D. hopiensis* KR-140^T(AY743262), *D. sonorensis* KR-87 ^{T}(AY743283).

Culture media used R2A and tryptic soy agars were from Becton, Dickinson and company, (Sparks, MD, USA).

Sampling, extraction and purification of DNA

Colored deposits and process water were sampled from paper and board machines in Europe and in North America (Table 1). The samples (≥5 g per site) were stored at −20 °C. DNA was extracted from 50 mg wet wt of the blended slime or deposit, from 80 mg of felt, from 80 mg of end product and from 3 ml of process water (pelleted at 10 000×*g*). Plate cultured bacteria (50–100 mg wet wt.) harvested from R2A (*Deinococcus*, *Meiothermus*) or from tryptic soy agar (the other strains). Extraction and purification of DNA was done as described by Ekman et al. [6].

Table 1 Target areas of the sampled machines

Sample	Machine and machine code	
	Paper machine	Board machine
Slimes and deposits[a]		
Miscellaneous (bentonite slurry mixer, storage tank for sizing starch slurry)	E, R	
Wire section, splash area	A, C, D, E, J, N, O R, USc, USA, USB	H, K, LK
Circulation water tank, krazer	J, N, R	
Filtrate tanks (filtrate, clear, super clear)	USB	H
Headbox	R, T, U	
Wire section of inner layer	U	H, L, No1
Broke thickener	R	H
Water tank (white water, shower, filtrate, cloudy filtrate tank)	A	K, R
Former inner wall		H, MK
Disc filter	R, T, USB	H
Lid of the feed tank of mineral pigment	T	
Screens	R, U	
Felts	V	K
Paper product		
End products with defects	A, R	L
End products (non-defect)	R	L
Process material		
Waters (wire water, shower water, circulation water, super clear filtrate tanks water)	A, R	
Raw material (carbonate, broke)	R	

[a] ≥5 g wet wt per sampling site collected from paper and board machines

Primers used and Quantitative real-time PCR (QPCR)

Primers targeted to 16S rRNA genes were used (Table 2). *D. geothermalis* specific primers were designed using DNAMAN-program Version 5.2.9 (Lynnon Biosoft, QC, Canada) and the ARB sequence database in ARB (ssujun02) including 17,217 checked bacterial 16S rRNA gene sequences [15].

The LightCycler Quantitative real-time PCR (QPCR) machine (Roche Diagnostics, Penzberg, Germany) was used. The reaction volume was 20 µl per capillary, including 2 µl of DNA suspension and 0.3 µM each primer. For the domain *Bacteria* (Table 2) the program described by Ekman et al. [6] was used. For *D. geothermalis* LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostic) (2 µl per reaction) was used with the temperature program of 10 min at 95 °C and 40 cycles of 10 s at 95 °C followed by annealing for 2 s at 65 °C and extension 12 s at 72 °C. After 40 cycles, melting temperatures of the amplicons were determined by raising the temperature to 98 °C at 0.1 °C s^{-1} and using the LightCycler software. For *Meiothermus*, the program is described by Ekman et al. [6]. QPCR kits SYBR Premix Ex Taq and LightCycler Faststart DNA Master SYBR Green I contained dNTPs, DNA polymerase enzyme, buffer and SYBR Green I fluorescent dye.

D. geothermalis E50051 DNA was used to prepare the calibration curves with the primers pE and pF′ and DgeF627a, DgeR866, three replicates. For the calculations *D. geothermalis* genome size of 3.27 Mb [18] was used and the copy number of 16S rRNA genes 2 per genome.

The data was processed using Origin 7.5 software (OriginLab Corp., USA). The floating bars express the highest and the lowest values, respectively. The median is indicated by vertical lines inside the bars.

SEM (scanning electron microcopy)

The images were prepared as described by Väisänen et al. [30]. Prior to exposure to mill water, the acid-proof steel coupons (AISI 316) were polished with water-proof silicon carbide paper (FEPA P1000) and then immersed at mill LK in the white water chest of the top wire for 9 days (45 °C) 30 cm below the surface and in the white water chest of the bottom wire (42 °C) 30 cm below the surface, pH 6.8. The coupons were examined with a Zeiss DSM 962 (Carl Zeiss, Oberkechen, Germany).

Results

Ninety colored slimes and deposits, 21 end products (paper, paperboard), 6 process waters, carbonate and broke from 24 paper and board machines in Europe and North America (Table 1) were analysed for the contents of bacteria. The method used was 16S rRNA gene targeted QPCR (real-time quantitative polymerase chain reaction) using the universal eubacterial primers pE and pF` (Table 2) with DNA isolated from the 120 mill samples as the templates.

The results compiled in Fig. 1 show that the deposits collected from 15 different areas of the paper or board machines contained between log 7 and log 12 copies of 16S rRNA genes per gram of the sampled deposit (wet weight). The median was very high, log 10 to log 11 16S rRNA genes per gram for the deposits collected from inside walls of tanks (containers), broke thickener, as well as in the headbox, wire areas and clogged felts, representing majority of the sampled deposits (69 out of 90). If the measured 16S rRNA gene numbers represent intact cells (rather than free DNA), an average of three rRNA operons per genome, the bacterial density in these 69 deposits would correspond to 3 to 30 × 10^9 cells g^{-1} wet wt. If an average cell weight is 10^{-11} g (wet weight) [10, 16] this cell density would contribute with 3–30% to the deposit volume or weight. Bacterial biomass, thus, is a major component in the slimes and deposits formed in the paper machine environment. Process waters and raw materials had a varying content of bacteria, ranging from log 4 to log 8.5 g^{-1} wet wt. The dispersal of 1 g of a deposit with 10^{10}–10^{11} of bacteria density could double the bacterial count in 1–10 m^3 of process water.

Table 2 16S rRNA gene targeted primers used for QPCR

Primer	Target	Nucleotide sequence (5′–3′)	Amplified region[a]
pE[b]	Bacteria	AAA CTC AAA GGA ATT GAC GG	908–928
pF`[b]	Bacteria	ACG AGC TGA CGA CAG CCA TG	1,053–1,073
DgeF627a	*D. geothermalis*	GGA GTG GGT TGG AGA CTG GCT	627–647
DgeR866	*D. geothermalis*	CCA GGC GGC ACG TTT CTC GC	866–885
MeioF692[c]	Genus Meiothermus	GAA ATG CGC AGA TAC CGG A	692–711
MeioR821[c]	Genus Meiothermus	TGT CGG ACA CCC AGC ACT	821–839

The amplicons generated by the primers were 165 bp (pE, pF`), 256 bp (DgeF627a, DgeR866) and 147 bp (MeioF692, MeioR821)

[a] *E. coli* numbering

[b] Edwards et al. [5]

[c] Ekman et al. [6]

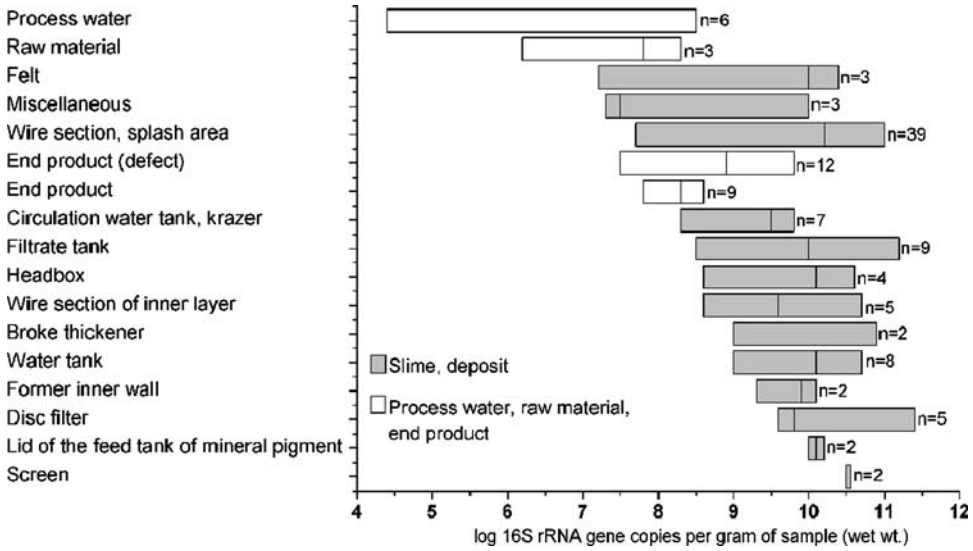

Fig. 1 Densities of bacterial 16S rRNA gene copies g^{-1} (wet wt.) at different sites of 18 paper machines and 6 board machines. The *floating bars* indicate the highest and the lowest values, respectively, and the *vertical lines* inside the *bars* indicate the median of the values. For the cases where only two samples were analysed, the *line* indicates the average value

From three mills, parallel sampling was arranged for defective paper (i.e. with colored spots or other visible defects) and good quality paper from the same machine (Table 1). The median bacterial content was almost ten-fold higher (log 9 bacterial ribosomal gene copies per gram of dry paper product) in the defect products than in those of good quality (log 8 g^{-1}) indicating a role of bacteria in the generation of the product defects.

Deinococcus geothermalis and *Meiothermus* spp. are known causative agents of colored slimes [6, 12, 14]. To quantitate *D. geothermalis* in the deposits, species-specific primers and QPCR were designed. The primers DgeF627a and DgeR866 (Table 2) amplified DNA of the type strain (DSM11300^T) and strains isolated from paper machines (E500551, E50053) yielding a single product of the 256 bp. This product, melting at 90 ± 0.5 °C, was obtained from 50 fg to 5 ng DNA (28 to 3 × 10^5 16S rRNA gene copies) within 12–32 cycles (Fig. 2a). Specificity of the primers was tested spiking 1 ng of DNA from type strains of *D. proteolyticus*, *D. radiophilus*, *D. murrayi*, *D. grandis*, *D. radiopugnans and D. radiodurans*, respectively, to 0 or 0.1 ng of DNA from *D. geothermalis* E50051. *D. geothermalis* (0.1 ng) DNA produced amplicons with the same crossing point at (20 cycles) whether or not 1 ng of DNA of these non-target species was present in the reaction (Fig. 2b). These non-target strains had ≥3 mismatches with the forward primer DgeF627a and 2 mismatches with the reverse primer DgeR866. In the absence of *D. geothermalis* DNA (0 ng), one or multiple amplicons were produced by these and other seven nontarget taxa, including two species of *Meiothermus* (listed in "Materials and Methods") but the crossing point of 1 ng per reaction exceeded 30 cycles, and the product(s) deviated in the melting temperature by 0.5–3 °C from that of *D. geothermalis*. It was concluded that when a product with the correct melting temperature was obtained with the primers DgeF627a and DgeR866 within 30 cycles, this could reliably be used to calculate the density of *D. geothermalis* in paper machine deposits.

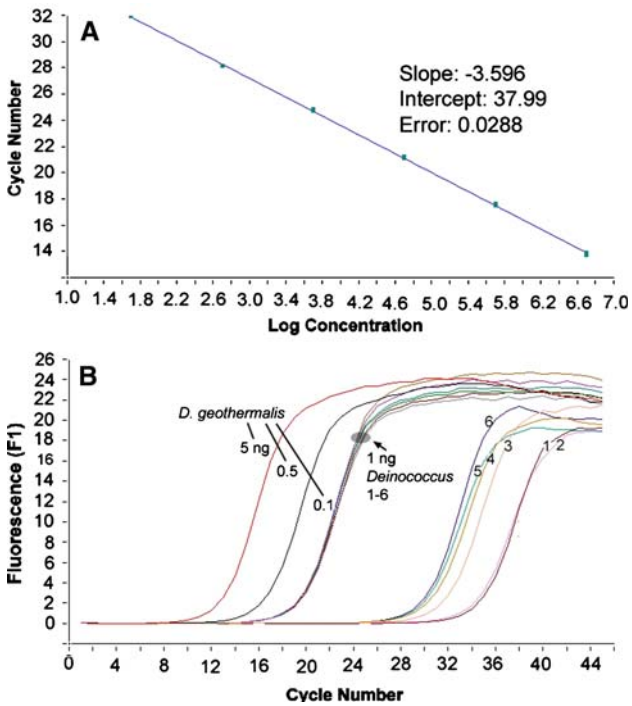

Fig. 2 **a** Calibration curve for quantitation of 16S rRNA gene copies of the samples. The *curve* was formed of a dilution series (with three replicates) 50 fg to 5 ng (28–280,000 16S rRNA gene copies) of genomic DNA from *D. geothermalis* E50051. The *crossing points* were between 13.8 and 32. **b** Amplification curves of *D. geothermalis* E50051 (0.1 ng) in the presence or absence of DNA of other species of *Deinococcus* (1 ng DNA). Type strains of *1 D. proteolyticus*, *2 D. radiophilus*, *3 D. murrayi*, *4 D. grandis*, *5 D. radiopugnans*, *6 D. radiodurans*. The *arrow* marks the amplification curves obtained from 0.1 ng of *D. geothermalis* in the presence of 1 ng DNA of the non-target *Deinococcus* species 1–6 (1 ng)

Out of the 120 process samples and end products tested for *D. geothermalis* with the Dge primers, 16 were found positive and 9 could be quantitated (Table 3). Four deposit samples, one clogged felt, one sample of process water and one defect paper product gave a correct melting point but could not be quantitated due to late crossing point (≥30 cycles, equivalent to $\leq 10^5$ *D. geothermalis* genomes per gram of sample). Out of the sampled 24 machines, *D. geothermalis* was found in 9 machines. The deposits, felts and waters, positive for *D. geothermalis*, all contained high density of bacteria (log 7 to log 10.5 16S rRNA genes per gram, Table 3) but only a minor portion of the bacterial biomass consisted of *D. geothermalis*, 0.001–1.3% in any of the machines. When *Meiothermus* spp, a pink slime former of paper machines, was analysed from the same samples using the genus specific primers MeioF692 and MeioR821 (Table 2) a very different result was obtained, 0.5–100% of the bacterial deposit contained rRNA gene copies consisted of *Meiothermus* spp. We conclude that when *D. geothermalis* was present, it was a minor component, whereas *Meiothermus* species were a major, and sometimes the dominant bacterial component in those machines that contained *D. geothermalis*.

A large number of samples were collected from two mills, paper machine R (46 samples at several occasions) board machine H (13 samples). In those mills, the proportion of *D. geothermalis* positive samples was minor (R) or none (H). Multiple *D. geothermalis* positive samples were obtained from only one board machine (K) and one paper machine (A). Most samples ($n = 5$) from these machines samples were *D. geothermalis* positive and, in addition, the spots in the end products of paper machine of mill A were *D. geothermalis* positive, indicating that when present, *D. geothermalis* endangers product quality.

Figure 3 shows a SEM micrograph of a biofilm colony growing on a steel coupon placed in a process water of a board machine with a pink slime problem. The primary biofilm in Fig. 3, adhering directly to the steel, strongly resembles *D. geothermalis* or other similar looking *Deinococci* in cell morphology as well as in its thread like appendages connecting the cells to steel and to each other. The biofilm looks like a monospecies colony. When such a biofilm grows in thickness to volumes that can be sampled in grams (like was done in this study, Table 1), the primary biofilm layer may become covered by other types of bacteria.

Discussion

Several strains of *D. geothermalis*, *Meiothermus silvanus* and *M. ruber* have been isolated from colored deposits collected from paper and board machines and their potency of forming pertinent biofilms on nonliving surfaces in pure and mixed culture has been shown by laboratory experiments [12, 13, 30]. These species, whose natural habitat is geothermal wells and soils [24, 29], are moderately thermophilic organisms known to produce red, pink, and brownish pig-

Table 3 Densities of bacteria in the nine machines positive for *D. geothermalis*

Analysed sample (machine)	16S rRNA gene copies		
	Total bacterial log g^{-1}	% of the total bacterial identified as	
		D. geothermalis	*Meiothermus* spp.
Deposits			
Wire section splash area (A)	9	0.05	0.5
Wire section splash area (MK)	8.4	0.03	2
Wire section splash area (D)	9.9	1.3	1
Wire section of inner layer (M)	9.7	<0.001*	
Shower water tank (K)	10	0.001	
Circulating water tank (N)	8.3	<0.001*	100
Cloudy filtrate tank (K)	10.5	<0.001*	67
Cloudy filtrate tank (R)	9.9	0.005	8
Reject storage tank (R)	9.6	0.05	7
AES screen splash area (USc)	10.5	0.03	13
Bow screen (R)	10.5	0.007	11
Not known, slime (E)	10	<0.001*	0.5
Press felt (K)	7	<0.001*	
Water tank, process water (A)	8.3	<0.001*	
End products			
Defect paper 1 (A)	9.7	0.2	
Defect paper 2 (A)	7.2	<0.001*	

The quantifications were done by QPCR using primers (Table 2) designed to amplify the 16S rRNA gene of Domain *Bacteria* (pE, pF`), *D. geothermalis* (DgeF627a, DgeR866) and *Meiothermus* spp. (MeioF692, MeioR821). Samples ($n = 104$) from the 14 other investigated machines tested negative for *D. geothermalis* and are not included in the Table. The machine codes behind the samples are explained in Table 1

* Non-quantifiable amount

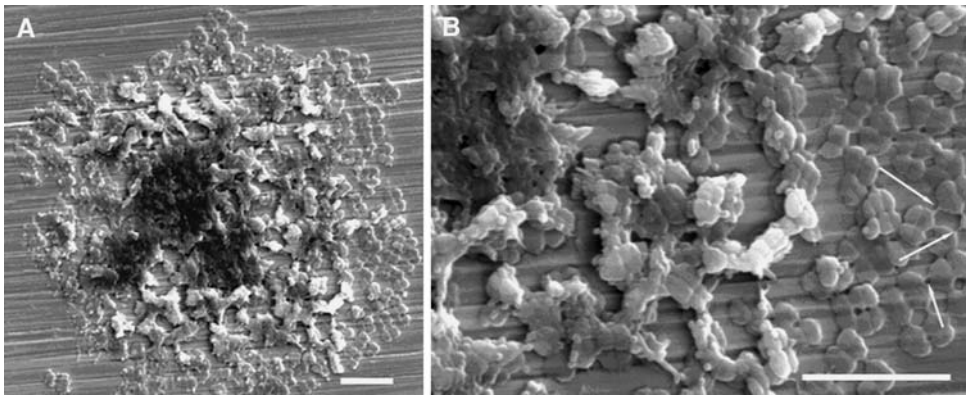

Fig. 3 Scanning electron microscopy (SEM) images (**a**, **b**) of a bacterial biofilm on stainless steel coupons immersed in the white water chests of a board mill for 9 days. **a** Bacterial microcolony on the steel surface. **b** Greater magnification of the same steel coupon. Bacteria formed a primary cell layer on the surface and showed growing as two and four cells clusters. Cell-to-cell interaction in the monolayer is mediated with thin adhesion threads, shown with *white arrows*. *Scale bars* 10 μm

ments [7, 21]. *Meiothermus* species were reported of being a dominant coloniser at the storage system of spent nuclear fuel [19] and recently shown by quantitative DNA based methods to occur in high density in the deposits and defective paper products collected from many paper machines [6].

D. geothermalis tolerates desiccation, IR and UV radiation [4, 18], oxidative stress [4] and biocides [12] predicting success in manufactured industrial environments. Its role and the quantitative contribution of *D. geothermalis* in the bacterial biomass of machine deposits and slimes have not been investigated prior to the present paper.

Mills water temperature is similar to optimum growth temperature of *D. geothermalis* (45–50 °C), and paper machine waters substrate suitable for *D. geothermalis* which possess multiple genes for the utilization of hemicellulose and xylose [18]. *D. geothermalis* was found in the deposits of nine machines, mainly from splash areas in the wire areas, screens and inside walls of tanks containing white water filtrates. Splash areas offer oxygen but require tolerating towards hot temperatures and desiccation. In *D. radiodurans* desiccation tolerance has been related to radiation resistance [20]. At all sites where measurable amount ($\geq$0.001% of the total bacterial burden) of *D. geothermalis* was found, *Meiothermus* spp. was also found, in quantities exceeding 10–100,000 fold those of *D. geothermalis*. We measured the total density of bacterial 16S rRNA gene copies in the slimes and deposits from 24 paper and board machines as log 8.3 to log 10.5 per gram wet-weight. This matches well with the findings of Claus and Müller [2] who, based on microscopic counting, reported 1.5×10^9 to 6.6×10^{10} bacterial cells per ml of paper machine biofilm. *D. geothermalis* thus is a minor constituent of machine colored deposits, whereas *Meiothermus* often is a bulk constituent [6]. In laboratory grown biofilms, *D. geothermalis* colonised nonliving surfaces in a patchy manner, forming individual colonies rather than mats on steel and glass surface, usually only 5–30 cells in height [14, 22]. The images in this paper show biofilm grown on steel coupons immersed under mill conditions [11], resembling the colony morphology of laboratory grown monospecies *D. geothermalis* biofilms [14, 22, 27]. Such a pattern of growth would explain why *D. geothermalis* (by criterion of species specific QPCR) was found only as minor constituent of the thick, mature biofilm deposits collected from paper and board machines for the present study.

The significance of *D. geothermalis* in the biofilm industrial deposits may be higher than appears from its minority presence, if its laboratory-shown properties also apply for mill conditions: (1) ability to adhere to steel or glass surface so firmly that the biofilm cannot be removed by hot NaOH (pH 12) or SDS wash [14]; (2) *D. geothermalis* colonised steel surface attracts several paper machine *Bacillus* species, including the undesirable pathogen *B. cereus* [23, 25, 31, 32], which otherwise would not produce biofilm in paper machine waters [13]; (3) *D. geothermalis* biofilm cells adhere to each other and the nonliving surface by large number of glycoproteins of type IV pili [27] but can nevertheless spread along the nonliving surface by gliding motility [14, 22, 27]; (4) *D. geothermalis* biofilm colonies do not produce loosely attached slime or capsule [7, 14], but, instead, are protected by a number of tightly attached glycoconjugates; [22] (5) the colonies are nonporous [22] and may thus exclude biocides and other microbe killing agents [12]. The industrial survey executed in the present study showed that *D. geothermalis* is in paper industry a frequent coloniser, not occurring in bulk quantity. This in line with the roles indicated for *D. geothermalis* by laboratory results, indicating that this species operates as a pedestal enabling other bacteria to adhere and grow into bulky biofilms. Such multilayered biofilm structure could explain the persistent, though quantitatively minor, contribution of *D. geothermalis* in the biofilms and end products of some mills.

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