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Structure and on-site formation of biofilms in paper machine water flow

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Paper machine biofilms formed *in situ* on stainless steel surfaces were studied. A robust flow cell was fitted to side stream (1.8 m s^{-1}) of the spray water circuit of a paper machine. This on-site tool allowed for assessing the efficacy of antifoulants and the adequacy of steel polishing under mill conditions. A rapid fluorescence-based assay was developed to quantify the biomass of shallow biofilms on machine steel. The fluorescence matched the ATP content measured for the same biofilms. Electrolytic polishing reduced the tendency of biofouling of 500 grit surface steel. Biofilm grew under machine conditions as clusters on the steels, showing uniformly coccoid, filaments or short rods; only one cell type in each cluster. The biofilm clusters excluded latex beads of 0.02 μm with hydrophilic or with hydrophobic surfaces from penetrating more than three to four layers of cells. Under the high hydraulic flow at the machine (1.8 m s^{-1}), the biofilm grew in 7 days 6–10 μm thick. The high flow rate guided the shape of the biofilm clusters emerging after the primary attachment of cells. Adhered individual bacteria were the platform on steel to which solids such as paper machine fines then accumulated.
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Introduction

Undesirable biofilm formation occurs in all industrial processes using water [3,4,11]. Ten to 150 m^3 of water is used per metric ton of manufactured paper, depending on the degree of mill closure and water recycling. The bulk raw materials for papermaking are natural products and therefore biodegradable. Deterioration is presently controlled by dispensing biocides into the raw materials and at multiple sites of the process, typically up to 1 kg of each active substance per ton of paper produced. This is not in line with the general tendency in this branch of industry toward sustainability and minimal resource usage. To reduce the need for biocide usage and to analyse the efficacy of non-biocidal antifouling strategies, the biology of biofilm growth on paper machines needs to be better understood.

Biofilm properties change in response to environmental conditions, especially speed of flow and nutrient availability [4,9,12]. To develop a successful antifouling strategy, biofilm formation must be studied under authentic conditions. Large-scale processes like papermaking with high hydraulic flow are difficult to simulate in the laboratory. Therefore, *in situ* experiments are needed.

The aim of this work was to analyse biofilms formed in the paper mill on machine steel surfaces. To this end, we designed flow cells applicable for studying biofilm formation in the mill connected to flow of the process waters. We report the salient properties of biofilms formed on stainless steel in the paper mill.

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Materials and methods

The paper machine

Experiments were conducted at a printing paper machine producing 150 000 tons of publication paper per annum with a water consumption of 10 m^3 ton^{-1}. The machine runs at acid pH (4.9–5.5) at 41–48°C. The spray water recycled in the wire area contained (mg l^{-1}, ±20%): total P 2.6, total N 5.7, SO_4^{2-} 1100, Cl^- 22, Ca 240, Mg 9.6, Fe 1.1 and TOC 270.

Experimental setup

Flow cells were large-sized (30×10×920 mm) modified Robbins devices constructed of stainless steel and polyacryl (Figure 1) holding 16 steel coupons 25×15 mm. The device had a void volume of 276 cm^3. Two identical flow cells were placed in series and fed (32 l min^{-1}, flow 1.8 m s^{-1}) from the spray water circuit at machine pressure. The temperature of the spray water was recorded at 30-min intervals with a thermocouple connected to a datalogger. The average values during exposure periods are indicated in the figure legends.

In addition to Fe, the stainless steel coupons of SIS 2343 [UNS S31600] contained (weight percentage): 0.03 C, 0.56 Si, 1.51 Mn, 0.04 S, 16.9 Cr, 10.7 Ni and 2.6 Mo. The coupons were polished to a grit of 100 (still showing turning grooves), 120 and 500, with or without subsequent electrolytic polishing as indicated below. The coupons were washed with detergent, rinsed with sterile water and cleaned with acetone in a sonic bath (2 min) before placing them into the flow cell.

Spray water fed to the flow cell contained biocides and other additives routinely used at the machine at the time of the experiment.

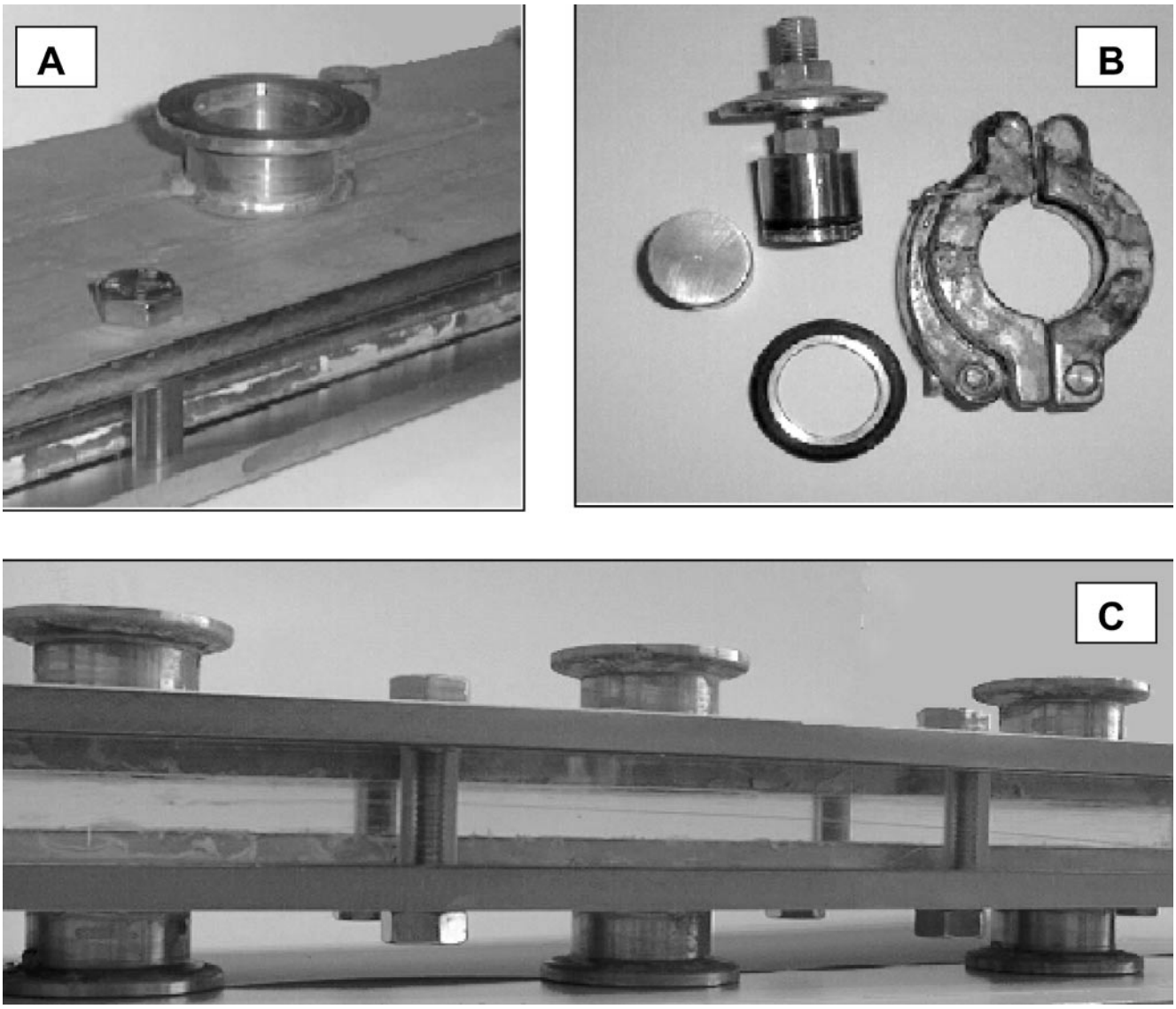

Figure 1 Flow cell designed for in-the-mill use. The cell was 920 mm in length with two walls of steel and two of polyacryl for transparency (A,C). The walls were connected with bolts and nuts. The stainless steel coupons (∅ 25 mm) were fitted to steel walls with two gaskets (B) and placed in the cell with cover and locking devices.

The additional antifouling chemical used in some tests was a biofilm inhibitor, Spectrum™ NT2001 (Hercules Inc.) containing sulfosuccinate as the active component, continuously dosed to 20 ppm.

Analytical protocols

For measuring ATP activity within the biofilm, the coupons were rinsed with Tris EDTA buffer (Tris 0.1 M, EDTA 2 mM, pH adjusted to 7.8 with acetic acid). They were then placed in 4 ml of boiling Tris–EDTA buffer with glass beads, the biofilm facing downwards. After boiling for 5 min, the tubes were cooled rapidly in an icebath. One hundred microliters of the cooled extract, 100 μl of ATP monitoring reagent (luciferin–luciferase of firefly; Thermo-Labsystems, Helsinki, Finland) and 100 μl of ATP standard solution (internal standard; 10^{-7} M in sterile distilled water; Thermo-Labsystems) were added in the order given. A luminometer (Bio-Orbit, Turku, Finland) reading was taken after the addition of each reagent. ATP content of the extract was calculated based on the response obtained for the internal standard.

Relative fluorescence units (rfu)

For direct fluorescence measurements of the biofilms, the stainless steel coupons were stained with syto 9 (nucleic acid stain, Molecular Probes Inc., Eugene, OR, 3.34 μM in water, 20 min). Fluorescence of the coupon was then measured with a scanning fluorometer (Fluoroskan Ascent, Thermo-Labsystems, Helsinki, Finland) using a filter of 485 nm (bandwidth 14±2 nm) for excitation and an emission filter of 538 nm (bandwidth 25±3 nm). Epifluorescence of the coupons was recorded as the sum of rfu from 137 points covering the coupon surface (∅ 25 mm). Background fluorescence of the stainless steel coupons was measured for each coupon and for each experiment.

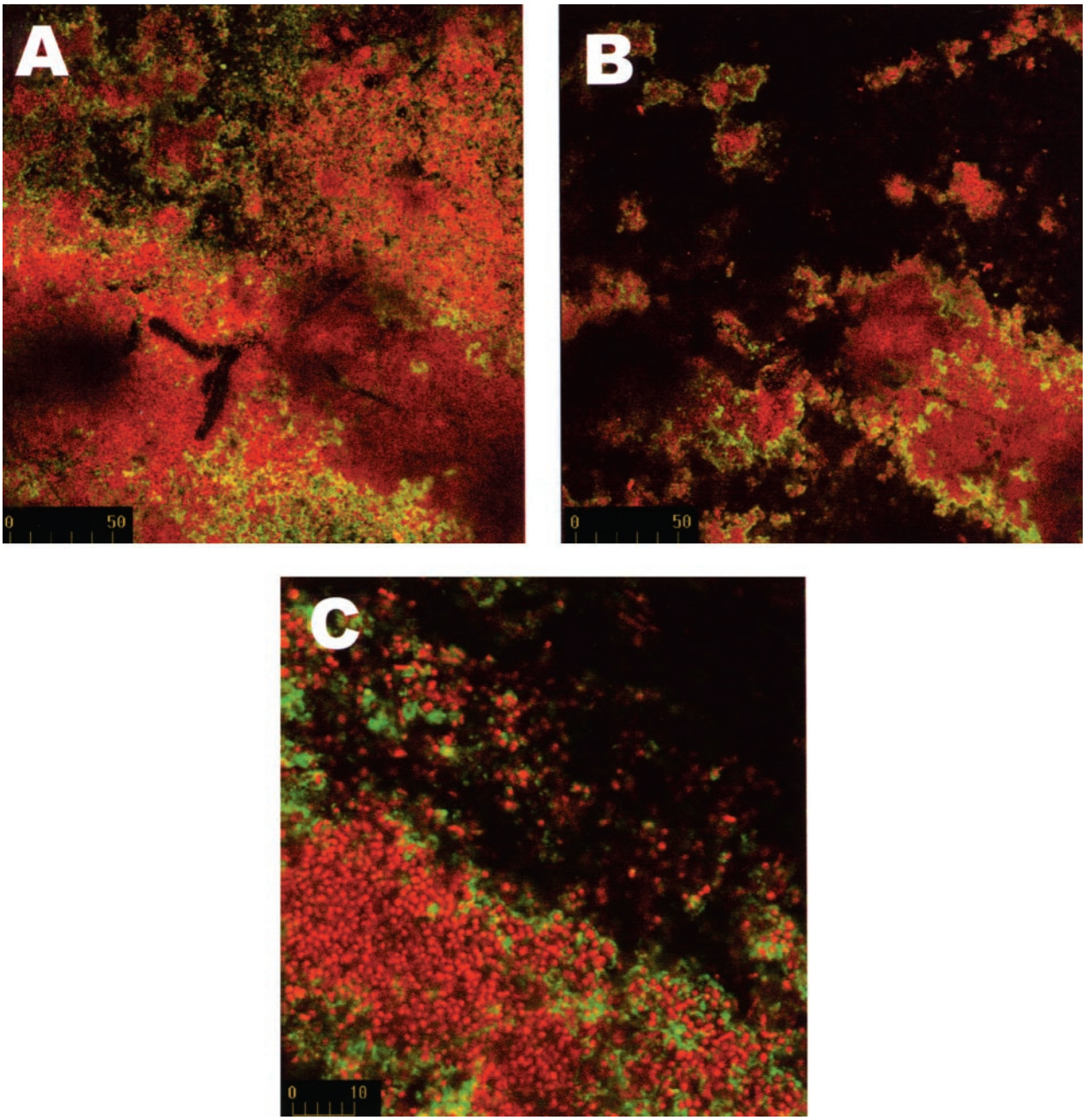

Figure 2 Optical sections (1 μm) of the CLSM image of a biofilm on stainless steel coupon grown in-mill for 7 days in papermill spray water stream (1.8 m s^{-1}). The biofilm was stained with EtBr (red) and carboxylate-modified fluorescent beads (green, diameter 0.02 μm). Panel A shows steel surface almost fully covered with EtBr-stained bacteria. Dark areas near the upper edge of panel A are bare steel surface. Panel B shows a single optical section of the biofilm at the distance of 6 μm from the steel surface. Panel C is a zoom up of panel B displaying the attachment sites of the fluorescent beads on the EtBr-stained bacterial cells. It shows that the void areas between cells were accessible to the 0.02-μm beads in the biofilm periphery to the depth of three to four cells only. Scale bar is in micrometers.

Scanning electron microscopy (SEM)

Coupons taken from the flow cells were allowed to air dry and then gold shadowed. SEM (JMS-840, Jeol Ltd., Tokyo, Japan) was performed as described by Väisänen *et al* [17].

Confocal laser scanning microscopy (CLSM)

CLSM (BioRad MCR-1024 with Zeiss Axiovert 135M using a KrAr-laser) was performed as described by Kolari *et al* [9]. Carboxylate-modified (diameter 0.02 μm±15.8%, 9×10^{12} beads ml^{-1}, surface hydrophilic) and aldehyde-sulfate-modified (diameter 0.029 μm±20.1%, 3×10^{12} beads ml^{-1}, surface hydrophobic) FluoSpheres® were used for assessing void volumes inside biofilm clusters. Bead staining was conducted by face down shaking (150 rpm) for 20 min followed by a wash with sterile filtered spray water. Ethidium bromide (EtBr; 100 μg ml^{-1}) staining for 20 min was conducted after the staining with fluorescent beads. Staining with syto 16 (20 μM in water) was done for 20 min followed by tetramethylrhodamine-tagged

concanavalin A (con A; 20 μg ml^{-1} in water) for 20 min. LiveDead® *Bac*Light™ bacteria viability stains (syto 9, live/green, 3.34 μM and propidium iodide, dead/red, 20 μM) were applied simultaneously, 20 min. All dyes were from Molecular Probes, Eugene, OR.

Results

Flow cell

Flow cells intended for *in situ* mill experiments must tolerate the mill conditions, that is, temperatures of up to 55°C, hydraulic flow of up to 2 m s^{-1}, papermaking chemicals as well as periodic cleaning with 1–2% NaOH (pH>12). Heavy-duty flow cells fulfilling these requirements were constructed of stainless steel and polyacryl, holding the volume as small as possible to save on chemicals that were going to be dispensed (Figure 1). The flow cell had modular structure allowing cleaning. Components were welded or joined with bolts and nuts.

Microscopic structure of the biofilm formed in paper machine water flow

The flow cells were fitted with stainless steel coupons (Figure 1B,C) and the cells were connected to the spray water circuit at the paper machine wet end. Biofilms formed *in situ* in paper machine spray water were analysed by CLSM.

Figure 2A shows biofilms on the steel coupons exposed *in situ* to the paper machine water flow for 7 days. The steel surface was covered by coccoid bacteria of uniform size (1.5×1.5 μm) and

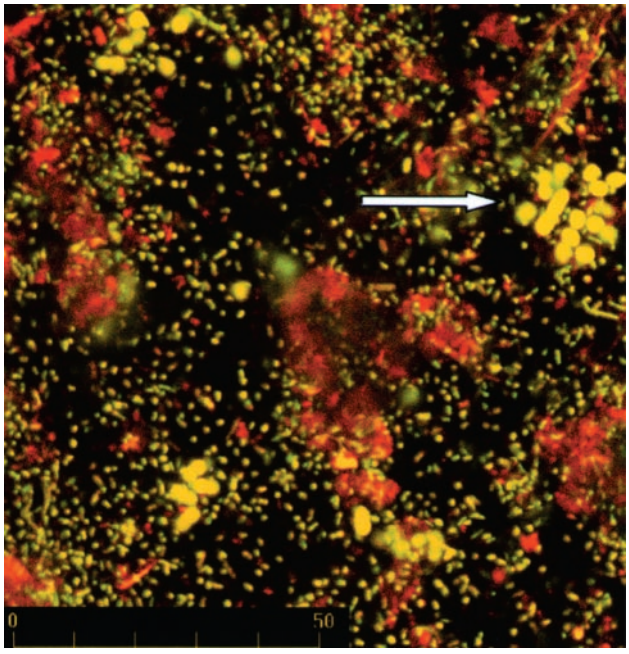

Figure 3 An optical section (1 μm) of a CLSM image of syto 16 (green) and tetramethylrhodamine-labelled con A (red)-stained biofilm on a stainless steel coupon grown for 5 days in the spray water circuit of a papermill. The figure shows a section at the 3-μm distance from the steel surface. The attached bacteria are stained green by syto 16. The larger sized yeast-shaped cells (arrow) fluoresce yellowish due to a lectin stain, con A, indicating the presence of glucose or mannose residues. Con A stain (red) also labelled syto 16 negative (i.e., void of DNA) amorphous fines (of pulping origin). Scale bar is in micrometers.

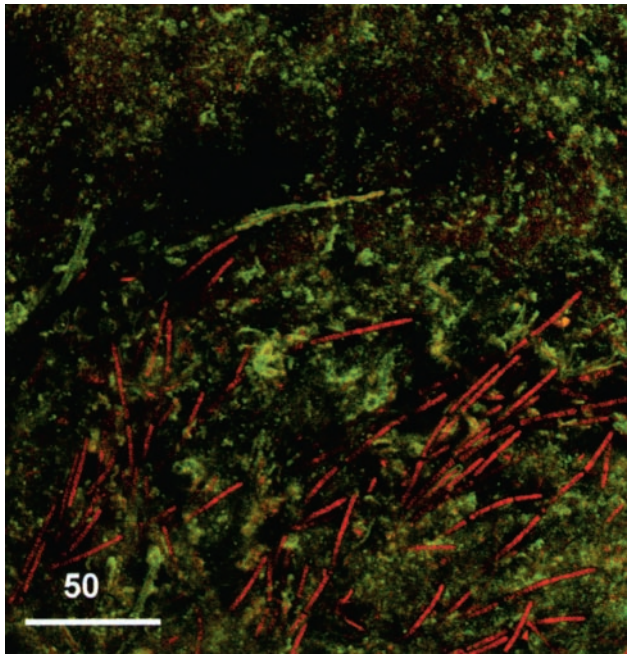

Figure 4 An optical section (1 μm) of the CLSM image of a 7-day-old biofilm stained with EtBr (red) and aldehyde sulfate-coated fluorescent beads (green, diameter 0.02 μm) on stainless steel coupon grown in the spray water circuit of a papermill. The section represents a view at a distance $\leq$1.5 μm from the steel surface. The large EtBr-stained filamentous bacteria (red) attracted no fluorescent latex beads indicating lack of hydrophobic components on cell surfaces. Scale bar is in micrometers.

shape and with a surface that attracted the carboxylate-modified fluorescent latex beads (green). The dark areas near the upper left corner of panel A are bare steel surface. The optical section in Figure 2B, at a distance of 6 μm above the steel surface, shows a much smaller coverage, indicating that the major part of this biofilm was less than 6 μm thick. The staining pattern obtained with carboxylate-modified fluorescent latex beads (green, diameter 0.02 μm) indicates low penetration of the beads into the interior parts of the biofilm. The zoom up in Figure 2C shows details of the sites where the fluorescent latex beads adhered on the EtBr-stained bacterial cells. The void space between bacterial cells was accessible to the 0.02-μm beads in the biofilm periphery to the depth of only three to four cells. The bacterial cell surfaces and the intercellular space thus attracted carboxylate-modified (hydrophilic) latex beads, indicating the presence of substances with accessible positive charges such as proteins.

Figure 3 shows a biofilm grown for 5 days on a steel coupon, stained with syto 16 and the lectin stain con A. It is an optical section at a distance of 3 μm from the steel surface. Rod-shaped bacteria (nucleic acid stain, green) are visibly attached to the steel surface as single cells. Larger sized mannose–glucose-specific con A-stained positive cells with yeast-like morphology (arrow) appear as sporadic microcolonies of 10–20 cells per optical section. The red con A stain adhered not only to the yeast-like cells (resulting in yellow fluorescence with syto 16) but also to structureless patches representing amorphous fines of ground wood origin. The bacterial cells did not stain with the lectin, indicating absence of mannose- or glucose-containing polymers on the their surfaces.

Figure 4 shows an optical section of an EtBr and aldehyde-sulfate latex bead (green, hydrophobic)-stained biofilm on the

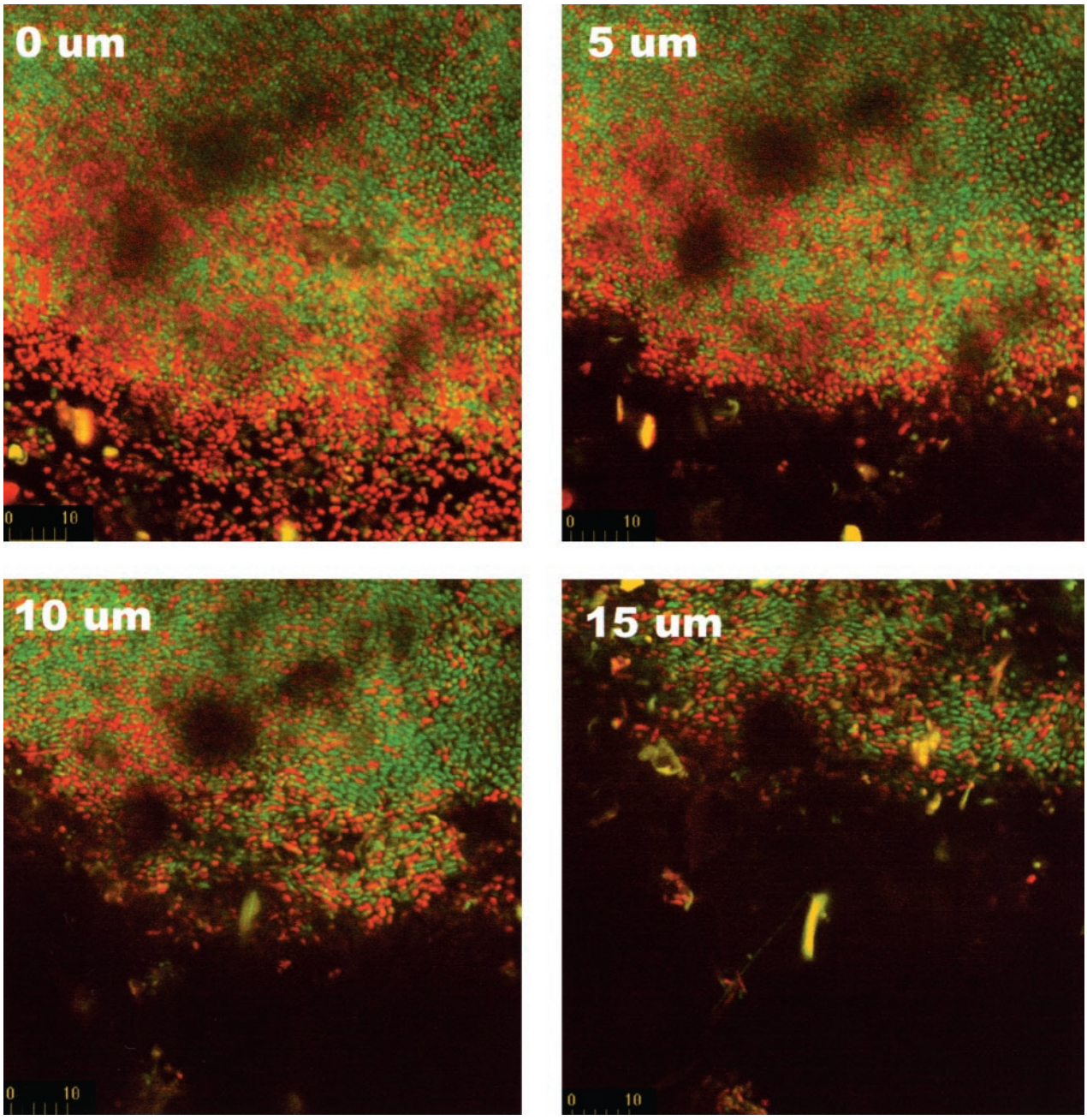

Figure 5 Optical sections (1 μm) of the CLSM image of viability-stained biofilm on a stainless steel coupon grown for 5 days in the spray water circuit of a papermill. The biofilm was stained with syto 9 (live/green) and propidium iodide (dead/red). The panels show optical sections of the biofilm cluster taken at 0, 5, 10 and 15 μm, respectively, distances from the steel surface. The biofilm clusters consist of bacteria of uniform size and shape, rods $\leq$2 μm in length. The red stained bacteria locate mainly in the peripheral areas of the cluster and near the steel surface. Scale bar is in micrometers.

coupon surface. EtBr stained large filamentous cyanobacteria-like cells (red, length up to 50 μm) not attracting the hydrophobic beads. The filaments appear septated like chains of cells.

Figure 5 shows serial optical sections of a viability-stained biofilm at increasing distances from the steel coupon surface. The biofilm consists of uniform rod-shaped bacteria $\leq$2 μm in length. Dead bacteria (red) were located mainly in peripheral areas of the biofilm cluster and closest to the steel surface. The core of the biofilm cluster is live, that is, green fluorescing, indicating that the biocides dosed into the wire showers of the paper machine affected the outermost cell layers only.

Figure 6 presents serial optical sections lateral to the steel surface of an EtBr and aldehyde sulfate fluorescent bead-stained biofilm. The core of the biofilm aggregate shows weak staining by EtBr (red) and contains no particles with bacteria-like morphology. The core is amorphous, resembling an emulsion droplet of resin or oil, coated by bacteria that have attracted the aldehyde sulfate latex beads (green). The bacteria are uniform-looking rods. The

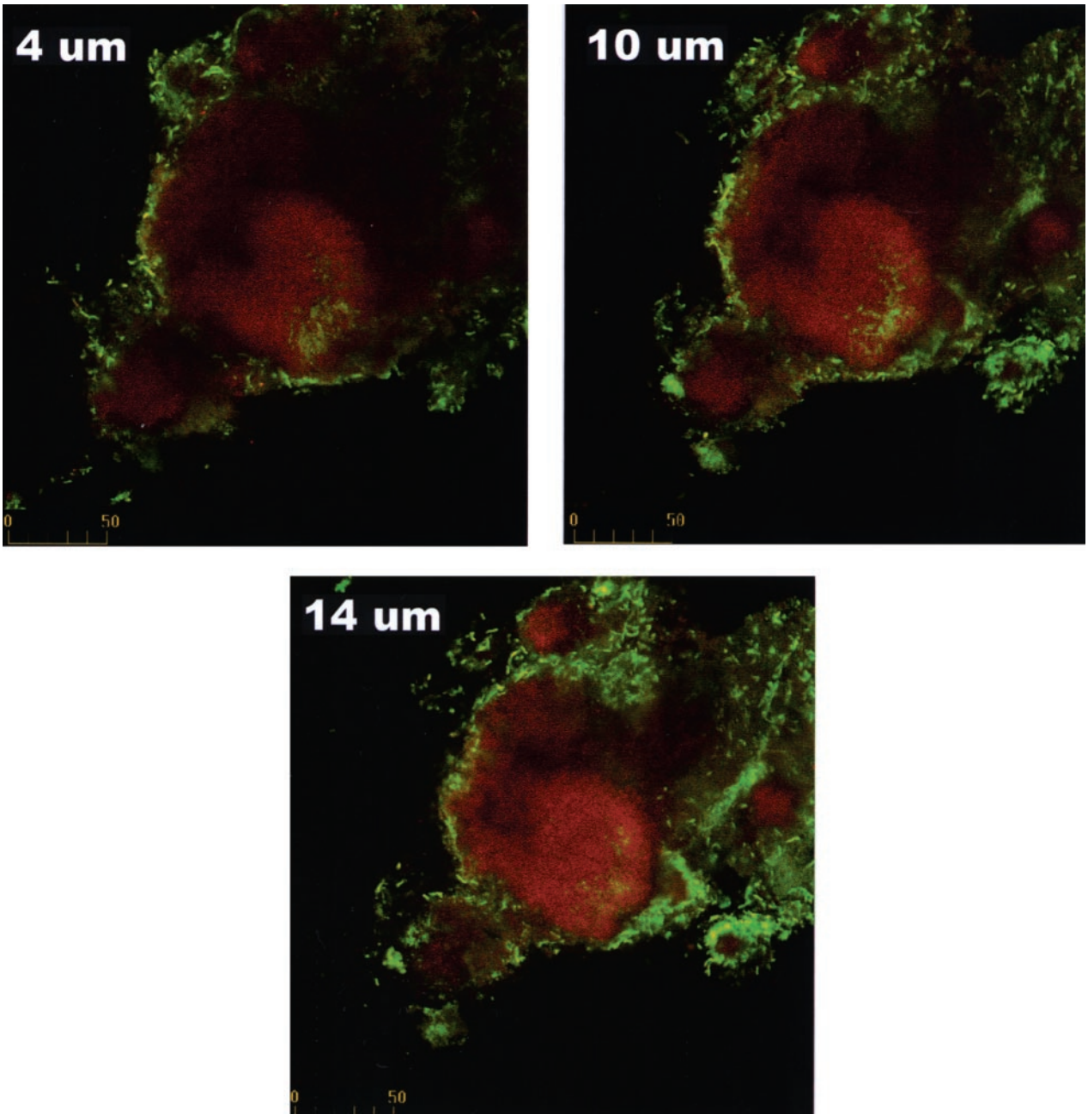

Figure 6 Optical sections (1 μm) at 4- to 6-μm steps of a CLSM image of EtBr (red) and aldehyde sulfate-coated fluorescent beads (green, diameter 0.02 μm) stained biofilm on stainless steel coupon grown for 5 days in the spray water circuit of a papermill. The panels show an amorphous emulsion droplet (red) covered by bacteria coated with the fluorescent latex beads. The distances of the optical sections from the steel surface are indicated in the figures. Scale bar is in micrometers.

hydrophobic surface of the bacteria, demonstrated by the aldehyde sulfate bead staining, may have promoted adherence of bacteria to the emulsion droplet.

Figure 7 shows a grooved landscape of the steel surface diagonal to the figure, underneath the stained biofilm. The grooved surface structure seems to have guided the biofilm orientation. Individual optical sections parallel to the steel surface in Figure 7 show that only bacteria (syto 16 stained, green) were present in the layer closest (panels 0 and 2 μm) to the steel surface. Lectin con A-stained pulping fines (red, also visible in Figure 3) were present in sections more distal to the steel surface than the bacteria (panel 6 μm). This architecture of the biofilm indicates that the biological slime deposit was built on a basement primarily of adhered bacteria to which non-living particles from the water flow then attached.

Figure 8 displays a series of optical sections of a viability-stained biofilm on a steel coupon surface. The direction of flow is from right to left. Single cells or layers of bacteria find shelter against the flow in the grooves of the steel surface. But when the bacterial cluster exceeded in height the top of the groove, the cluster

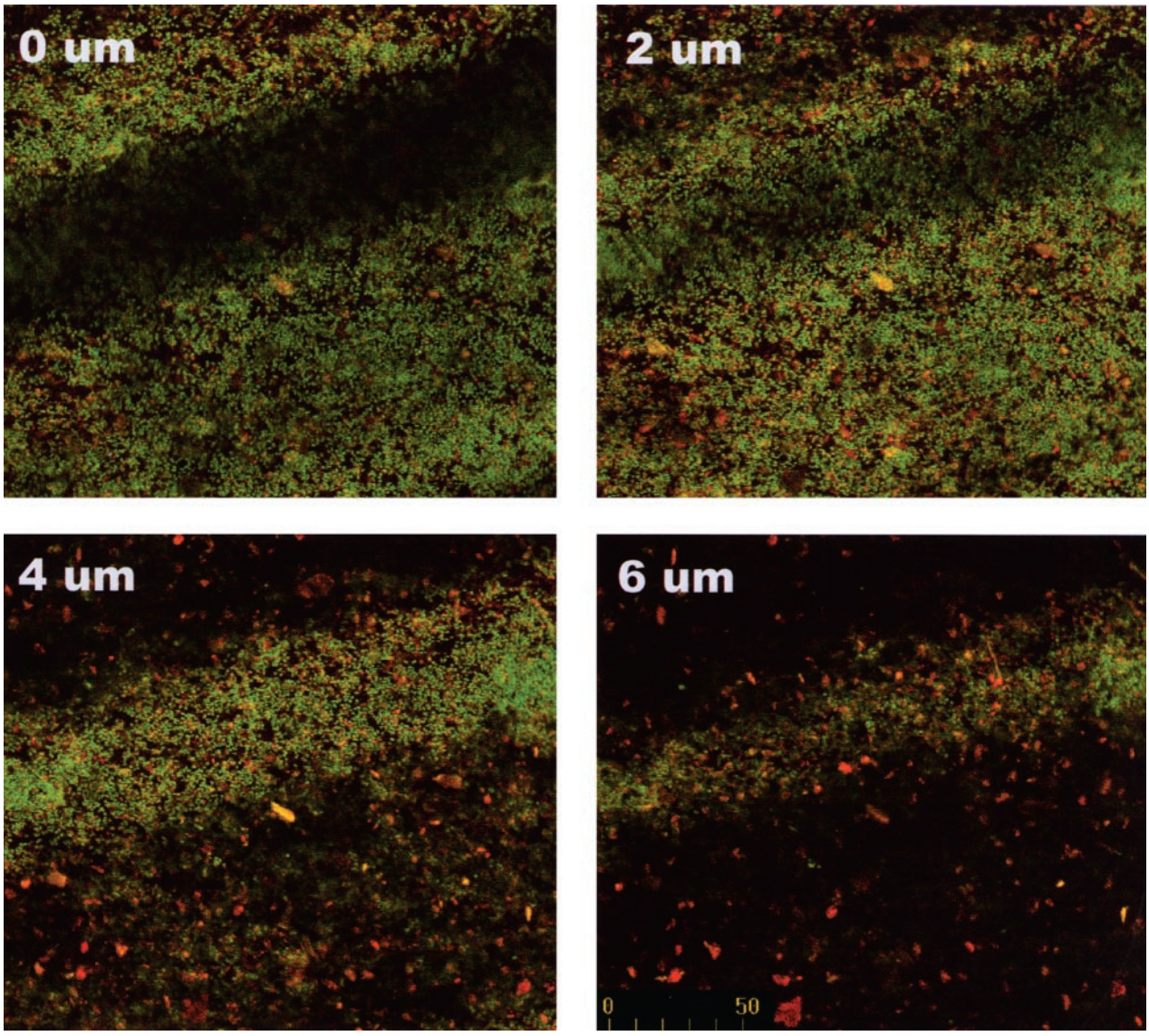

Figure 7 Optical sections (1 μm) of the CLSM image of biofilm on a stainless steel coupon grown for 5 days in the spray water circuit of a papermill. The biofilm was stained with syto 16 (green) and tetramethylrhodamine-labelled con A (red). The distance of the respective sections from the steel surface is indicated in the figures. Grooves on the steel surface are visible diagonal to the image. Scale bar is in micrometers.

was reorganised parallel to the direction of flow. On layers less than 3 μm thick, most bacteria were dead (red), but inside the aggregates, the bacteria stained with the live-stain (green). This may reflect biocide usage in the paper machine, lethal to single cell layers but allowing multiple cell layers to survive. The bacteria were uniform-looking cocci of 1.5 μm size.

Influence of surface finishing on biofouling of stainless steel

Stainless steel coupons with different surface finishings were analysed for biofouling in the flow cell (Figure 1) in-mill. The accumulated biofilm was quantified by ATP content and by uptake of the nucleic acid stain syto 9. The results shown in Figure 9 indicate that the steel coupons finished to grit 120 accumulated twice as much biofilm in 6 days as those finished to grit of 500 with or without electrolytic polishing. After an exposure of 12 days, the amount of biofilm had increased but the difference persisted: 125 pmol of ATP cm^{-2} on steels of 120 grit compared to 63–73 pmol of ATP cm^{-2} on steels of 500 grit, with or without electrolytic polishing. Fluorescence emission from 6-day biofilms stained with syto 9 were lower (210 rfu cm^{-2}) for the steels finished to 500 grit and electrolytically polished than for the same brand of steel not electrolytically polished (Figure 9). This indicates that electrolytic polishing may further reduce the already low tendency of biofouling observed for steels of 500 grit.

ATP content of the biofilm on stainless steels increased from 4–10 pmol cm^{-2} to 60–130 pmol in 12 days (Figure 9). Dry weight of the paper machine biofilm bacteria was 5.5×10^{-14} g $cell^{-1}$ and the ATP content was 111 μg g^{-1} of biomass (dry weight) [16]. Based on this, the accumulated biofilm would have contained 0.18–0.48 g of bacteria (dry weight) m^{-2} of steel surface after 6 days and 10 times more after 12 days.

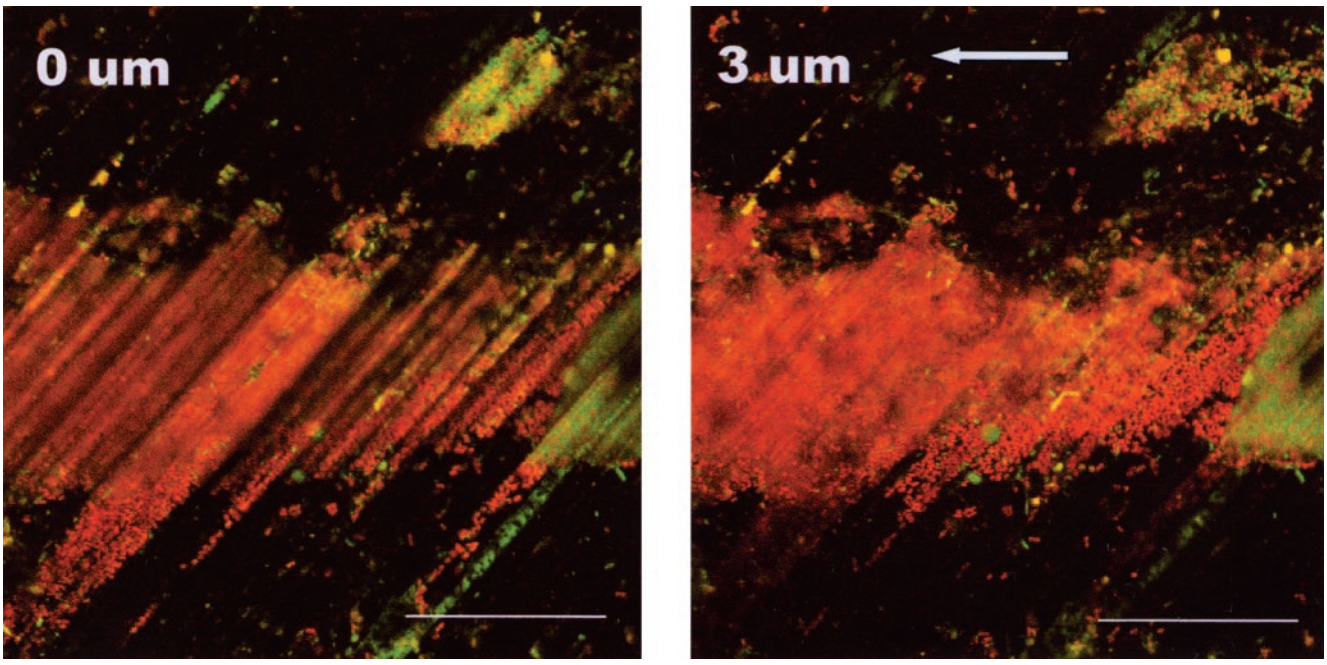

Figure 8 Optical sections (1 μm) of CLSM image of viability-stained biofilm grown for 5 days on a stainless steel coupon in the spray water circuit of a papermill. The viability stain consisted of syto 9 (live/green) and propidium iodide (dead/red). The distance of each optical section from the steel surface is shown in the respective panel. The arrow shows the direction of flow. The bar is 50 μm.

These results show that the two different methods, the emission of fluorescence by syto 9-stained biofilms and the ATP content, gave parallel results for quantifying the biomass up to 12-day-old paper machine biofilms. The limit of detection was 10^2 cells cm^{-2} for the fluorescence method. Fluorescence measurements can therefore be used as an indicator for the degree of biofouling when the biofilm contains between 5 and 100 pmol of ATP cm^{-2} of steel surface, equivalent to 5–100 g (wet weight) of biofilm m^{-2}.

SEM was used to inspect details of bacterial attachment to differently polished grades of stainless steel. Figure 10 shows that the steel surface finished to 120 grit had grooves sheltering one to three layers of bacteria, whereas the steel polished to 500 grit sheltered only a monolayer of bacteria. Electrolytic polishing further flattened the grooves on the steel surface so that bacterial attachment no longer was guided by the polishing grooves (bottom panel in Figure 10). Figure 10 also shows that the 120 grit surface allowed growth of bacterial clusters on top of the grooves, whereas the 500 grit with or without electrolytic polishing accommodated only small clusters and mostly single bacterial cells.

The SEM results thus confirm the conclusions based on the CLSM of fluorescently stained biofilms (Figure 7) on the same steels.

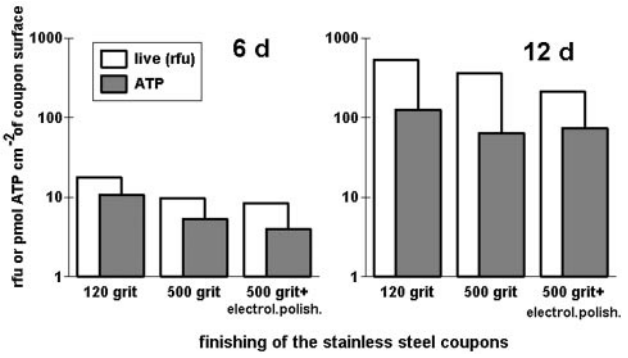

Figure 9 The ATP content and fluorescence emission (relative fluorescence units, rfu) of syto 9-stained biofilms on surfaces of stainless steel coupons with different finishings. The steels were finished to 120 and 500 grit with or without electrolytic polishing. The biofilms were grown on coupons placed in the flow cell (Figure 1) connected to the wet end spray water circuit of a paper machine for 6 or 12 days. Average temperature of the spray water was 45°C (6 days) and 44°C (12 days). Note the semi-logarithmic scale.

Using the flow cell for in situ testing of the efficacy of an antifoulant

Two flow cells were connected in series to the wire spray water circuit of a paper machine. An antifoulant, Spectrum™ NT2001 (Hercules Inc.; containing sulfosuccinate as the active component), was dosed (continous 20 ppm) into the downstream flow cell, the upstream flow cell serving as reference for biofilm formation at this site of the printing paper machine.

Biofilms that formed on the stainless steels in the dosed and in the undosed flow cells are shown in Figure 11. There was 54% more biofilm ATP on the coupons of the undosed as compared to the dosed flow cell. Efficacy of the antifoulant persisted beyond 7 days of exposure (Figure 11). The coupons were inspected with SEM for details of the bacterial colonisation. Micrographs taken after 3 days of exposure (Figure 12) show large numbers of attached bacteria on the stainless steels, similarly on the coupons in the dosed or the undosed flow cells. Coupons exposed for 7 days in the flow cell to machine spray water were more intensively covered by biofilm in the absence of the antifoulant compared to coupons in the flow cells dosed with 20 ppm of the selected antifoulant (sulfosuccinate). Bare areas of steel were visible on steels exposed

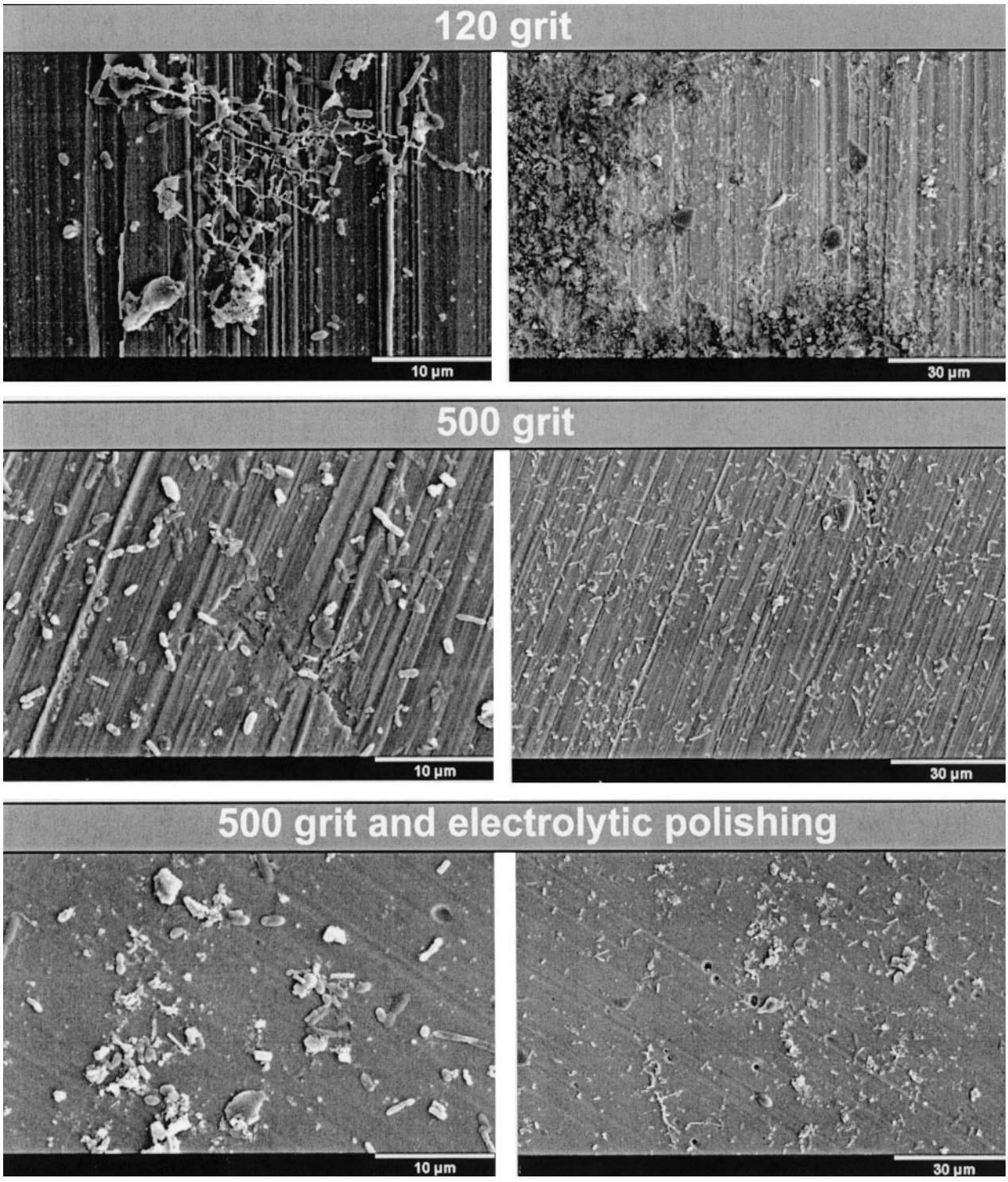

Figure 10 SEM micrographs of surfaces of stainless steel coupons with different finishings after 12 days of exposure to paper machine wet end spray water circuit in the flow cell. Micrographs of steels polished to 120 grit show larger communities than the more extensively polished steels (compare top panel to lower panel). The surface of the steels with higher quality finishing carried fewer clusters and more individually attached cells. The mill biocide used at the time of the experiment was glutaraldehyde.

in the dosed flow cell (bottom panel in Figure 12). Fines and fibers adhering to the biofilm were more abundant on the coupons exposed in the undosed flow cell. The coupons shown in Figure 12 were finished to approximately 100 grit while the Figure 10 surfaces were 120–500 grit. The more intensive growth in the experiment displayed in Figure 12 as compared to Figure 10 reflects the greater roughness of the steel coupons. The mill biocide was glutaraldehyde during the experiment in Figure 10. It is known to affect a larger spectrum of microorganisms than 2,2-dibromo-3-nitrilopropionamide, which was used in the mill during the

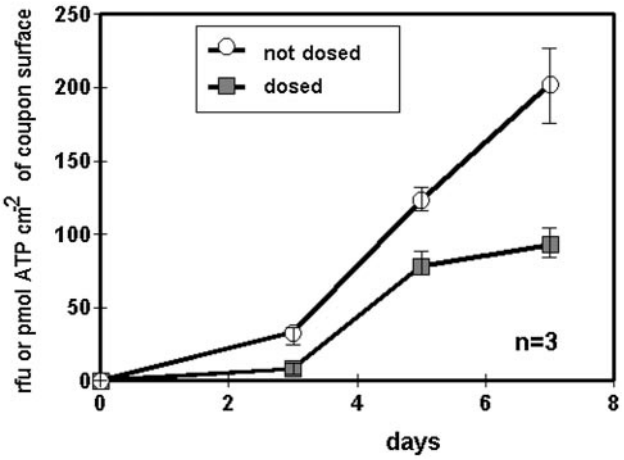

Figure 11 Effect of antifoulant dosing on the ATP contents of biofilms accumulating on stainless steel coupons in flow cells connected to paper machine wet end spray water. One of the two identical flow cells (Figure 1) was experimentally dosed with an antifoulant, Spectrum NT2001, of which the active component was sulfosuccinate. The figure shows average ATP contents with the standard deviations. The mill main biocide used at the time of this experiment was 2,2-dibromo-3-nitrilopropionamide. Average temperature of the spray water was 42.5°C.

experiment in Figure 12. This situation may have contributed to the lesser biofouling in Figure 10 as compared to Figure 12.

Discussion

The biofouling studies reported here were executed under authentic conditions inside a paper machine and under a high hydraulic flow of genuine mill water. Earlier studies involving paper machines were conducted in the laboratory using "slime inoculum" of paper machine origin [2,13]. Klahre and Flemming [8] reported an on-line *in situ* monitoring of biofouling in process waters from a paper mill. They analysed slime or biofilm formation by differential turbidity and pressure drop measurements in the mill, giving on-line information of total slime formation but not allowing for analysis of the biofilm architecture.

This research demonstrates that the construction chosen for the flow cells was sufficiently robust to allow continuous use under the varying conditions of flow and chemical usage prevailing in the paper mill. Microscopic inspection revealed that the clusters in 5- to 7-day-old biofilms consisted of clusters of morphologically similar cells but the cell type varied from cluster to cluster. The high hydraulic flow at the machine (1.8 m s^{-1}) may explain why the thickness of the biofilm clusters grown in 7 days was often limited to 6 to 10 μm. The biofilm clusters may thus represent colonies originating from a single adhered cell or several cells identical in size and surface properties. The visual diversity of bacteria observed in the present study was lower than in the study by Elvers *et al* [5] who found a diversity of bacteria and fungi in biofilms of the photo processing industry. We observed only three morphologically distinguishable types of biofilm bacteria: uniformly coccoid cells, filaments or short rods. We were not able to confirm whether the long filamentous microorganisms (Figure 4) were truly bacterial in nature. Väisänen *et al* [17,16] showed that the diversity of bacteria in a papermaking process appeared lower when the samples were cultivated at 50°C than the diversity obtained at 28°C.

The interior of the biofilm clusters excluded both the hydrophobic and the hydrophilic latex beads of 0.02 μm in diameter. This is similar to what was observed for Baltic Sea water-grown biofilms [9]. We used tetramethylrhodamine-conjugated con A to label glucose- or mannose-containing exopolysaccharides, and found con A-reactive yeast like cells but no biofilm bacteria. In addition, con A reacted with the amorphous paper machine fines. The presence of mannose and glucose was to be expected in the fines as cellulose and hemicellulose contain these sugars. Lindberg *et al* [10] found glucose and mannose in deposits collected from the same paper machine as in this study. Our results (Figure 3) indicate that these sugars may have been contributed by the fines of wood origin adhering or sorbed into the slime, not necessarily being of microbial origin. Laboratory-grown slime from pure cultures of *Burkholderia cepacia* isolated from this printing paper machine contained around 50% of mannose and glucose of the total carbohydrate [10]. Based on the biofilm anatomy *Burkholderia* spp. were unlikely to be a major coloniser of the mill-grown biofilms (Figures 2–8).

We observed that the high flow (1.8 m s^{-1}) strongly guided the shape of the biofilm clusters emerging after primary attachment. Scheuerman *et al* [15] observed that the presence of grooves perpendicular to the flow resulted in preferential attachment of cells on the downstream edges of the groove and to lesser extent on the upstream edges. They found that when the flow rate was increased from 0.02 to 0.08 m s^{-1}, the rate of attachment increased, with a decrease in the importance of position relative to the grooves. Our results show similar positioning of the bacteria with respect to grooves, although the grooves (1–5 μm) in our 120 and 500 grit polished stainless steel coupons were smaller than the 10- to 40-μm-wide grooves analysed by Scheuerman *et al* [15]. Continuous high-velocity flow (>1.5 m s^{-1}) has been used to minimise the settling out of solids and the concern for microbe-induced corrosion [6]. The flow rates we used were much higher than those by Scheuerman *et al* [15] and near the lower limit of Geesey [6], but our findings on the patterns of attachment were similar. The high hydraulic flow at the machine (1.8 m s^{-1}) may explain why the biofilm thickness seemed limited to 6–10 μm.

We show that the biofilm ATP could be extracted directly from the biofouled stainless steel surface and used as an indicator of biomass in paper machine biofilms. Gracia *et al* [7] compared ATP yields obtained by different methods of extraction from biofilms on polystyrene plates and decided that dimethyl sulphoxide was superior. Use of dimethyl sulphoxide requires the extract to be diluted before ATP assay to avoid luciferase inhibition. The ATP contents of our steel coupons were low because of the thin biofilm, not allowing dilution. Boiling with Tris buffer not only extracted ATP but also mechanically demolished the tightly steel-bound cell clusters.

The results obtained with one antifoulant indicated that the flow cell (Figure 1) used in this study is an excellent tool for investigating the efficacy of antifoulants under mill conditions. The same antifoulant as used here was earlier tested in the side stream of a tissue mill with an 80% reduction in biofilm biomass [14]. This is close to our result (54%). The novel flow cell also proved an efficient tool for investigating the relation between steel surface smoothness and the tendency to biofoul, revealing the effects of roughness (500–100 grit) and of electrolytic polishing. Arnold and Bailey [1] reported with SEM inspection that the biofilm formation was lower for steels with an electrolytically

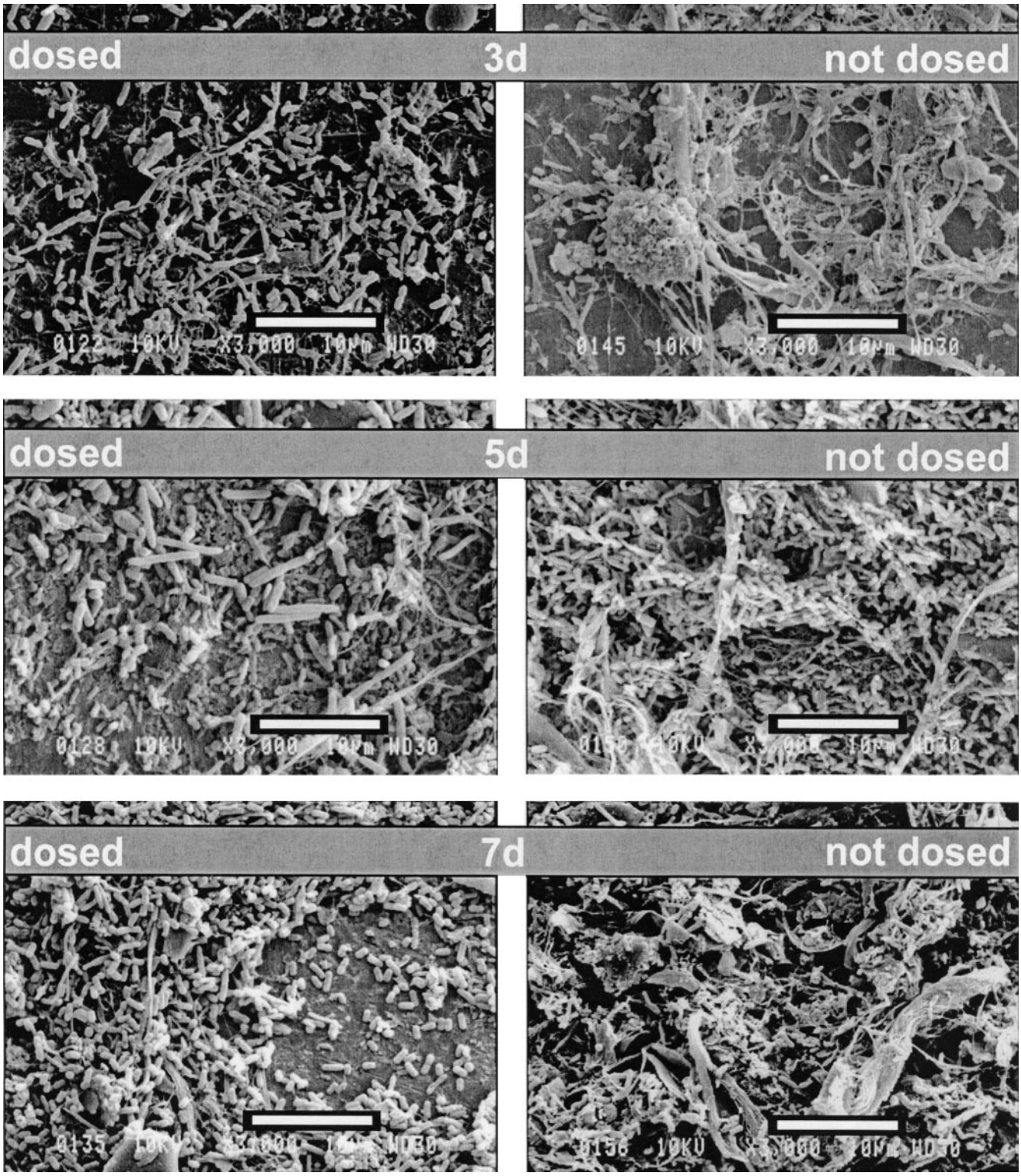

Figure 12 SEM micrographs of surfaces of stainless steel coupons placed in the flow of paper machine wet end spray water in mill for 3, 5 and 7 days. The down stream flow cell was experimentally dosed with 20 ppm (continuous) of the antifloulant Spectrum NT2001 (active component sulfosuccinate). In addition, the spray water to both flow cells contained the biocide 2,2-dibromo-3-nitrilopropionamide routinely used in the mill at the time of the experiment. The bar in all graphs is 10 μm.

polished surface than for non-polished. They used poultry process inoculum in the growth media and no flow, very different from the low nutrient and high flow applied in our experiments.

A further innovation in this study was the use of a scanning fluorometer, marketed for monitoring microtiter plates, for direct scans of fluorescently stained biofilm on coupons of stainless steel.

This method allowed rapid gathering of quantitative and qualitative information on the live biofilm still on its original surface, by using specific stains.

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