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ORIGINAL PAPER

Biofilm-forming bacteria with varying tolerance to peracetic acid from a paper machine

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Abstract Biofilms cause runnability problems in paper machines and are therefore controlled with biocides. Peracetic acid is usually effective in preventing bulky biofilms. This study investigated the microbiological status of a paper machine where low concentrations (≤15 ppm active ingredient) of peracetic acid had been used for several years. The paper machine contained a low amount of biofilms. Biofilm-forming bacteria from this environment were isolated and characterized by 16S rRNA gene sequencing, whole-cell fatty acid analysis, biochemical tests, and DNA fingerprinting. Seventy-five percent of the isolates were identified as members of the subclades *Sphingomonas trueperi* and *S. aquatilis*, and the others as species of the genera *Burkholderia* (*B. cepacia* complex), *Methylobacterium*, and *Rhizobium*. Although the isolation media were suitable for the common paper machine biofoulers *Deinococcus*, *Meiothermus*, and *Pseudoxanthomonas*, none of these were found, indicating that peracetic acid had prevented their growth. Spontaneous, irreversible loss of the ability to form biofilm was observed during subculturing of certain isolates of the subclade *S. trueperi*. The *Sphingomonas* isolates formed monoculture biofilms that tolerated peracetic acid at concentrations (10 ppm active ingredient) used for antifouling in paper machines. High pH and low conductivity of the process waters favored the peracetic acid tolerance of *Sphingomonas* sp. biofilms. This appears to be the first report on sphingomonads as biofilm formers in warm water using industries.

Keywords *Sphingomonas trueperi* · *Sphingomonas aquatilis* · Biofilm · Peracetic acid · Paper machine

Introduction

Peracetic acid, the peroxide of acetic acid, is widely used as a disinfectant. Its antimicrobial action is presumably based on the oxidation of thiol groups in proteins [3, 21], disruption of membranes [3, 21, 39], or damage to bases in DNA [3], and peracetic acid was reported to increase the sensitivity of bacterial spores to heat [27]. It is effective at temperatures from −40 to +85°C and degrades to acetic acid, hydrogen peroxide, and water, leaving no toxic residue [3]. The efficacy and environmental safety of peracetic acid make it an attractive disinfecting agent for industrial use.

The moisture, warmth, and supply of nutrients in paper machines are suitable for many bacteria. Bacterial biofilms on wet machine surfaces can cause runnability problems and therefore are controlled with antifouling agents (biocides). Peracetic acid is used for disinfecting patient-care equipment, such as dialyzers and endoscopes, where water volumes are low and high concentrations of biocide can be used; e.g., a Centers for Disease Control and Prevention

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guideline [40] recommends the use of peracetic acid at a concentration of 0.2%. Peracetic acid concentrations below 10 ppm were reported to be effective for disinfecting wastewaters [21, 52]. Low concentrations (up to 10 ppm) of peracetic acid are commonly used in paper machines, but its efficacy in this or other industrial environments has not been reported.

This paper reports on the microbial status of a paper machine in which a low concentration (≤15 ppm of the active ingredient) of peracetic acid was used continuously. Biofilm formation by *Sphingomonas* spp. and peracetic acid tolerance of *Sphingomonas* monoculture biofilms in paper machine process waters are described.

Materials and methods

Culture media and chemicals

R2A and Brain Heart Infusion (BHI) agars were from BD (Franklin Lakes, NJ, USA). Tryptic soy broth agar (TSBA) was tryptic soy broth (BD, Franklin Lakes, NJ, USA) supplemented with agar 15 g l^{-1}. White water broth was authentic paper machine white water from the sampled machine supplemented with soluble potato starch 1 g l^{-1} (Sigma–Aldrich, St. Louis, MO, USA) and yeast extract 0.5 g l^{-1} (Biokar Diagnostics, Beauvais, France). White water agar was white water broth with 15 g of agar l^{-1}. R2 broth was prepared in natural spring water (Tuus-Lähde, Kerava, Finland) as described by Hunt and Rice [18] without agar addition. The other media were prepared in Milli-Q (Millipore, Billerica, MA, USA) ultrapure water. The pH of acidified agars was adjusted with HCl.

Peracetic acid tolerance of biofilms was tested by using a technical product, Fennosan PAA (Kemira, Oulu, Finland), that contains 15% (w/w) peracetic acid and 13–16% (w/w) hydrogen peroxide. The other chemicals were of analytical grade and obtained from local suppliers.

Paper machine sampling

Biofilms were harvested from wet surfaces of an acidic fine paper machine with a production capacity of 30,000 tons/year where 50–100 ppm of a peracetic acid-containing biocide was continuously dosed in the process water. The sampled surfaces were in the wire area (two different surfaces), the broke storage tank and the water flotation tank. Temperatures of the sampled surfaces, measured with a non-contact infrared thermometer with laser sighting (Raynger® ST™, Raytek, Santa Cruz, CA, USA), were from 28 to 40°C. The pH of the white water ranged from 3.5 to 5.6. Process waters were sampled from the headboxes of two acidic fine paper machines that used a peracetic acid-containing biocide. The samples were stored at −20°C until used. Redox potential was measured with a Pt electrode with Ag/AgCl as the reference (Thermo Electron, Waltham, MA, USA), conductivity with a PW 9529 meter (Philips, Eindhoven, Netherlands), dissolved organic carbon (DOC; 0.2 µm filtered) with a total organic carbon analyzer TOC-5000 (Shimadzu, Kyoto, Japan), and ATP luminometrically (Lumitester C-100, Kikkoman, Chiba, Japan) using the ATP Biomass Kit HS (BioThema, Handen, Sweden). Al, Fe, Mn, Cu, Cr, Ni, and Ti were measured with an inductively coupled plasma spectroscopy analyzer (Perkin Elmer Plasma 3200, Wellesley, MA, USA).

Isolation and characterization of biofilm-forming bacteria

Isolates from biofilm-derived inocula were obtained by using oligotrophic media, as reported previously [23]. The media were R2A agar pH 7.2 or acidified to pH 5.5 and white water agar (pH 5.5), grown for 3–5 days at 37°C. Rich medium (BHI agar, acidified to pH 5.5) was used to detect contaminants. Biofilm formation of the isolates was tested in R2 or white water broth (pH 5.5) on 24-well polystyrene plates (Nunc, Roskilde, Denmark) incubated for 3 days at 37°C, 160 rpm. The biofilm yield was visualized by staining with crystal violet as described by Kolari et al. [22].

For whole-cell fatty acid analysis, the isolates were grown on TSBA for 3 days at 28°C. Fatty acid methyl esters were prepared and analyzed by using the MIDI Sherlock Microbial Identification System with the TSBA 50 library version 4.5 (MIDI, Inc., Newark, DE, USA) as instructed by the manufacturer.

Full length 16S rRNA gene sequencing was done by using universal primers pA, pD, pE, pD′, pF′, and pH′ of Edwards et al. [13], 518F (5′ → 3′ CCAGCAGCCGCGGTAATACG; Macrogen Inc., Seoul, South Korea), and 800R (5′ → 3′ TACCAGGGTATCTAATCC; Macrogen Inc., Seoul, South Korea). The sequences were assigned to genus using the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/) release 10.11 Classifier tool (naïve Bayesian rRNA classifier, release 7.8; http://rdp.cme.msu.edu/ [49]) with a bootstrap value of 80% as the confidence threshold value. Closest matching sequences were searched for from the nucleotide (nr/nt) sequence database using the megablast program (optimized for highly similar sequences) of the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/) release 2.2.24 [53] of the National Center for Biotechnology Information. The sequences were deposited in the GenBank sequence database under accession numbers HM484354–HM484373.

Ribopattern analysis of whole-cell DNA was done as described by Busse et al. [7] with biomass grown on R2A agar (pH 7) for 3 days at 37°C. Restriction with *Eco*RI and

fragment analysis were done with an automated ribotyper (RiboPrinter® Microbial Characterization System, DuPont Qualicon, Wilmington, DE, USA) using the RiboExplorer® software version 2.1.4216.0.

Gram staining was done according to the standard method described by Hunt and Rice [18] and catalase, oxidase, and hydrolysis of starch tested as described by McFaddin [30] with colonies grown on R2A agar (pH 7, supplemented with 10 g of soluble starch l^{-1} for starch hydrolysis test) for 3–13 days at 37°C. Motility was examined with phase contrast microscopy. Biochemical reactions were tested with API 20 NE test strips (bioMérieux, Marcy l'Etoile, France). Tolerance to 3% NaCl was tested in R2 broth supplemented with 30 g of NaCl l^{-1} for 5 days at 37°C and 160 rpm.

Microplate biofilm assays and biocide tolerance of the biofilms

Biofilm production by the pure isolates was quantified on 96-well polystyrene microplates (cell culture treated F96 MicroWell™ Plates, Nunc, Roskilde, Denmark) in acidified R2 broth (pH 5.5). The wells of the plates, grown for 1–3 days at 37°C and 160 rpm, were emptied, washed, stained, and measured by the method of Kolari et al. [22, 23, 36]. The absorbance ($A_{595nm} \leq 0.1$) of non-inoculated control wells, caused by well-adhered crystal violet, was subtracted.

Biocide tolerance of the pure culture biofilms was tested with biofilms pre-grown on the spikes of 96-well microwell plate lids (Nunc-Immuno™ TSP, Nunc, Roskilde, Denmark) from inocula of 1,300 ± 100 cfu/well. Biofilms on the spikes were exposed to 0, 25, 50, 75, or 260 ppm of the biocide (technical product) in wells of a second microplate filled with paper machine process waters or sterilized spring water for 4 h at 20 ± 2°C, no shaking. After exposure, the viability of the biofilm on the spikes was tested at 37°C in wells of a third microplate filled with sterile R2 broth at pH 7.0 or acidified to pH 5.5. After 18–20 h, the resulting regrown biofilm was measured as above.

Statistical analysis

Principal component analysis of the whole-cell fatty acid compositions was carried out with the Sherlock Command Center software (version 4.5, Midi Inc., Newark, DE, USA). A linear regression model was used to analyze the associations of the chemical features of the process waters with increased peracetic acid tolerance of the biofilms. Tolerance to the concentration of biocide dosed at the onset of exposure was used as the response variable. Pearson correlations between the variables were also calculated.

Results

Biofilms were harvested from surfaces of an acidic (pH 3.5–5.6) fine paper machine where 50–100 ppm of a biocide product containing 15% (w/w) of peracetic acid and 13–16% (w/w) of hydrogen peroxide was regularly dosed into the white water. Pure cultures ($n = 60$) were isolated from the biofilms on acidified (pH 5.5) R2A agar and white water agar. All isolates were gram-negative rod-shaped bacteria. On the basis of principal component analysis of the fatty acid compositions of whole cells, the isolates formed six groups (Table 1). The isolates of groups A ($n = 39$) and B ($n = 6$) were superior as biofilm formers compared with the other groups when tested as pure cultures (Fig. 1). Groups A and B were rich in 2-hydroxy-tetradecanoic acid (7–18%) and contained no 3-hydroxy acids (Table 1). These are features typical for sphingomonads [6]. The colonies of groups A, B, and F on R2A agar were yellow, group C pale yellow, group D pink, and group E grayish white. *Sphingomonas* species often grow as yellow or orange-pigmented colonies [51], although is not a characteristic feature of all sphingomonads [6]. The tested isolates ($n = 19$) of groups A–E were catalase-positive. None of the fatty acid compositions of the isolates were similar to those of the genera *Deinococcus*, *Meiothermus*, or *Pseudoxanthomonas*, even though the growth media used for isolation were suitable for these [11, 14, 23], indicating that these genera were not prevalent in the sampled paper machine.

The complete 16S rRNA gene of 20 of the isolates was sequenced. The RDP Classifier tool showed with 100% confidence that the isolates of groups A and B were members of the genus *Sphingomonas*. The isolates of group A had highest sequence similarity (>98.5% in BLAST) to type strains of *S. trueperi*, *S. pituitosa*, and *S. azotifigens* (Table 2) and also to "*Sphingomonas elodea*" ATCC 31461 (species name not approved). Sequences of the group B isolates were most similar (>97% in BLAST) to the type strains of *S. aquatilis*, *S. panni*, and *S. desiccabilis* and a strain of *S. melonis* (Table 2). Phenotypic characteristics of these strains were compared with those of the respective type strains (Table 3). The results in Tables 2 and 3 show that the isolates of groups A and B (Table 1) belonged to the *S. trueperi* and *S. aquatilis* subclades of the genus *Sphingomonas*.

Isolates of group C (Table 1) were classified as members of the genus *Burkholderia* with 100% confidence by the RDP Classifier and had highest 16S rRNA gene sequence similarity to the following species of the *B. cepacia* complex: *B. cepacia*, *B. cenocepacia*, *B. multivorans*, and *B. vietnamiensis* (≥99.0% in BLAST; Table 2). The whole-cell fatty acid compositions of the group C isolates (Table 1) show that 2-hydroxy-tetradecanoic acid was

Table 1 Whole-cell fatty acid compositions of biofilm-forming bacterial isolates from biofilms of a paper machine treated with peracetic acid

Isolates	Percent (w/w) of fatty acid[a] (range)											
	14:0	14:0 2OH	14:0 3OH/ 16:1 iso I[b]	16:0	16:0 3OH	16:1 w7c/ 15:0 iso 2OH[b]	17:1 w6c	18:0	18:0 3OH	18:1 w7c	11 methyl 18:1 w7c	19:0 cyclo w8c
Group A ($n = 39$)	0.4 (<0.1–2.3)	9.7 (7.4–12.7)	<0.1 (<0.1)	20.4 (12.0–24.1)	<0.1 (<0.1)	0.2 (<0.1–1.2)	0.7 (<0.1–3.4)	1.3 (<0.1–2.3)	<0.1 (<0.1)	60.0 (49.7–66.3)	5.2 (1.6–7.3)	<0.1 (<0.1–0.9)
Group B ($n = 6$)	1.0 (0.5–1.6)	16.0 (13.8–18.1)	<0.1 (<0.1)	29.3 (25.0–36.0)	<0.1 (<0.1)	1.0 (0.8–1.2)	1.0 (0.5–2.0)	1.4 (1.0–1.8)	<0.1 (<0.1)	41.6 (35.9–47.8)	6.1 (5.1–6.8)	0.9 (0.6–1.4)
Group C ($n = 9$)	3.9 (2.9–4.3)	0.3 (<0.1–1.4)	4.6 (4.0–5.5)	28.2 (25.5–32.8)	4.4 (3.8–5.5)	18.2 (14.1–23.1)	<0.1 (<0.1)	1.2 (0.7–1.8)	<0.1 (<0.1)	29.9 (23.0–33.9)	<0.1 (<0.1)	3.2 (<0.1–7.7)
Group D ($n = 3$)	<0.1 (<0.1)	<0.1 (<0.1)	4.5 (4.0–4.9)	3.4 (2.2–5.4)	<0.1 (<0.1)	0.7 (0.5–0.9)	<0.1 (<0.1)	5.3 (4.7–6.0)	2.5 (2.3–2.7)	82.8 (80.3–84.5)	<0.1 (<0.1)	0.9 (0.6–1.2)
Group E ($n = 2$)	<0.1 (<0.1)	<0.1 (<0.1)	3.2 (1.8–4.5)	6.2 (6.2)	5.4 (3.7–7.0)	0.8 (0.4–1.1)	<0.1 (<0.1)	5.2 (1.4–8.9)	1.7 (1.6–1.8)	70.3 (69.9–70.8)	1.1 (0.7–1.4)	2.5 (2.2–2.8)
Group F ($n = 1$)	0.6	2.0	<0.1	6.2	<0.1	0.8	9.1	0.6	<0.1	70.3	3.0	<0.1

The average and range (min–max) are given. The division into groups A–F was based on principal component analysis (not shown)

[a] IUPAC names of the fatty acids are: 14:0, tetradecanoic acid; 14:0 2OH, 2-hydroxy-tetradecanoic acid; 14:0 3OH, 3-hydroxy-tetradecanoic acid; 16:1 iso I, iso branched pentadecenoic acid (isomer unknown); 15:0 iso 2OH, 2-hydroxy-13-methyltetradecanoic acid; 16:1 w7c *cis*-9-hexadecenoic acid; 16:0 3OH, 3-hydroxy-hexadecanoic acid, 17:1 w6c, *cis*-11-heptadecenoic acid; 18:0, octadecanoic acid; 18:0 3OH, 3-hydroxy-octadecanoic acid; 18:1 w7c, *cis*-11-octadecenoic acid; 11 methyl 18:1 w7c, 11-methyl-*cis*-11-octadecenoic acid; 19:0 cyclo w8c, *cis*-11-cyclopropylnonadecenoic acid

[b] Reported as a summed feature due to imperfect peak separation during chromatography

practically absent but 3-hydroxy-tetradecanoic acid and 3-hydroxy-hexadecanoic acid were abundant (4–5%). This matches with the identification of the isolates as members of the betaproteobacterial *B. cepacia* complex species other than *B. pyrrocinia*, *B. ambifaria*, or *B. vietnamiensis* [25].

The 16S rRNA gene sequence of isolates PVL19 (group D), R2S6 (group E), and PVL18 (group F) revealed no close similarity (>98% in BLAST) to any validly described species of bacteria (Table 2). The fatty acid profile of isolate PVL18 (presence of 2-hydroxy-tetradecanoic acid and absence of 3-hydroxy acids; Table 1) and the RDP Classifier result (*Sphingomonas* with 99% confidence) supports its classification as a *Sphingomonas* sp. The strain PVL18 was catalase-negative, whereas sphingomonads generally are catalase-positive [51]. The isolates of group D were members of *Methylobacterium* and those of group E were members of *Rhizobium*, as defined by the RDP Classifier (confidence 100%).

DNA fingerprinting analysis was done to shed light on intraspecies diversity among the biofilm-derived isolates. Four different ribopatterns were found for ten isolates identified as members of the subclade *S. trueperi* (Fig. 2). Three isolates of the *S. aquatilis* subclade (R2S2, R2S8, PVS14) were identical in ribopatterns. Three isolates belonging to the *B. cepacia* complex (R2S13, R2S14, PVL20) all had different ribopatterns (Fig. 2). None of the ribopatterns matched those of the respective type strains of *Sphingomonas* or of the species of the *B. cepacia* complex.

Conclusions based on the results obtained with the different tools used for identifying 20 isolates from the peracetic acid-tolerant biofilms of the sampled paper machine are as follows. Eleven isolates represented four different genotypes of the alphaproteobacterial genus *Sphingomonas*, *S. trueperi* subclade. Three isolates were indistinguishable from each other by sequencing or fingerprinting and belonged to the *S. aquatilis* subclade. Three isolates were members of the betaproteobacterial *B. cepacia* complex. The remaining isolates represented novel alphaproteobacterial species related to the genera *Sphingomonas*, *Methylobacterium*, or *Rhizobium*.

Seven of the densely yellow isolates of the *S. trueperi* subclade shed progeny that grew as translucently yellow colonies when pure cultures were subcultured on R2A agar (pH 5.5 and pH 7; S1) or on acidified BHI (pH 5.5) agar. The bright yellow colony and its translucent counterpart (S1) were mucoid, possessed identical whole-cell fatty acid compositions, 16S rRNA gene sequences (Table 2), and ribopatterns (Fig. 2), and were identical in biochemical (Table 3) microscopic properties. The bright yellow but not the translucent type formed biofilm on a polystyrene microplate in R2 broth (pH 5.5) (Fig. 1). Changes in the colony morphology and the loss of biofilm-forming ability were concurrent and irreversible but both colony types were

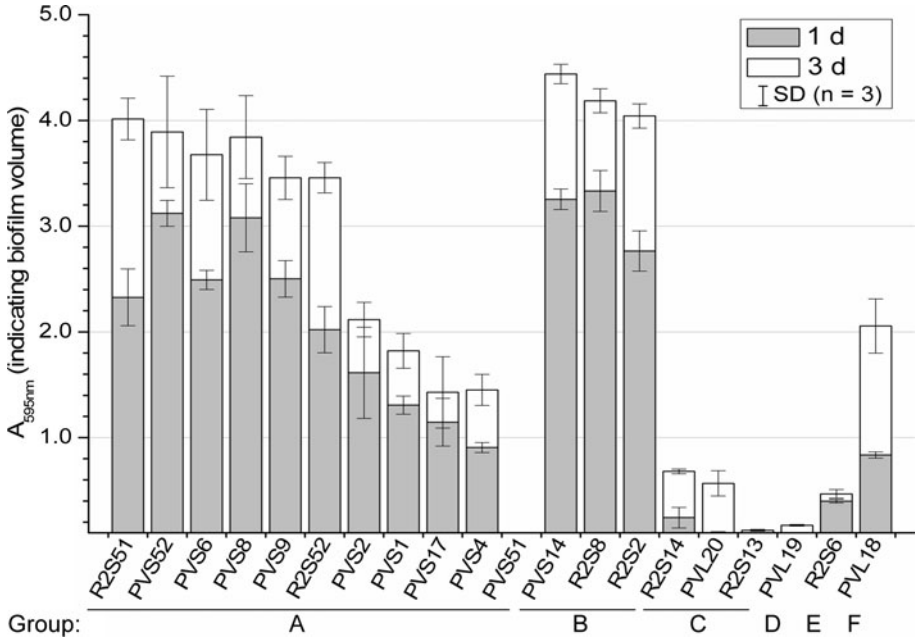

Fig. 1 Biofilm formation by isolates from biofilms of a paper machine treated long term with peracetic acid. Biofilm formation was measured in R2 broth (acidified to pH 5.5) on polystyrene microplates at 37°C (1–3 days, 160 rpm), quantified after staining with crystal violet. The groups are as shown in Table 1. The non-biofilm-forming isolate PVS51 was isolated as a variant of the isolate PVS52 (Fig. 3)

equally mucoid (Electronic supplementary material, Fig. S1). The results show that these sphingomonads can readily undergo notable phenotypical changes and lose the ability to form biofilm when subcultured in the laboratory.

The isolates representing different genotypes of *Sphingomonas* (five different ribopatterns, Fig. 2) were tested for peracetic acid tolerance when grown as monoculture biofilms. The pre-grown biofilms were exposed to 0–260 ppm of the biocide product (0–40 ppm of peracetic acid) in sterile spring water. The results (Fig. 3) show that one of the isolates (PVS17) survived 260 ppm of the biocide (ca. 40 ppm peracetic acid) when exposed as a pre-grown biofilm. All representatives of the different genotypes ($n = 5$) survived exposure to 75 ppm of the biocide product (approximately 10 ppm of peracetic acid, initial pH 4.7) as pre-grown biofilms. The results (Figs. 1, 3) show that the isolates that produced largest volumes of biofilm were not those that were most resistant to peracetic acid. When the peracetic acid tolerance of one pre-grown monoculture biofilm was investigated in 30 independently sampled paper machine white waters (Table 4), the tolerance to peracetic acid was influenced by the water used for diluting the biocide. Figure 4 shows that the biofilm (A_{595nm} of pre-grown biofilm 0.33) of *Sphingomonas* sp. R2S2 tolerated 25, 50, or 75 ppm of the peracetic acid-based technical product (approximately 4, 7.5, or 10 ppm of peracetic acid) in 11 out of 15 waters sampled on different days from paper machine 1 (Fig. 4a), whereas the biofilm (A_{595nm} of pre-grown biofilm 0.41) survived similar peracetic acid treatments only in 4 out of the 15 waters sampled from paper machine 2 (Fig. 4b). Twelve different chemical parameters were measured from these process waters (Table 4) to find an explanation for this difference. Table 4 shows that the waters from both machines were highly oxidized, redox potentials ranging from 200 to 300 mV vs. Ag/AgCl (equivalent to ca. 160–260 mV vs. $Hg/HgCl_2$). This is natural because both machines used peracetic acid for antifouling. One parameter, conductivity, was found to differentiate the two machines. The conductivity of waters collected from paper machine 1 averaged 333 μS cm^{-1} (range 240–430 μS cm^{-1}) compared with 472 μS cm^{-1} (range 340–750 μS cm^{-1}) of waters from paper machine 2. This indicates that low salt content favored tolerance of the *Sphingomonas* monoculture biofilm to peracetic acid. Also high pH of the waters was associated ($P < 0.05$) with increased tolerance to peracetic acid, possibly reflecting the lower biocidal effect of the dissociated form of peracetic acid as compared with the protonated form. A one unit increase in pH indeed corresponded approximately to a 100 ppm increase in the tolerated concentration of biocide, but the difference in pH only explained 20% of the observed results ($r^2 = 0.18$). The Pearson correlation between pH and the tolerated concentration of biocide was 0.42 ($P < 0.05$), which supports the results obtained by using the linear regression model. The other four chemical parameters (redox, dissolved organic carbon, Al, Fe) that differed between machines 1 and 2 showed no statistically significant correlation to peracetic acid tolerance in the linear regression model.

Discussion

This study showed that biofilm-forming bacteria from a paper machine treated with peracetic acid were mainly sphingomonads. Only thin biofilms accumulated at a few

Table 2 16S rRNA gene sequence comparison data of biofilm-forming bacterial isolates from biofilms of a paper machine treated with peracetic acid

Sequence accession number and length (nt) in GenBank			16S rRNA gene sequence comparison	
Isolate	Acc. no.	nt	Alignment length nt	Closest match as similarity % in BLAST search to species[a]
Group A				
R2S51	HM484355	1,373	1,354	99.7: *Sphingomonas azotifigens*; 99.5: *S. pituitosa*, *S. trueperi*
R2S52	HM484356	1,299	1,300	99.7: *S. azotifigens*; 99.6: *S. trueperi*, *S. pituitosa*
PVS1	HM484361	1,404	1,404	99.1: *S. azotifigens*, *S. trueperi*; 98.8: *S. pituitosa*
PVS2	HM484362	1,411	1,405	99.5: *S. pituitosa*, *S. trueperi*; 99.4: *S. azotifigens*
PVS4	HM484363	1,368	1,370	99.3: *S. azotifigens*; 99.2: *S. trueperi*; 99.0: *S. pituitosa*
PVS51	HM484364	1,379	1,379	99.5: *S. azotifigens*, *S. pituitosa*, *S. trueperi*
PVS52	HM484365	1,393	1,399	99.1: *S. azotifigens*, *S. pituitosa*, *S. trueperi*
PVS6	HM484366	1,407	1,407	99.1: *S. pituitosa*, *S. azotifigens*, *S. trueperi*
PVS8	HM484367	1,413	1,405	99.7: *S. pituitosa*, *S. trueperi*; 99.6: *S. azotifigens*
PVS9	HM484368	1,426	1,405	99.7: *S. pituitosa*, *S. trueperi*; 99.6: *S. azotifigens*
PVS17	HM484370	1,369	1,372	99.2: *S. azotifigens*, *S. trueperi*; 99.0: *S. pituitosa*
Group B				
R2S2	HM484354	1,422	1,408	97.9: *S. aquatilis*; 97.8: *S. melonis*; 97.6: *S. panni*; 97.5: *S. desiccabilis*
R2S8	HM484358	1,363	1,359	98.1: *S. aquatilis*, 98.0: *S. melonis*; 97.9: *S. panni*
PVS14	HM484369	1,418	1,409	97.7: *S. aquatilis*, *S. melonis*; 97.5: *S. panni*; 97.4: *S. desiccabilis*
Group C				
R2S13	HM484359	1,461	1,462	99.8: *Burkholderia multivorans*; 99.5: *B. vietnamiensis*, *B. cepacia*; 99.2: *B. cenocepacia*
R2S14	HM484360	1,475	1,469	99.7: *B. multivorans*; 99.3: *B. vietnamiensis*, *B. cepacia*; 99.2: *B. cenocepacia*
PVL20	HM484373	1,451	1,455	99.2: *B. multivorans*
Group D				
PVL19	HM484372	1,384	1,364	97.7: *Methylobacterium isbiliense*; 97.4: *M. nodulans*; 97.0: *M. aquaticum*
Group E				
R2S6	HM484357	1,346	1,338	97.2: *R. cellulosilyticum*; 96.8: *R. huautlense*, 96.7: *R. galegae*
Group F				
PVL18	HM484371	1,283	1,289	95.8: *S. pituitosa*; 95.4: *S. azotifigens*, *S. trueperi*

Groups are as shown in Fig. 1 and Table 1

[a] Strain codes and sequence GenBank accession numbers of the species used for comparison: *S. azotifigens* NBRC 15947^T, AB217471; *S. pituitosa* EDIV^T, NR_025363; *S. trueperi* LMG 2142^T, NR_026338; *S. aquatilis* JSS-7^T, NR_024997; *S. melonis* MPU95, AB334774, *S. panni* C52^T, AJ575818; *S. desiccabilis* CPID^T, AJ871435; *B. multivorans* ATCC 17616, AP009387; *B. vietnamiensis* G4, CP000614; *B. cepacia* ATCC 55792, AY741359; *B. cenocepacia* HI2424, CP000459; *M. isbiliense* DSM17168^T, AB302929; *M. nodulans* ORS2060^T, CP001349; *M. aquaticum* GR16^T, NR_025631; *R. cellulosilyticum* CCBAU85045, EU256433; *R. huautlense* SO2^T, NR_024863; *R. galegae* 59A2, AF025853

sites in this machine, indicating that the biocide was effective. The genera reported to be prevalent in biofilms of paper machines and known to grow abundantly on the R2A medium have been described as species of the genera *Deinococcus*, *Meiothermus*, *Bacillus*, *Paenibacillus*, and *Pseudoxanthomonas* [11, 14, 17, 23, 33, 35, 37, 44, 47]. None of these genera were found in the paper machine studied in the present work. *Sphingomonas* and other alphaproteobacteria as well as *Burkholderia* were reported to occur in paper machine waters and slime deposits [47] but not as the dominant biofilm formers. It therefore appears that biofilm formation by the common paper machine biofoulers was inhibited by peracetic acid in the studied paper machine.

The majority of the *Sphingomonas* spp. isolates from the biofilms in the present work tolerated peracetic acid to ca. 10 ppm (active ingredient) as pre-grown monoculture biofilms. This is within the concentration range used at the mill (50–100 ppm of the technical product containing 15 wt.% of peracetic acid). Seventy-five percent of the biofilm

Table 3 Phenotypic characteristics of selected isolates (in bold) from biofilms of a paper machine treated with peracetic acid

Characteristics	Strain												
	PVS1	**PVS2**	**PVS51**	**PVS52**	**PVS17**	*S. azotifigens* NBRC 15497^T	*S. pituitosa* EDIV^T	*S. trueperi* LMG 2142^T	**R2S2**	*S. aquatilis* JSS-7^T	*S. melonis* DAPP PG-224^T	*S. panni* C52^T	*S. desiccabilis* CP1D^T
Motility	+	+	+	+	+	+	+	+	+	+	−	−	−
Catalase	+	+	+	+	+	+	+	+	+	+		+	+
Oxidase	+	+	+	+	+	+	−	+	−	+	+	−	+
Growth in 3% (w/v) NaCl	−	−	−	−	−	−		+	−		+		+
Growth at 45°C	w	w	w	w	−	−	−		−			−	−
Nitrate reduction	−	−	−	−	−	−	−		−	−			+
β-Galactosidase	+	+	+	+	+	+	+	+	w	+	+	+	+
Hydrolysis of													
Starch	+	+	w	w	+	+	−		+		−		+
Esculin	+	+	+	+	+	+	+	+	+	−	+		−
Gelatin	w	−	−	−	−	+	−	+	−	−	−		+
Assimilation of													
Glucose	+	+	+	+	+	+	+	+	+	+	+	−	+
Arabinose	+	+	+	+	+	+	+	+	+	+	+	−	+
Mannose	+	+	+	+	+		+	+	+	−	+	w	+
Mannitol	−	−	−	−	−		−	−	−		−		−
N-Acetyl-glucosamine	+	−	−	−	+		+	+	w	+	+	−	
Maltose	+	+	+	+	+	+	+	+	+	+	+	w	+
Gluconate	−	−	−	−	−	−	−	−	w	−	−	−	
Caprate	−	−	−	−	−			−	−	+	−		
Adipate	−	−	−	−	−	−	−	−	−	−	−		
Malate	−	−	−	−	−	+	+	−	w	−	+		
Citrate	−	−	−	−	−	−	−	−	−	−	−	+	−
Phenylacetate	−	−	−	−	−		−	−	−	−	+		

Data for *Sphingomonas*-type strains with highest 16S rRNA gene sequence similarity to the paper machine isolates are quoted from published sources as follows: *S. azotifigens* [50], *S. pituitosa* [10], *S. trueperi* [19], *S. aquatilis* [26], *S. melonis* [5], *S. panni* [8], and *S. desiccabilis* [38]. All isolates and type strains were negative for the production of indole, arginine dihydrolase, and urease
+ positive; − negative; *w* weakly positive

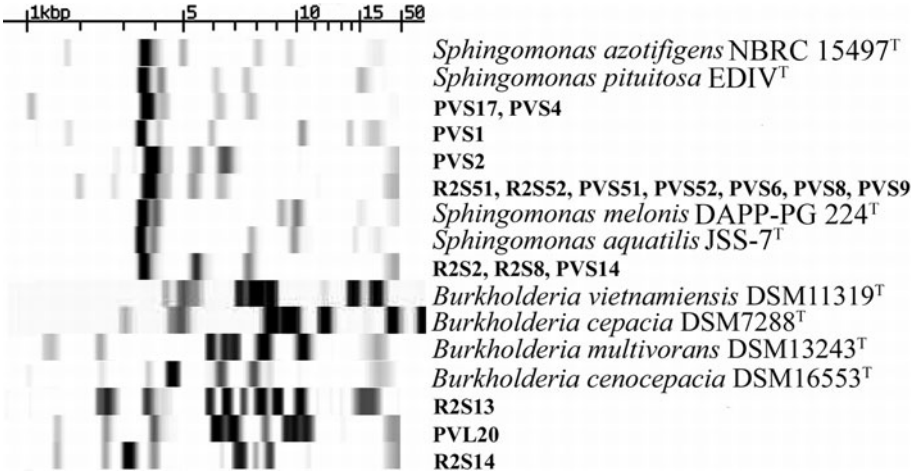

Fig. 2 Ribopatterns obtained with *Eco*RI from the genomic DNA of 17 paper machine biofilm isolates of *Sphingomonas* sp. and *Burkholderia* sp. (*in bold*) and of reference type strains. The reference strains were selected on the basis of similarity of the 16S rRNA gene sequences (Table 2). The *scale bar* indicates fragment sizes (kbp)

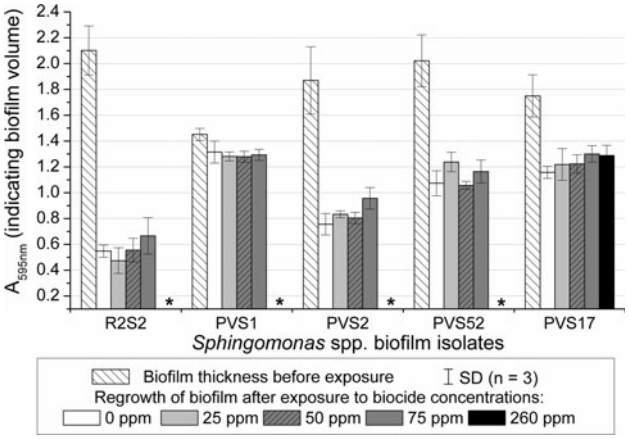

Fig. 3 Tolerance of monoculture biofilms of *Sphingomonas* spp. paper machine isolates to peracetic acid. Biofilms were grown in wells and on spikes on the lid of a microplate with R2 broth. The first column for each isolate indicates the biofilm volume on the walls of this first microplate, measured with the crystal violet staining method. The spikes with the pre-grown biofilms were exposed to peracetic acid (0–260 ppm in autoclaved spring water) by immersion in wells of a second microplate for 4 h at 20 ± 2°C. Subsequently, the spikes with the peracetic acid-exposed biofilms were immersed in R2 broth (no biocide) on a third microplate. After incubation of 18 h at 37°C, the biofilm volumes were measured. Columns 2–6 for each isolate in the figure show how much biofilm grew from the peracetic acid-exposed inocula on the spikes. *Asterisks* indicate biofilm volume below the detection limit ($A_{595nm} \leq 0.1$)

isolates were members of the subclades *S. trueperi* ($n = 39$) and *S. aquatilis* ($n = 6$). The *S. trueperi* subclade contains three phylogenetically closely related but phenotypically diverse species, *S. trueperi*, *S. pituitosa*, and *S. azotifigens*, which are distinguished from each other only by DNA–DNA relatedness [50]. *S. pituitosa* was first described from a eutrophic fountain and produces large amounts of the unique and highly viscous sphingan PS-EDIV [10, 41]. "*S. elodea*", a non-approved species closely similar to *S. trueperi*, produces high quantities of gellan, a sphingan used as a gelling, stabilizing, and suspending agent in many industrial applications [15]. Exopolysaccharide production is believed to be important in biofilm formation [43], e.g., in *S. paucimobilis* (the type species of *Sphingomonas*), exopolysaccharides were reported as crucial for biofilm formation on glass surfaces [2]. It is also possible that the sphingomonads isolated in this study owe their persistence to exopolysaccharide production.

In contrast to the subclade *S. trueperi*, biofilm formation by the subclade *S. aquatilis* was not reported hitherto. Species of this subclade were isolated from water (*S. aquatilis* [26]), brown spots on melons (*S. melonis* [5]), roots of trees and soil (*S. desiccabilis* [38]). So far there is no report on exopolysaccharides produced by this subclade.

The mechanisms of bacterial tolerance to oxidizers, such as peracetic acid, are not yet fully understood. Acute exposure to a sublethal concentration of peracetic acid reportedly increased the hydrogen peroxide tolerance of *Escherichia coli* O157 [55], but tolerance or resistance of biofilms to prolonged exposure of low concentrations of peracetic acid has not been reported. Cross-resistance against peracetic acid was reported for vegetative cells of *Bacillus subtilis* after prolonged exposure to chlorine dioxide [28]. An oxidase-negative *Sphingomonas* sp. isolate capable of adhering to steel was found from a dairy plant after fog disinfection with peracetic acid [4]. Suspended cells of that isolate were sensitive to peracetic acid, which led to the conclusion that the sphingomonad had survived the thorough disinfection because of its ability to rapidly attach to surfaces and to form biofilm rather than to an actual resistance towards the disinfectant [4]. *Sphingomonas* biofilms were isolated from drinking water distribution networks continuously exposed to oxidizing chemicals such as ozone and chlorine-based products [16, 24] and also from waterlines of dental units where hydrogen peroxide was used for disinfection [32, 45]. As a result of the unstable nature of peracetic acid, technical biocides containing peracetic acid are always in equilibrium with

Table 4 Characteristics of process waters sampled from two paper machines and used as the media for testing the tolerance of *Sphingomonas* monoculture biofilms towards the peracetic acid-containing biocide as described in Fig. 4

Sampling date	Measured parameters of the waters								Highest concentration of biocide tolerated by the strain R2S2[b] (mg l^{-1})
	pH	Redox[a] (mV)	Conductivity (µS cm^{-1})	ATP content (fmol ml^{-1})	DOC (mg l^{-1})	Al (mg l^{-1})	Fe (mg l^{-1})	Ti (mg l^{-1})	
Paper machine 1									
July 6	4.9	230	296	130	279.6	17	1.0	0.93	75
July 8	5.4	210	296	120	161.0	9.8	0.95	<0.5	50
July 12	5.1	216	283	50	113.0	9.8	0.61	<0.5	50
July 14	5.1	215	310	40	63.1	3.2	0.83	<0.5	25
July 18	5.0	213	426	20	102.4	2.4	0.65	<0.5	25
July 20	5.0	214	364	130	90.4	4.2	0.73	<0.5	0
July 22	5.4	204	378	90	93.1	5.2	0.88	<0.5	25
July 25	4.9	206	380	30	86.5	5.0	0.83	0.68	25
July 27	4.9	205	360	70	120.1	6.6	0.55	<0.5	50
July 29	4.8	320	310	60	127.0	4.1	<0.5	0.50	0
August 1	5.4	277	238	–	77.7	2.9	<0.5	0.51	50
August 3	4.8	278	353	230	88.7	4.9	0.55	<0.5	25
August 5	5.0	292	312	9	97.4	12	0.77	<0.5	0
August 8	5.8	263	316	–	84.0	12	0.68	<0.5	75
August 10	4.9	261	373	120	80.5	2.6	0.57	<0.5	75
Paper machine 2									
July 6	4.8	234	552	30	209.8	15	0.63	2.3	0
July 8	5.0	226	392	20	99.7	3.0	<0.5	<0.5	0
July 11	4.8	225	420	670	137.5	16	0.55	2.5	0
July 13	4.8	219	522	50	161.3	4.4	0.50	<0.5	0
July 15	5.0	222	346	250	81.4	4.4	0.50	<0.5	0
July 18	5.7	216	354	30	99.7	<2	<0.5	<0.5	0
July 20	4.9	235	543	20	99.0	6.8	0.50	0.50	25
July 22	4.8	246	549	10	111.5	6.6	<0.5	1.0	25
July 25	4.8	242	746	4	239.7	18	0.60	61	0
July 27	4.8	245	622	20	192.4	13	0.58	2.8	0
July 29	4.9	237	350	520	85.8	28	0.95	6.3	50
August 1	4.2	253	516	8	110.2	7.7	<0.5	3.8	0
August 3	4.7	244	373	170	81.8	3.1	<0.5	<0.5	0
August 5	5.7	216	337	190	79.4	8.5	0.58	<0.5	75
August 8	5.0	232	458	700	121.6	13	0.66	2.6	0
					Sterile spring water				0

The highest biocide concentration which did not prevent biofilm regrowth of *Sphingomonas* sp. R2S2 within 18 h at 37°C in each process water is indicated (last column). Concentrations of Mn, Cu, Cr, and Ni were measured but remained below the detection limit in all waters: Mn < 0.2 mg l^{-1} and Cu, Cr, and Ni < 0.5 mg l^{-1} each

DOC dissolved organic carbon, – not determined

[a] versus Ag/AgCl

[b] Highest of tested biocide concentrations (0, 25, 50, 75, and 260 ppm of the biocide product containing 15% peracetic acid, 4 h exposure) which permitted biofilm regrowth after the lid with the exposed biofilm on spikes was transferred in R2 broth (pH 5.5) free of peracetic acid

hydrogen peroxide and acetic acid. The present study showed that the sphingomonads isolated from biofilms that survived peracetic acid treatment were strongly catalase-positive. Two reports indicated that *Sphingomonas* spp. and positive *S. trueperi* in dental unit waterlines using hydrogen peroxide for disinfection persisted as biofilms and increased in prevalence in some of the waterlines over time [32, 45]. Sphingomonads are known to degrade a wide range of recalcitrant natural compounds and xenobiotics, and it has been proposed that they could adapt to changes in

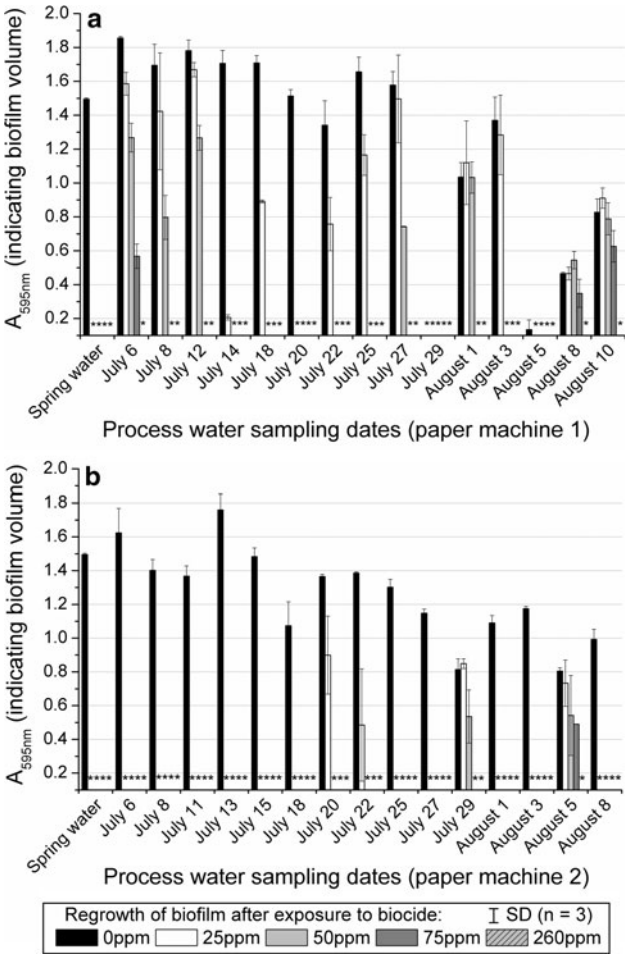

Fig. 4 Tolerance of monoculture biofilms of the *Sphingomonas* sp. isolate R2S2 to different concentrations of peracetic acid diluted in paper machine process waters. Biofilms were grown in R2 broth in wells and on the spikes on the microplate lid inoculated with 1,300 ± 100 cfu/well. When the biofilm had grown to A_{595nm} 0.33 ± 0.02 (**a**) or 0.41 ± 0.04 (**b**), the microplate lid with biofilm on the spikes was transferred to a second microplate containing peracetic acid (0–260 ppm) in process water. After exposure of 4 h at 20 ± 2°C, the peracetic acid-exposed biofilms on the spikes were immersed in R2 broth (no biocide) on a third microplate. After incubation of 18 h at 37°C, the biofilms in the wells of this third microplate were measured with the crystal violet staining method. The figure shows how much biofilm grew from the peracetic acid-exposed inocula on the spikes. *Asterisks* indicate biofilm quantity below the detection limit ($A_{595nm} \leq 0.1$). The process waters were sampled over 30 days from two paper machines and their compositions are presented in Table 4

the chemical composition of their environment more rapidly than other bacterial genera [42]. The results of this present study suggest that sphingomonads, especially members of the subclades *S. trueperi* and *S. aquatilis*, may be more capable than bacteria of other genera in adapting to peracetic acid in warm water. It also shows that these subclades contribute to biofilm formation in warm water using industry.

Several isolates of the *S. trueperi* subclade showed an irreversible change in colony morphology from bright yellow to translucent on solid growth media. This change in colony morphology was linked to the loss of the ability to form biofilm, thus resembling the phenomenon reported for the gammaproteobacteria *Pseudomonas aeruginosa* [12], *Vibrio cholerae* [48], *Vibrio alginolyticus* [9], *Escherichia coli* [46], and the gram-positive taxa *Listeria monocytogenes* [31], *Staphylococcus epidermidis* [54], *Streptococcus pneumoniae* [1], and *Bacillus subtilis* [20]. Many studies found that the colony type that formed more biofilm was also more resistant against antibiotics and biocides than the other colony type. For example, the phenotype of *E. coli* and of *V. cholerae* that formed larger amounts of biofilm was more tolerant towards hydrogen peroxide than the phenotype with weaker biofilm formation [46, 48]. It has been suggested that these changes were caused by phase variation of cell surface structures like outer membrane polysaccharides and proteins [1, 9, 12, 46, 48, 54]. There is no published report on phase variation in *Sphingomonas*, but spontaneous phenotypical variation, indicated by loss of the mucoid colony morphology of the gellan-producing "*Sphingomonas elodea*" ATCC 31461, was noted to be connected to loss of gellan production caused by repressed enzymatic activity [29]. However, our observations indicate that the loss of biofilm-forming ability did not involve reduction of exopolymer production.

The composition of the white water in which the peracetic acid biocide was dosed was shown to affect the peracetic acid tolerance of a pre-grown *Sphingomonas* monoculture biofilm. Elevated pH was statistically shown to promote tolerance, although it only explained 20% of the changes. Because peracetic acid has a pK_a of 8.2, it can be expected to be more permeant at acid pH than in its dissociated form. The microbicidal action of peracetic acid is optimal at pH 2.5–4 [34], and the sporicidal effect of peracetic acid is known to decrease as pH increases [3]. Oxidized and reduced transition metal ions have been shown to enhance the bactericidal action of peracetic acid towards vegetative cells [3]. Our results showed higher efficacy of peracetic acid against biofilms in paper machine white waters with high salt content than that in low conductivity white waters.

The present study appears to be the first one where sphingomonads were the main biofilm formers in warm water using industry. The prevalence and peracetic acid tolerance of sphingomonads in biofilms when peracetic acid was used suggests that this genus is capable of adapting to oxidizing aqueous environments.

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