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Degradation of paper mill water components in laboratory tests with pure cultures of bacteria

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Abstract

The degradation of dissolved and colloidal substances from thermomechanical pulp (TMP) by bacteria isolated from a paper mill was studied in a laboratory slide culture system. *Burkholderia cepacia* strains hydrolysed triglycerides to free fatty acids, and the liberated unsaturated fatty acids were then degraded to some extent. Saturated fatty acids were not notably degraded. However, the branched anteiso-heptadecanoic fatty acid was degraded almost like the unsaturated fatty acids. About 30% of the steryl esters were degraded during 11 days, increasing the concentrations of free sterols. Approximately 25% of the dehydroabietic, and 45% of the abietic and isopimaric resin acids were degraded during 11 days. The degree of unsaturation seemed to be of greater importance for the degradation of fatty acids than the molar mass. No degradation of dissolved hemicelluloses could be observed with any of the nine bacterial strains studied. *Burkholderia cepacia* strains and one *Bacillus coagulans* strain degraded monomeric fructose and glucose in winter TMP water, but in summer TMP water, with much lower sugar concentrations, also other *Bacillus* strains degraded monomeric sugars.

Introduction

In the production of wood-containing printing paper grades, large amounts of dissolved and colloidal substances, mainly composed of hemicelluloses, wood extractives, lignans and lignin-related substances, are released from mechanical pulps and dissolved or dispersed into the process water (Holmbom and Örså 1993). In paper mill water systems the common temperatures (30 to 60 °C) and pH (4.8 to 7.5) are suitable for microorganisms. Bacterial deposits may adversely affect paper machine runnability and paper quality, and is controlled in the mills by dosing biocides. Moreover, the mills strive to decrease water use by recirculation of process waters. Since environmental concerns at the same time call for restrictions of biocide usage, it is important to find other means of controlling bacteria. Knowledge of the dependence of bacterial growth on process water quality, especially the amount and composition of dissolved and colloidal substances, is needed.

Hallaksela et al. (1992) studied bacteria inhabiting xylem of *Picea abies* and found that Gram-positive bacteria were generally able to utilise cellulose and hemicellulose. Väisänen et al. (1998) showed that *Bacillus licheniformis*, *Brevibacillus brevis*, *Burkholderia cepacia*, and *Microbulbifer*-like strains, later identified as *Thermomonas* gen. nov. (Busse et al.), possessed carbohydrate-degrading activities. All dissolved and colloidal substances in paper mill waters do not necessarily serve as carbon source for bacteria. Resin acids, collectively classified as pimaranes (pimaric, sandaracopimaric and isopimaric acids) and

abietanes (abietic, levopimaric, palustric, neoabietic and dehydroabietic acids), are known to have antimicrobial activity, and are toxic to aquatic organisms (Owens 1991). Degradation of abietic, dehydroabietic, and isopimaric acid by bacteria was reported by Mohn et al. (1999) who noticed that this ability was distributed among different phylogenetic groups of bacteria.

The aim of this work was to analyse the degradation of dissolved and colloidal substances from thermomechanical pulp (TMP), especially the wood extractives, by bacteria isolated from a paper mill.

Materials and methods

Bacterial strains

The studied strains were isolated from a paper mill in Finland producing mineral-coated magazine paper from mechanical and chemical pulp and identified as described by Väisänen et al. (1998). The studied strains and their sampling sites are shown in Table 1. The strains were stored at −70 °C in Nutrient broth (Difco, Detroit, MI, USA) containing 15 vol-% glycerol. For inoculation of the test waters, the strains were grown on Plate count agar (Difco, Detroit, MI, USA) at 40 °C or 45 °C for 1–2 days. One loopful of biomass was suspended in 1 mL of 0.1% sterile peptone water, 1 g peptic digest of meat (Biokar Diagnostics, Beauvais, France) in 1 L of distilled water, and 150 μL of the suspension was added to each of the test waters.

Preparation of paper mill model water

A paper mill model water (TMP water) was prepared essentially as described by Ekman et al. (1990). TMP from Norway spruce (*Picea abies*), taken from a Finnish paper mill at about 35% consistency, "winter TMP" during the cold period and "summer TMP" during the warm period of the year. The TMP was suspended in distilled water to 20 g L^{-1} (w/w) and agitated with 150 rpm at 60 °C for 1.5 h. The fibres were separated by suction-filtering (glass filter funnel number 2, Schott, Germany). To simulate paper mill conditions of low water usage, the filtrate was recycled once with fresh TMP (20 g L^{-1}), except for the 11-day experiment where 10 g L^{-1} was used and the filtrate was recycled twice. The resulting TMP water was autoclaved (130 °C, 15 min) before use.

Cultivation of bacteria

Autoclaved TMP water (150 mL) in flasks (500 mL) holding microscope glass slides (Menzel-Gläser 76 × 26 × 1 mm) or stainless steel coupons (77 × 20 × 0.5 mm) of Outokumpu Polarit 757 (= AISI 316) was inoculated with the selected bacteria (Table 1). Glass slides or steel coupons were used in order to provide surfaces for biofilm formation. However, the role of biofilms for the degradation was not specifically assessed in the present work. New slides (washed with ethanol) were used for each experiment. The cultures were incubated on an orbital shaker (125 rpm, GFL 3015, Burgwedel, Germany). The contained liquid was sampled after removal of the slides and analysed for monomeric sugars, total carbohydrates, and wood extractives. The cultivation temperature was 40 °C for the strains of *Burkholderia cepacia* and *Burkholderia pickettii*, and 45 °C for the strains of the other species.

Analysis of dissolved and colloidal substances in TMP water

Total carbohydrates in the TMP water were analysed by gas chromatography (GC) after acid methanolysis, as described by Sundberg et al. (1996). Monomeric sugars were analysed similarly as the carbohydrates, except that the methanolysis step was omitted. Water samples (2.0 mL) were freeze-dried in 10-mL pear-shaped flasks. The residue was dissolved in 100 μL of pyridine, and 150 μL of hexamethyldisilazane (Fluka 52619) and 70 μL of trimethylchlorosilane (Fluka 92360) were added. The silylation was allowed to proceed for 4 h at room temperature. The supernatant was transferred to a screw-capped vial and the samples were injected into the GC with an autosampler. The repeatability of the analyses was within 5%.

Analysis of wood extractives was performed on methyl *tert*.-butyl ether (MTBE) extracts by GC, as described by Örså and Holmbom (1994).

For analysis of individual wood extractive components, alkaline hydrolysis was done before extraction with MTBE. A volume of 2.0 mL 0.5 M KOH in 90% ethanol was added to 4 mL of TMP water. After 2 h at 70 °C, the sample was acidified to pH 3.5 with 30% phosphoric acid (H_3PO_4). After that, 2.0 mL of the internal standard containing 0.02 mg mL^{-1} of each heneicosanoic acid, betulinol, cholesteryl heptadecanoate and 1,3-dipalmitoyl-2-oleoyl glycerol was added, and wood extractives were extracted into MTBE as described by Örså and Holmbom (1994). The individual wood extractives were analysed

Table 1. The bacterial strains and their sampling sites in the paper mill.

Bacterial strains	Sampling site
Burkholderia cepacia strains F45L5, F45193, and F453DL1	Biofilm on steel surface in paper machine wire water
Burkholderia cepacia strains A2843 and B2842	Carboxymethyl cellulose slurry
Burkholderia pickettii strain A28161	Hot water
Bacillus coagulans strain E50L1	Wire water (steel surface)
Bacillus coagulans strain B50211	Machine chest
Bacillus atrophaeus strain A28111	Dry starch container

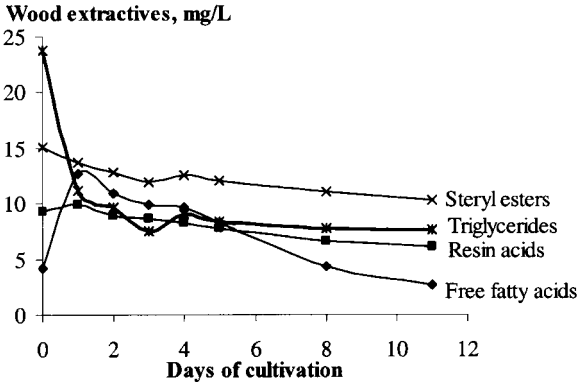

Figure 1. Wood extractives remaining in summer TMP water after glass-slide culturing of *Burkholderia cepacia* strain F45L5. The initial pH was 7.3 and the temperature 40 °C.

by GC. The sample (1.0 μL) and a 2.0 μL solvent plug of toluene were injected with an autosampler (Varian 8200, Walnut Creek, California) using a split-injection technique. Hydrogen was used as carrier gas (2 mL min^{-1}). The column coated with crosslinked dimethyl polysiloxane (HP-1, Hewlett Packard) was 25 m long and 0.20 mm in i.d., and had a film thickness of 0.11 μm. The injector temperature was 260 °C. The column temperature was initially 150 °C, and programmed to 230 °C at a rate of 7 °C min^{-1} and then to 290 °C at 10 °C min^{-1}. The detector (FID) temperature was 290 °C. The repeatability of the analyses was within 5%.

Results

We used bacterial strains isolated from a paper mill as tools to elucidate the microbiological degradation of dissolved and colloidal substances in paper mill process water. Water extract of thermomechanical spruce wood pulp was used as model paper mill water. The strains used are listed in Table 1.

Burkholderia cepacia strains F45L5, A2843, and F453DL1 were cultivated for 5 days in summer TMP water and winter TMP water, containing different levels of monomeric sugars, after which the wood extractives remaining in the water were analysed. There was no major difference in the degradation of wood extractives between summer and winter TMP water (Table 2). All *Burkholderia cepacia* strains degraded wood extractives (6–20%). F45L5 hydrolysed triglycerides resulting in an increase in the amount of free fatty acids. Also A2843 hydrolysed triglycerides, but less than F45L5. Also free fatty acids and resin acids were degraded by A2843. F453DL1 hydrolysed hardly any triglycerides, but seemed to degrade fatty and resin acids. The degradation of wood extractives was strain-specific.

The time course of degradation of the wood extractives was studied when a culture of *Burkholderia cepacia* strain F45L5 was incubated in summer TMP water for 11 days. The triglycerides in the TMP water were hydrolysed to free fatty acids, and this initially (day 1) increased the total amount of fatty acids (Figure 1). Also steryl esters were partially (30%) transformed during 11 days. Figure 2 shows the amounts of free fatty acids, resin acids, and sterols in the medium. After day 1, the liberated unsaturated pinolenic, linoleic and oleic fatty acids were degraded (Figure 2A), whereas the straight-chain saturated fatty acids showed little or no degradation (Figure 2B). However, the branched anteiso-heptadecanoic fatty acid was degraded almost like the unsaturated fatty acids (Figure 2B). Approximately 25% of the dehydroabietic, and 45% of the abietic and isopimaric resin acids were degraded during 11 days (Figure 2C). Due to hydrolysis of steryl esters, the concentrations of free sitosterol, sitostanol and campesterol increased during the entire test period (Figure 2D), indicating no degradation of the liberated sterols. The pH decreased from 7.3 to 6.2 (day 1) and after day 2 slowly increased to 7.4

Table 2. Wood extractives (mg L^{-1}) in TMP water after exposure to cultures of *Burkholderia cepacia* strains F45L5, A2843 and F453DL1.The initial pH was 6.9 and the temperature 40 °C. Stainless steel coupons were used. The repeatability of the experiments for strain F45L5 was within ± 1.5% for free fatty acids, ± 5.2% for sitosterol, ± 3.5% for steryl esters, and ± 3.9% for triglycerides.

Burkholderia cepacia strain	Time	Free fatty acids	Resin acids	Sitosterol	Steryl esters	Triglycerides	Σ	Changes* in contents of	
								Triglycerides	Free fatty acids
Winter TMP water									
F45L5	0 d	4.4	10.0	3.3	16.7	40.6	74.9		
F45L5	5 d	9.4	10.1	4.4	12.5	23.8	60.2	−16.6	+17.8
A2843	0 d	5.1	12.3	3.6	16.7	40.2	78.0		
A2843	5 d	3.3	10.7	3.5	14.2	34.9	66.6	−5.2	−6.4
F453DL1	0 d	5.1	12.1	3.6	16.2	38.1	75.1		
F453DL1	5 d	4.4	10.9	3.3	14.6	37.0	70.3	−1.1	−2.5
Summer TMP water									
F45L5	0 d	4.9	11.3	3.3	14.8	32.1	66.4		
F45L5	5 d	6.4	10.9	4.1	12.9	19.2	53.5	−12.7	+5.3
A2843	0 d	4.7	10.8	3.1	14.8	32.2	65.5		
A2843	5 d	2.5	9.8	3.1	13.6	30.0	59.0	−2.2	−7.8
F453DL1	0 d	5.3	12.1	3.3	15.6	33.1	69.4		
F453DL1	5 d	3.4	9.9	3.0	13.6	31.4	61.4	−1.7	−6.8

* − = μmol L^{-1} degraded; + = μmol L^{-1} formed. An average molar mass of 1014 g mol^{-1} for triglycerides and 280 g mol^{-1} for free fatty acids was used.

(Figure 2A). The initial rapid decrease in pH during the first day (Figure 2A) may have been due to the initial formation of free fatty acids from the triglycerides (Figure 1). When the fatty acids then were degraded, the pH was restored. The different behaviour of fatty acids, resin acids and sterols can also be seen in the chromatograms presented in Figure 3. The decrease in unsaturated fatty acids can be seen, as well as the increase in sitosterol. The total amount of lignans did not change. Only a pH-induced, chemical transformation of lignans was noticed. Figure 4 shows the impact of the molar mass on the degradation of fatty and resin acids. The degree of unsaturation (Figure 2A and 2B) seemed to be of greater importance for the degradation of fatty acids than the molar mass (Figure 4). Resin acids were decreased to a different extent (Figure 2C) although they all have a molar mass of 300–302 g mol^{-1} (Figure 4).

Table 3 summarises the amounts of the various wood extractives found in summer and winter TMP water after 3 days of culturing of other bacterial species prevalent in paper mills. In addition to *Burkholderia cepacia* strain F45L5 degrading triglycerides and steryl esters, also *Bacillus atrophaeus* strain A28111 degraded wood extractives. Triglycerides, steryl esters and resin acids in winter TMP water were degraded, but no increase in fatty acids followed, indicating that the fatty acids had been degraded already during the 3 days of cultivation. The other studied *Bacillus* strains (*Bacillus coagulans* strains E50L1 and B50211) had no lipolytic activity towards wood extractives. In summer TMP water, no significant degradation occurred in addition to the degradation of triglycerides to free fatty acids by *Burkholderia cepacia* strain F45L5. In conclusion, the degradation of TMP wood extractives by the bacteria was generally less than 10%.

No degradation of dissolved hemicelluloses (500–600 mg L^{-1}) in TMP water could be observed with any of the nine bacterial strains studied. However, monomeric sugars were removed by most of the bacteria. Since the TMP prepared from Norway spruce harvested in winter contained much more monomeric sugars than TMP from summer-harvested trees, we analysed the degradation of arabinose, xylose, galactose, glucose, mannose, fructose, glucuronic acid and galacturonic acid in winter and summer TMP waters. The results obtained after exposure for 3 days of culturing are compiled in Table 4. The content of glucose and fructose was about 20-fold higher in winter than in summer TMP water. *Burkholderia cepacia* strains and one *Bacillus coagulans* strain degraded monomeric fructose and glucose in winter TMP water, but in summer TMP water also other *Bacillus* strains degraded monomeric sugars. The reason why also *Bacillus coagulans* strain B50211 degraded monomeric sugars in summer TMP water, but not in winter

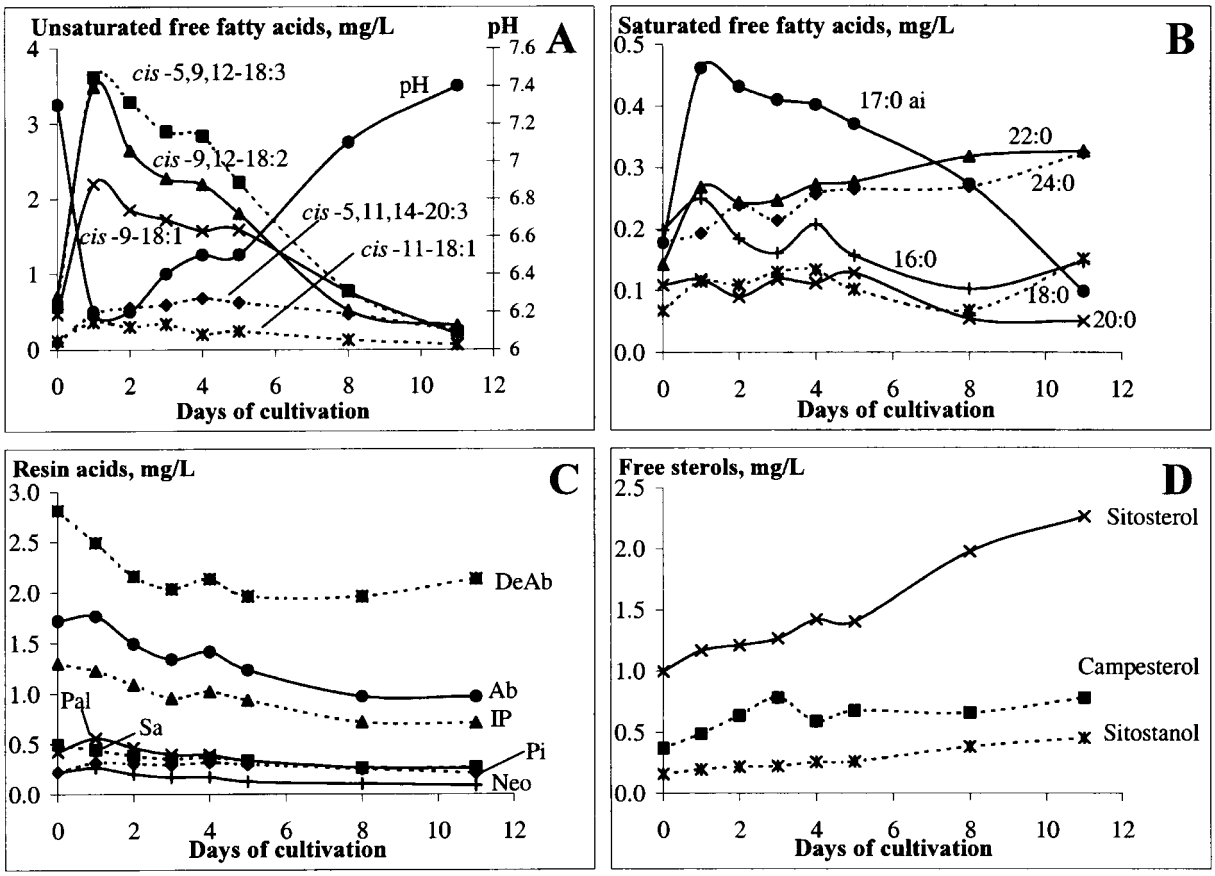

Figure 2. The time course of the formation and degradation of fatty acids, resin acids and free sterols in summer TMP water when *Burkholderia cepacia* strain F45L5 was cultivated on glass slides. The initial pH was 7.3 and the temperature 40 °C. A. Unsaturated free fatty acids. Changes in pH during cultivation are shown on the secondary y-axis. B. Saturated free fatty acids. C. Resin acids. D. Free sterols. For abbreviations, see Table 5.

TMP water, may have been the low monomeric sugar content in the summer TMP water.

Discussion

In this study the degradation of water-extractable wood components by paper mill bacteria was analysed. *Burkholderia cepacia* and *Bacillus coagulans* have been found frequent on machine surfaces in that environment. *Burkholderia cepacia* strains (F45L5, A2843, and F453DL1) were chosen because of their ability to degrade wood extractives (Väisänen et al., 1998; Hallaksela et al., 1992). Also *Bacillus atrophaeus* strain A28111 was found to be lipolytic towards wood extractives (Table 3).

The results in this study show that an explanation for the prevalence of *Burkholderia cepacia* in paper mill process waters may be their ability to utilise many different types of wood extractives. The wood extractives supply a constant source of carbon, promoting stable residential colonisation of the paper machine. Bacterial communities solely relying on monomeric sugars or sucrose as carbon source would be starving during summer time, when the concentrations of monomeric sugars are low (Ekman et al., 1990). Some bacteria degraded triglyceride-derived unsaturated fatty acids, but straight-chain saturated fatty acids accumulated, indicating that they were not degraded. However, it is possible that some acids may have been degraded immediately after the triglycerides were hydrolysed and therefore were not found in the medium.

It is noteworthy that the bacteria in this study did not degrade hemicelluloses. When present, glucose and fructose were preferably used by the bacteria. The selected bacteria thus changed the TMP water com-

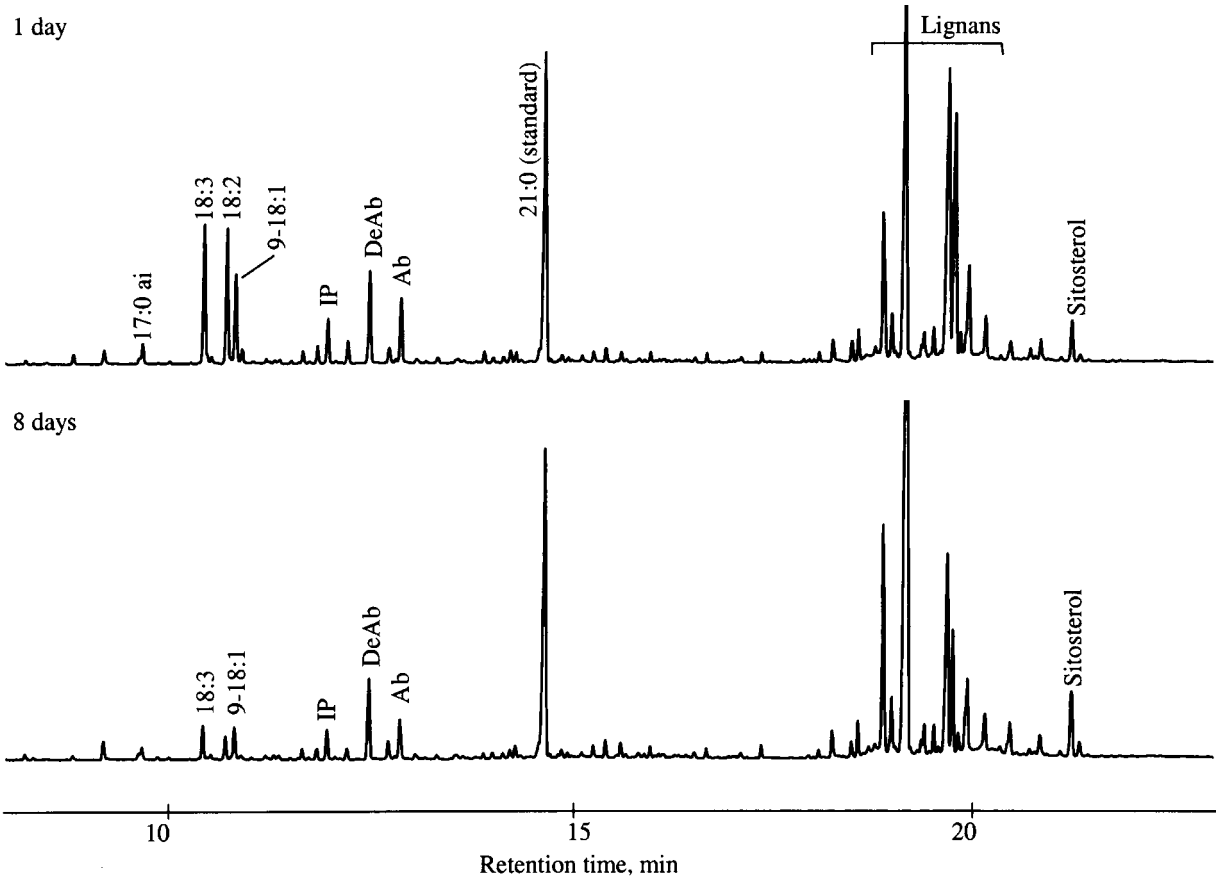

Figure 3. Chromatograms for wood extractives in summer TMP water after 1 day (above) and 8 days (below) of glass-slide culturing of *Burkholderia cepacia* strain F45L5. The initial pH was 7.3 and the temperature 40 °C. The amount of internal standard was 40.0 μg. For abbreviations, see Table 5.

Table 3. Wood extractives (mg L^{-1}) in TMP water after exposure to each of the nine indicated bacterial strains for 3 days. The initial pH was 6.0 and the temperature 40 °C or 45 °C. Glass slides were used.

	Free fatty acids	Resin acids	Sito-sterol	Steryl esters	Trigly-cerides	Σ	Free fatty acids	Resin acids	Sito-sterol	Steryl esters	Trigly-cerides	Σ
T = 40 °C	Winter TMP water						Summer TMP water					
Sterile	5.4	11.9	3.1	15.0	33.9	69.3	6.1	10.9	2.7	15.0	30.7	65.4
Burkholderia cepacia strain F45L5	8.0	12.0	3.2	13.3	23.2	59.7	8.3	11.9	3.2	14.0	22.6	60.0
Burkholderia cepacia strain F45193	4.9	11.8	3.0	14.8	34.6	69.1	4.2	10.4	2.6	14.9	30.2	62.4
Burkholderia cepacia strain A2843	3.7	11.3	3.0	15.1	34.2	67.2	3.3	10.3	2.5	14.7	30.2	61.1
Burkholderia cepacia strain F453DL1	4.7	10.7	2.9	14.9	33.7	67.1	5.3	11.0	4.2	14.8	31.1	66.4
Burkholderia cepacia strain B2842	3.9	11.9	2.9	15.3	33.0	67.0	4.0	10.7	2.6	15.0	30.2	62.5
Burkholderia pickettii strain A28161	7.9	12.4	2.9	15.4	34.6	73.3	6.2	10.4	2.6	14.8	31.4	65.5
T = 45 °C												
Sterile	5.5	11.3	2.7	12.7	27.6	59.7	6.0	10.7	2.5	14.4	28.9	62.5
Bacillus coagulans strain E50L1	6.5	12.6	2.6	13.7	29.8	65.2	4.0	10.7	2.5	14.7	29.5	61.3
Bacillus coagulans strain B50211	5.4	12.4	2.7	13.8	31.3	65.6	5.8	10.8	2.4	14.9	30.3	64.2
Bacillus atrophaeus strain A28111	3.9	8.4	2.2	9.3	20.0	43.8	3.4	9.1	2.5	15.1	31.3	61.4

Table 4. Monomeric sugars (mg L^{-1}) in TMP water after exposure to cultures of each of the nine indicated bacterial strains for 3 days. The initial pH was 6.0 and the temperature 40 °C or 45 °C. Glass slides were used. N.A. = not analysed. Other sugars include xylose, galactose, mannose, glucuronic acid, and galacturonic acid.

	Arabinose	Glucose	Fructose	Other sugars	Σ	Arabinose	Glucose	Fructose	Other sugars	Σ
T = 40 °C	Winter TMP water					Summer TMP water				
Sterile	5.7	24.8	85.4	6.0	121.8	5.9	1.2	4.6	2.1	13.8
Burkholderia cepacia strain F45L5	4.8	0.6	52.0	6.4	63.8	0.0	0.7	0.0	2.6	3.2
Burkholderia cepacia strain F45193	5.8	0.7	0.0	2.2	8.7	5.9	0.7	0.0	1.9	8.5
Burkholderia cepacia strain A2843	5.4	0.6	67.3	6.3	79.6	4.7	0.7	0.0	2.1	7.4
Burkholderia cepacia strain F453DL1	5.2	0.7	20.4	4.2	30.4	0.0	0.7	0.2	1.9	2.8
Burkholderia cepacia strain B2842	0.0	0.6	45.3	5.3	51.2	0.0	0.7	0.4	2.2	3.4
Burkholderia pickettii strain A28161	5.8	0.3	49.3	7.7	63.1	4.9	0.8	1.2	3.7	10.6
T = 45 °C										
Sterile	5.7	24.4	81.2	6.3	117.6	6.1	1.6	4.9	2.0	14.6
Bacillus coagulans strain E50L1	5.2	0.8	25.7	4.7	36.3	0.0	0.8	0.5	2.7	4.0
Bacillus coagulans strain B50211	4.9	20.7	79.1	7.5	112.2	1.7	0.8	0.5	3.2	6.1
Bacillus atrophaeus strain A28111	N.A.	N.A.	N.A.	N.A.	N.A.	3.4	0.9	0.4	2.6	7.3

position by removing monomeric sugars, but leaving the hemicelluloses. The remaining TMP water contained percentually more recalcitrant dissolved and colloidal substances (hemicelluloses, sterols, certain resin acids, saturated fatty acids) than the original TMP water.

Water solubility can affect the biodegradability of substances in paper mill waters. Hemicelluloses, monomeric sugars and lignans occur in dissolved form. The wood extractives are present in paper mill waters mainly in colloidal droplets, except for free fatty and resin acids (pK_A values 5.5–6.4) that are partly dissolved in the water phase, depending on the pH (Ekman et al. 1990). The colloidal state of wood extractives could be an explanation to the limited degradation of extractives by most of the bacteria.

The retention time of papermaking materials in the machine varies from 2 h for starch in the starch container to approximately one week for broke in the broke container (Väisänen et al. 1998). Dissolved and colloidal substances do not leave the paper machine with the paper, unless they form complexes or aggregates, e.g., by using fixing agents. A part, mainly wood extractives, is fixed to fibre in the wet end before the headbox, and goes that way out with the paper. The paper web contains more than 35% of water after the press section, so about one third of the dissolved and colloidal substances will become incorporated in the paper when water is evaporated in the drying section. The remaining two thirds, if not adhered to fibres, leave only with the effluent. Because the process water is recirculated, as well as the broke part of the pulp (up to 25% of total), the retention time of non-aggregated/fixed dissolved and colloidal substances will be many hours or even days. Therefore, the degradation observed within the experiment time of 3 days and up to 11 days in the present work is of relevance.

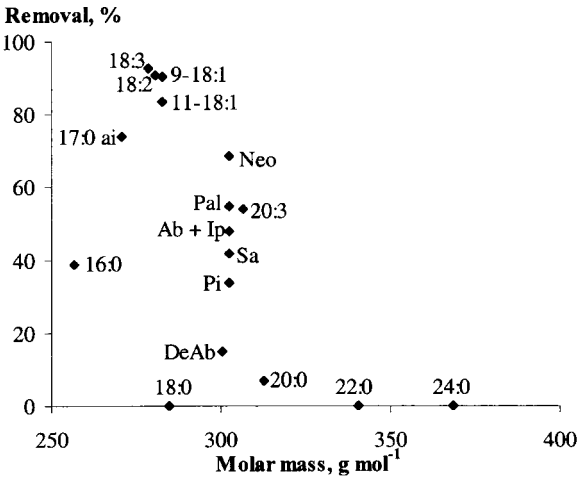

Figure 4. Impact of the molar mass on the degradation of wood extractive components during exposure of summer TMP water for 11 days to a glass slide culture of *Burkholderia cepacia* strain F45L5 at 40 °C and initial pH 7.3. For abbreviations, see Table 5.

Of the nine paper mill bacteria studied, only *Burkholderia cepacia* strains degraded wood extractives. Monomeric sugars, but not hemicelluloses, were de-

Table 5. Abbreviations for wood extractive components.

Abbreviation	Component
16:0	Palmitic acid
17:0 ai	Anteiso-heptadecanoic acid
18:3 (*cis*-5,9,12-18:3)	Pinolenic acid
18:2 (*cis*-9,12-18:2)	Linoleic acid
9-18:1 (*cis*-9-18:1)	Oleic acid
11-18:1 (*cis*-11-18:1)	*Cis*-11-octadecenoic acid
18:0	Stearic acid
20:3 (*cis*-5,11,14-20:3)	*Cis*-5-,*cis*-11-,*cis*-14-eicosatrienoic acid
20:0	Arachidic acid
22:0	Behenic acid
24:0	Lignoceric acid
Ab	Abietic acid
DeAb	Dehydroabietic acid
IP	Isopimaric acid
Pal	Palustric acid
Pi	Pimaric acid
Neo	Neoabietic acid
Sa	Sandaracopimaric acid

graded by most of the bacteria studied. Since the selected bacteria degraded typical paper mill water components to a different extent, the process water quality will affect the bacterial growth and flora in paper mills. When the composition changes, e.g., as a result of decreased raw water intake, the amount and kind of bacteria may be expected to change.

Acknowledgments

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