J Ind Microbiol Biotechnol (2008) 35:1165–1173
DOI 10.1007/s10295-008-0396-8

ORIGINAL PAPER

Prokaryotic microbiota of recycled paper mills with low or zero effluent

Charlotta Kanto Öqvist · Jukka Kurola · Jaakko Pakarinen · Jaakko Ekman · Satu Ikävalko · Jaakko Simell · Mirja Salkinoja-Salonen

Received: 21 March 2008 / Accepted: 7 July 2008 / Published online: 24 July 2008

Abstract The tendency in the paper industry is to close all water loops to save water. This leads to higher process temperatures and may increase the colloidal and dissolved material in the process circulation. Increase of nutrients in the water circuits may favor microbiological growth and fouling. In this paper the chemical and microbial compositions of water circuits and deposits were studied of two closed cycled paper/board mills, one mill totally closed (0 m^3 waste water t^{-1}), and the other low discharging (about 4 $m^3\,t^{-1}$). The zero discharge mill accumulated high amounts (>10 g C L^{-1}) of organic carbon in the circulation waters, about 40% of which composed of volatile acids (lactic, acetic, propionic and butyric acid). Water contents of sulfate, chloride, sodium and calcium increased to >1 g L^{-1} of each. q-PCR targeted on 16S rRNA genes indicated that the bacteria in water circuits were mainly viable cells. In both mills anaerobic growth (10^6–10^8 CFU mL^{-1}) equalled or exceeded aerobic growth, with odor problem but no actual slime problem. The major part (40%) of all identifiable bacterial sequences were closest but yet distant (<96%) to *Enterococcus cecorum* and in the 4 $m^3\,t^{-1}$ discharging mill also *Bacillus thermoamylovorans* and *Bacillus coagulans*. Slimes and deposits from the mills contained high amounts, $\geq 10^8$ g^{-1}, of archaean, but only the genus *Methanothrix* was identifiable from the cloned sequences. The findings indicate that closing the water circuits strongly limited diversity of the microbiota but allowed efficient mineralization of the dissolved and suspended matter.

Keywords Paper machine · Quantitative RT-PCR · 16S rRNA · Closed water loop · Recycled fibers · Archaea

C. Kanto Öqvist · J. Kurola · J. Pakarinen · J. Ekman · M. Salkinoja-Salonen (✉)
Department of Applied Chemistry and Microbiology, Biocenter, University of Helsinki,
P O. Box 56, 00014 Helsinki, Finland
e-mail: mirja.salkinoja-salonen@helsinki.fi

C. Kanto Öqvist
Ashland Deutschland GmbH, Fütingsweg 20,
47805 Krefeld, Germany

S. Ikävalko · J. Simell
Ashland Nordic Oy, Viikinkaari 6,
00790 Helsinki, Finland

Introduction

In the world wide paper industry the tendency due to environmental restrictions goes towards lower specific water consumption per ton paper or board produced [14]. This leads to higher process temperatures, increase in the colloidal and dissolved material in the process circulation [1]. The higher levels of nutrients in the water circuits may favor microbiological growth [15] inside the paper mill and thereby affecting the process and the product qualities. A well-known cause of quality and runnability problems in the papermaking process is deposit and slime formation [2, 7, 13, 16–19, 21, 22, 25, 26, 32], however real cases indicate that not the deposits is the dominating problem for a closed mill, but odor problems in the product and in the surroundings. Low water uses in the paper machine circuits makes retention time long, and therefore growth rate no longer is the main factor for the competitiveness between the different species of the microbiota. There appears to be no published work on the composition of microbiota in industrial closed or nearly closed systems.

In this work we used cultivation dependent and independent methods to gain understanding on what happens with

the paper machine microbiological population when the machine circuits close.

Materials and methods

Sampling

Two paper/board machines were studied, with equal papermaking processes, raw materials and product qualities. One mill called MHS, is 100% closed, the other, called MSS, uses ca. 4 m^3 of fresh water per ton of produced board or paper. Both mills use 100% recycled fiber as the raw material. Process samples were collected in 50 mL sterile bottles from two sites, the short circuit (called white water), and the long circuit (called clarified water). Slimes were searched for although neither mill actually had any slime problem. The slime collected from the mill MSS was from the clarified water chest. The deposits sampled from the mill MHS were solids in a floor channel where water circulates. Both sites represented stagnant phases in the systems. Subsamples for the DNA extractions were immediately frozen. For cultivation, the subsamples were sent per 24 h delivery to the laboratory for cultivations.

Chemical characterization of the circulating waters

Cations were analyzed by ion chromatography using DIONEX ICS 1000/Chromeleon, equipped with a conductivity detector, CG12A/Ion CS12A column with CSRS Ultra 4 suppressor and 20 mM methane sulfonic acid as the eluent. The ion chromatograph used for analyzing the anions was DIONEX DX-120/Chromeleon equipped with a conductivity detector, AG14/AS14 column with ASRS Ultra 2 suppressor and 3.5 mM Na_2CO_3 + 1.5 mM $NaHCO_3$ as the eluent. TOC (total organic carbon), AOX (adsorbable organic halogens), iron, manganese, aluminum and total phosphorus were analyzed photometrically by the Hach Lange CADAS 200 system. Volatile organic acids were analyzed by liquid chromatography using the Agilent 1100 system with UV detection and on EuroKat H ion exchanger column with 0.01 M H_2SO_4 as the eluent.

Analysis results are given as means of duplicate samples. Deviations between the duplicates were $\leq$20%.

The samples were filtrated using Whatman® GF/A filters when so indicated in the text.

Cultivations

Culture media used for the water samples: R2A for the enumeration of total aerobic bacterial count, Chromocult® Coliform agar for coliform bacteria and *E. coli, Pseudomonas* CFC Selective Agar (Merck KGaA, Darmstadt, Germany) with 1 l of 10 mg cetrimide, 10 mg fucidin and 50 mg cephalosporin for the enumeration of *Pseudomonads*, Sabouraud 4% dextrose agar with 100 mg L^{-1} chloramphenicol and 100 mg L^{-1} tetracycline (Merck KGaA, Darmstadt, Germany) pH 5.4 for fungi and yeasts. Iron–sulfite agar (Oxoid Ltd, Basingstoke, Hampshire, England) for the detection of thermophilic anaerobic organisms, and black colonies were counted as reducers of sulfite to sulfide. The plates were placed in an anaerobic jar and the oxygen was removed with Anaerogen™ Atmosphere Generation System (Oxoid Ltd, Basingstoke, Hampshire, England). Total anaerobic bacteria were determined as all colonies growing in the iron–sulfite agar.

The R2A plates were incubated for 2–4 days at 45 °C (relevant for warm paper machines) and the selective media for 1–4 days at 32 °C (for better differentiation). Total coliform bacteria and *E. coli* CFU were read from the same plates based on the clusteromorphic indicator, as instructed by the manufacturer. Yeasts and fungi were enumerated from the same plates based on colony morphology.

Isolation of DNA

All samples were stored at −20 °C. No pretreatment was applied before DNA extraction and the water samples were not centrifuged.

Method I (waters): the samples were lyzed in FastPrep® Matrix E tubes (Qbiogene, Irvine CA, USA) for 30 s in the FastPrep® Instrument for in a lysis buffer with Proteinase K from MagNa® Pure LC DNA Isolation Kit III Bacteria and Fungi (Roche diagnostics, Penzberg, Germany). The samples were then extracted according to standard protocol for MagNa® Pure with a phenol–chloroform, and then purified using the KingFisher (ThermoElectron, Waltham, MA, USA) instrument with lysing/binding buffer, magnetic particles in suspension and washing buffers I–III including elution buffer from the kit [10]. Programming was according to the manufacturer's instructions. Method II (waters and deposits): the samples were lysed as for method I, but using the FastDNA® Spin Kit special sodium phosphate buffer and MT buffer. When 1 mL of the sample was used for extraction, the amount of sodium phosphate buffer was reduced from 978 to 450 μL and the MT Buffer was increased from 122 to 125 μL. The extraction of the DNA in the FastDNA® SPIN Kit was done with a binding matrix suspension, and separated from the binding matrix with the help of a special SPIN™ filter, and an elution buffer according to the manufacturer's instructions.

The DNA concentration was measured fluorimetrically by the use of PicoGreen® ds DNA Quantization Reagent and Kits (Molecular Probes, Eugene, OR, USA) according to instructions of the kit. The DNA yield was about 30% higher with method II.

Quantitative real-time PCR (q-PCR) for the domains eubacteria and archaea

The LightCycler Quantitative real-time PCR machine (Roche Diagnostics Penzberg, Germany) was used for the amplifications. The total reaction volume was 20 μL, including 2 μL of the template and 0.3 μM of each primer for the domain Eubacteria, and 0.4 μM of each primer for the Archaea. For the domain Eubacteria the primers pE and pF′ [10] were used (product a size 167 bp), and for the archaean primers 1369F and 1541R [8] (product size 172 bp). In the reaction mix 10 μL SYBR® Premix Ex Taq were added (Takara Bio Inc., Shiga, Japan) and to the archaean analysis 0.4 μL of BSA buffer (10 μg mL^{-1}). Temperature program used for the eubacteria: 30 s at 95 °C, 40 cycles of 5 s at 94 °C followed by annealing and extension for 20 s at 60 °C [10], and for the archaean: 30 s at 95 °C, 40 cycles of 5 s at 95 °C, followed by annealing 10 s at 58 °C and extension 10 s at 72 °C. Melting curve analysis on the amplicon was done from 65–95 °C, 0.1 °C s^{-1}, with continuous measurement of the fluorescence signal. Positive control for the domain bacteria was *Deinococcus geothermalis* strain 50051 (own collection [31]), and for the domain archaea *Sulfolobus acidocaldarius* DSM 639 (German Collection of Microorganisms and Cell cultures, Braunschweig, Germany).

Preparation of DNA for the cloning and sequencing of the 16S rRNA gene

Eppendorf Master cycler was used with the primer pair pA and pH′ for the eubacteria [9], and the primer pair 21F and 958R [8] for the cloning of archaean. The reaction volume was 50 μL and contained 2 μL of the (eubacteria), or 1 μL (archaea) of the template, 5 μL of 10× PCR buffer (with or without tenside), 1 μL of dNTP (equals 0.2 mM), and 1 μL of DyNAzyme II Taq polymerase, all three from Finnzymes (Espoo, Finland).

Program for the eubacteria: 94 °C for 10 min, then 35 cycles of 94.0 °C for 1 min, 55.0 °C for 1 min, 72.0 °C for 1 min, extension and annealing 10 min for 72.0 °C, and for the archaea: 94.0 °C for 7 min, then 27 cycles of 94.0 °C for 30 s, 57.0 °C for 1 min, 72.0 °C for 1 min, extension and annealing 10 min for 72.0 °C. The obtained products were inspected for homogeneity and size with a standard agarose gel containing ethidium bromide.

Molecular cloning and sequencing of the PCR amplicon was purchased as paid service from University of Helsinki University Institute of Biotechnology DNA Laboratory. The PCR products were cloned into *E. coli* plasmid libraries using PCR Cloning Kit (Qiagen, Valencia, CA, USA). Plasmid DNA was extracted from the clones using MultiScreen 96PLASMID Plates (Millipore, Billerica, MA, USA), the inserts re-amplified with universal vector primers and purified with MultiScreen 384 PCR-plates. Sequencing was done using the primer pD′ [9] yielding an approximately 500 bp long product, BigDye Terminator cycle sequencing kit and ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

The 16S rRNA gene sequences were analyzed as described earlier [10], aligned and compared by ClustalW for equal sequences, and analyzed using the Seqmatch, Classifier [33] and the Tree-builder by the Ribosomal Data base project II (http://rdp.cme.msu.edu/). Sequences ≥97% identical were assumed to represent the same species.

The DNA sequences were deposited in the EMBL nucleotide database under the accession numbers FM162197–FM162231 and FM162352–FM162394.

Results

Characterization of the recycling waters of the two closed cycle paper mills

Table 1 shows the chemical characteristics of the clarified water and the white water of the two paper/board mills, illustrating the effects of mill closure. Compared to the MSS mill the MHS was more acid and accumulated high amounts of organic carbon (TOC), sulfate, chloride, sodium and calcium (>1 g L^{-1} of each). About 40% of the organic carbon composed of volatile acids (lactic, acetic, propionic and butyric acid). The content of TOC, the contents of sulfate, of the cations sodium, potassium, magnesium, iron, calcium, and organic acids (lactic, formic, butyric) were one 10-fold or more higher in the waters of the mill MHS than in those of the mill MSS. A closed mill has no outlet for nutrients or solids to leave other than with the paper/board and by evaporation from the drying section (organic acids, an odor problem). As can be seen in Table 1, the mill MHS waters represented an environment more extreme than MSS.

Cultured microorganisms

The clarified and white waters of the mills MHS and MSS were analyzed for cultivable bacteria and fungi (Figs. 1, 2). The densities of bacteria capable of anaerobic growth were high (10^7–10^8 CFU mL^{-1}) in the circulation waters, possibly explaining the odor problems in the mills. The mill MSS waters contained higher density of H_2S producing anaerobically growing bacteria. The ratio of anaerobic colony counts compared to aerobic was high in the waters of the mill MHS. The mill MSS waters contained about one ten-fold more coliform bacteria and H_2S producers than those of the mill MHS. No further difference was found

Table 1 Characterization of the short (white water) and the long (clarified water) circuit waters of two closed cycle paper/board mills

Mill	MHS		MSS	
Analyzed item	Clarified water	White water	Clarified water	White water
From crude samples				
pH	5.9	5.6	7.2	7
T (°C)	54	52	42	46
Conductivity (mS $cm^{-1)}$	40.4		8.5	
Total organic carbon (TOC) (mg P $L^{-1)}$	10,370	10,025	1,040	790
Adsorbable organic halogens (AOX) (mg L^{-1})	7.85	6.9	8.5	5.6
From sample filtered through 1.6 μm				
TOC (mg L^{-1})	10,080	10,325	920	540
AOX (mg L^{-1})	5.3	3.9	6.6	5.1
Chloride (mg L^{-1})	1,075	1,106	200	110
Nitrite (mg L^{-1})	<1	<1	<1	<1
Nitrate (mg L^{-1})	7	10	<1	<1
Sulfite (mg L^{-1})	<10	<10	<10	<10
Sulfate (mg L^{-1})	1,420	1,504	135	90
Phosphate (mg L^{-1})	2.5	<1	4	<1
3,6-Dimethyl-1,4-dioxan-2,5-dion (mg L^{-1})	190	117	3	4
Sodium (mg L^{-1})	1,205	1,220	180	110
Ammonium (mg L^{-1})	37	37	2	6
Potassium (mg L^{-1})	144	145	16	14
Magnesium (mg L^{-1})	137.5	139	17	11
Calcium (mg L^{-1})	2,510	2,627	315	210
Iron (mg L^{-1})	1.9	1.95	0.5	1.5
Manganese (mg L^{-1})	8	8	1.5	1.4
Aluminum (mg L^{-1})	5	5.5	<0.1	<0.1
Total phosphorus (mg L^{-1})	<0.5	<0.5	2.2	1.5
Lactic acid (mg L^{-1})	6,760	6,583	220	190
Formic acid (mg L^{-1})	330	258	<10	25
Acetic acid (mg L^{-1})	2,775	3,190	630	370
Propionic acid (mg L^{-1})	875	885	400	200
Iso-butyric acid (mg L^{-1})	<10	<10	<10	<10
Butyric acid (mg L^{-1})	520	585	15	15
Valeric acid (mg L^{-1})	<10	<10	<10	<10

between the mills to explain the different problems that they have.

Quantitation of eubacteria and archaea 16S rRNA genes in the paper/board mills deposits and waters

The 16S rRNA targeted q-PCR results for eubacteria matched closely with those of the cultivable bacteria in the white water (Table 2; Fig. 1, 2). This means that a major portion of the ribosomal gene copies detected originated from cultivable bacteria.

High density of archaean ribosomal gene sequences were found in the solid deposits from the mill MHS and in the slime deposit from the mill MSS, 10^7–10^9 gene copies g^{-1}. The clarified waters from these mills showed only traces (<10^4 mL^{-1}) of archaean DNA. It thus seems that an archaean population had accumulated at sites where there was little moving water.

Diversity of prokaryotic 16S rRNA sequences in the two paper/board mills

This was analyzed by cloning and sequencing of 16S rRNA genes from the mill samples. Waters from the clarifier, far away from any biocide dosage, were analyzed for the eubacteria, and a solid- and a slime deposit for the archaea. A total of 17 eubacterial clones of the 16S rRNA genes were sequenced from the MHS. Some were closely related,

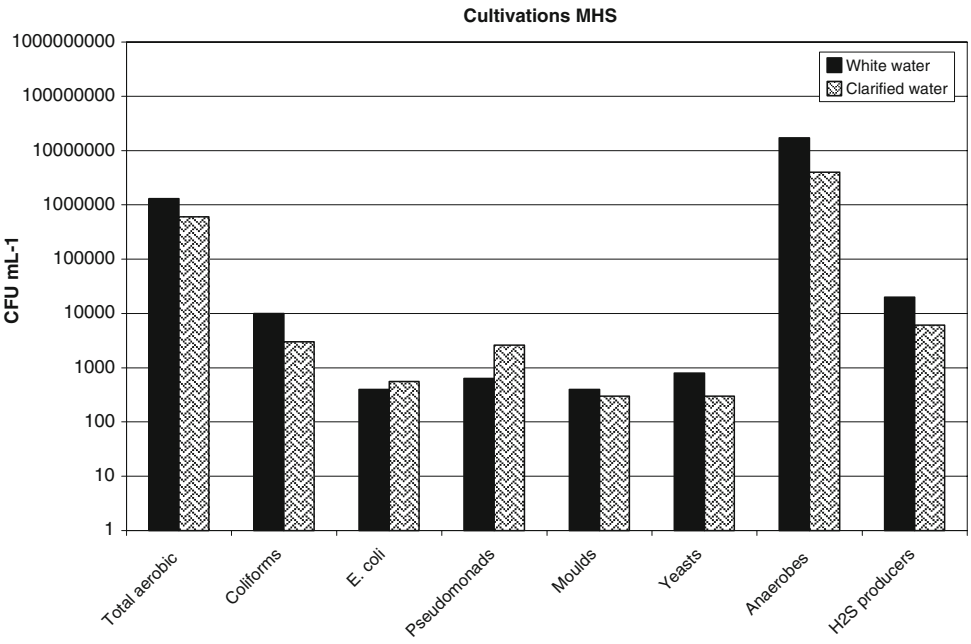

Fig. 1 Cultivation results obtained of white water and clarified water from closed cycle paper/board mill MHS. Nonselective medium (R2A) was used for the aerobic count and five selective agars for specific groups. The numbers represent cultivations from the mill during two sampling campaigns with over one month time distance. The figure shows average results. Differences between the samplings were ≤2 ten-folds

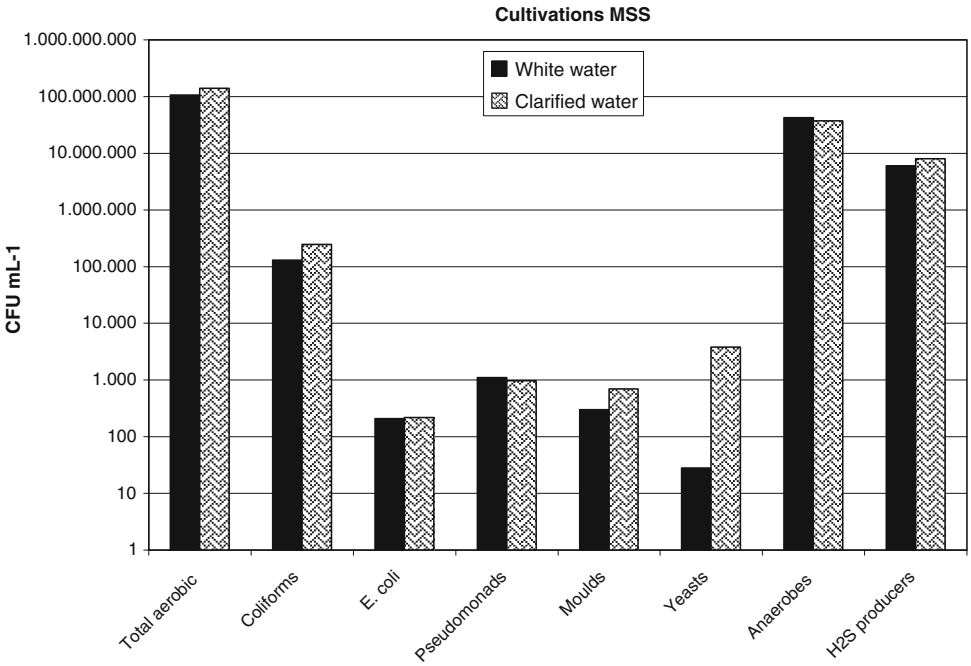

Fig. 2 Cultivation results obtained of white water and clarified water from closed cycle paper/board mill MSS. Nonselective medium (R2A) was used for the aerobic count and five selective agars for specific groups. The *numbers* represent cultivations from the mill during two sampling campaigns with over 1-month time distance. The figure shows average results. Differences between the samplings were ≤2 ten-folds

and 12 were unique and interpretable. From the mill MSS a total of 114 eubacterial 16S rRNA gene clones were retrieved for sequencing and similarity analysis. Forty-three of these were unique and interpretable. Twenty-three archaeal 16S rRNA gene sequences were obtained from the slime deposit of the mill MSS, all represented the genus *Methanothrix*. Although a 16S rRNA gene product of about 800 bp was obtained from mill MHS deposits with the archaean targeted primers, no cloned sequence was obtained, in spite of several attempts with different cloning conditions.

The bacterial sequences obtained from the mill MHS DNA grouped into four branches (Fig. 3). The branch C contained sequences resembling those reported from anaerobic reactors for brewery sewage treatments. Branch D contained sequences with a distant similarity (≤96%) to *E. cecorum*, with narrow diversity. Branch B was closest related but yet distant (≤93% similar) to *Clostridia* known to produce short organic acids. The fourth branch (A) had closest resemblance to a bacterial clone B9 (AY426453) reported from a full-scale anaerobic bioreactor treating paper mill wastewater [25]. Sequencing of the mill MHS

Table 2 Bacteria and archaea found in the two closed cycle paper/board mills MHS and MSS by Quantitative-PCR using primers for the16S rRNA gene

Mill	Sampling point	Copies of bacterial 16S rRNA gene (mL^{-1})	Copies of bacterial 16S rRNAgene (g^{-1})	Copies of archaeal 16S rRNAgene (mL^{-1})	Copies of archaeal 16S rRNAgene (g^{-1})
MHS	White water	1.8×10^6		n.d.	
	Clarified water	3.7×10^7		$<1.0 \times 10^4$	
	Solid deposit		4.4×10^8		7.2×10^7
MSS	White water	3.3×10^8		n.d.	
	Clarified water	3.1×10^8		$<1.0 \times 10^4$	
	Slime deposit		9.6×10^8		5.0×10^8

Description of the process waters in Table 1

The deposits (floor channel MHS and slime MSS) were collected during the same campaign as the waters in Table 1

n.d. not detected

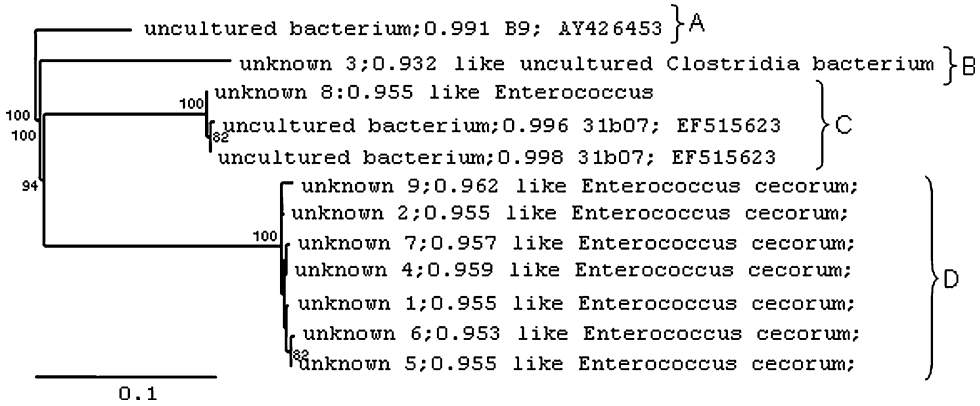

Fig. 3 Neighbor-joining phylogenetic tree of 16S rRNA gene sequences cloned from clarified water of the closed cycle zero effluent mill MHS. Sequence match is indicated before the strain code

bacterial clones revealed that most of the sequences belonged to uncultured or unknown species. This may reflect the extremophilic conditions in this machine environment (Table 1).

Forty-three of the obtained clones were retrieved from the mill MSS clarified water after multiple cloning efforts. According to the tree analysis (Fig. 4) these divided into four branches, where the group of 29 clones was found ≥99% similar to *B. coagulans* ATCC 7050 and are displayed as one. Five sequences were <97% similar to *B. coagulans* but yet closest to this species. The branch G represented another *Bacillus* group, similar to *B. thermoamylovorans.* These species are known to utilize starch at high temperatures [5, 30]. Interesting is that also the mill MSS contained unknown bacteria closest but distantly related to *E. cecorum*. The mill MSS, was without a problem of odors in the product but had sometimes problems with "rotten egg" odor that could have been explained by the H_2S producers in the water cycles (Fig. 2). Absence of the odor problems in the product may be due the high process pH (Table 1) and the lower amount of organic acid in the MSS circulation waters compared to the MHS circulation waters. None of the rRNA gene sequences (bacterial or archaeal) obtained from the mills MHS or MSS indicated relatedness to known sulphate reducing bacteria.

Discussion

The two closed cycle paper mills MHS and MSS studied in this paper have different problems in the process and in the products. The mill MHS suffered at times from bad odors in the products and complaints from the neighborhood due to a sour smell. The mill MSS had no product problem, but received sometimes complaints from the community due to "rotten egg" odor (H_2S). It is thinkable that at the mill MSS strictly anaerobic autotrophs converted sulphate to less oxidised forms of sulphur feeding the large community of sulphate nonreducing H_2S producers. Conditions at the mill MHS were less strictly anaerobic, explaining the domination of fermentative *Enterococci* and the organic acids—type of odor. The known sulphate converting autotrophs are hyperthermophilic *Archaea* (*Sulfolobus*), but evidence for mesophilic crenarchaeota was recently presented [3, 24]. The mill MHS had undertaken major efforts to keep up the product quality and to minimize the complaints from the

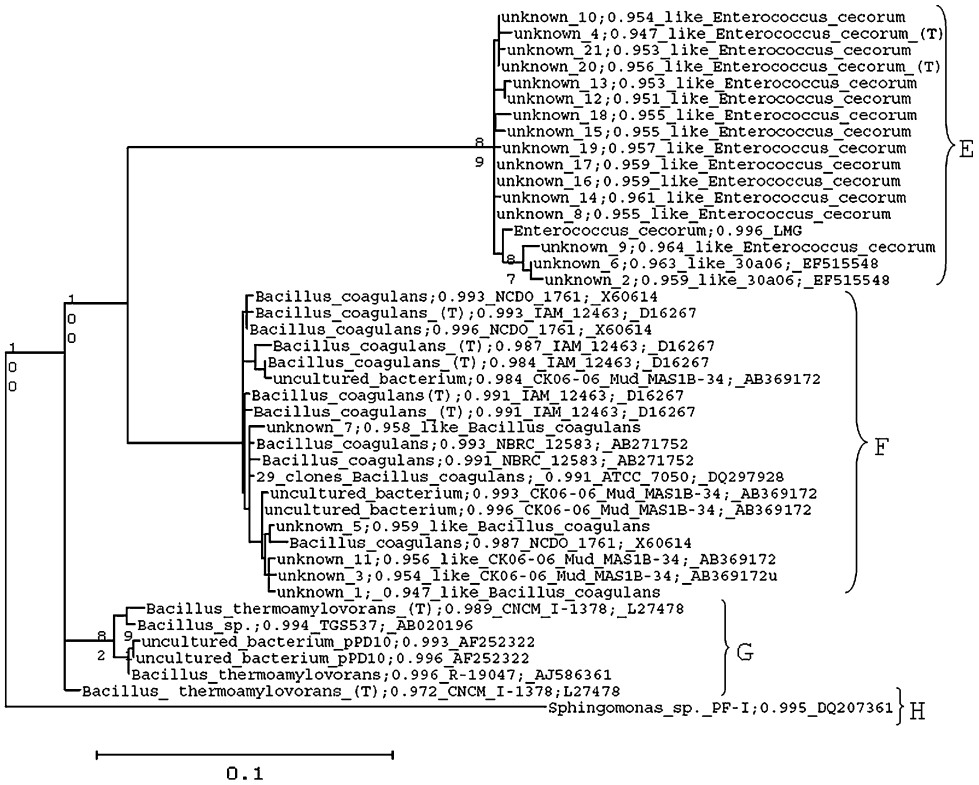

Fig. 4 Neighbor-joining phylogenetic tree of 16S rRNA gene sequences cloned from clarified water from the low effluent closed cycle mill MSS. Sequence match is indicated before the strain code

neighbors. This mill dispenses several biocidal products into their system, explaining the less strict anaerobicity.

The present preliminary study is to our knowledge the first report on the prokaryotic microbiota of paper mills discharging low amount or no waste water. The effluent free mill offered an extreme environment for microbes. Culture independent method yielded evidence of only five taxons in the effluent free mill, and seven in the low effluent mill. Traditional paper mills discharging 10–20 m^3 of effluent are known to possess high diversity when studied with culture independent methods [7, 20], or with cultivation methods [31, 32]. Results in this paper indicate that the reduction of effluent discharge from 4 to 0 m^3 made the mill water circuit somewhat less attractive for growth of cultivable aerobic bacteria, coliform bacteria, and H_2S producers. The sequencing result indicated that both the bacteria and the archaea in the 0 m^3 discharge mill represented taxa of yet undescribed species. Anaerobic bacteria were the most abundant culturable group in the 0 m^3 discharge mill. The circulation waters of the 0 m^3 discharge mill were characterized by organic carbon content of ten times that found in the 4 m^3 discharging mill and about 100 times higher than open mills [30, 31]. The closed mill water circuit contained at least 20 times more of lactic and butyric acids than the 4 m^3 discharging mill. The high content of these organic acids, low inorganic P and N content may explain the low diversity of bacteria. Lactic acid and lactates (p*K*a 3.79), propionic acid and propionates (p*K*a 4.87) are synergistically active in inhibiting growth of bacteria, moulds and yeasts (including gram-positive cocci, Clostridia, Enterobacteriaceae, Pseudomonas), concentrations 0.1–5%, being most effective at acid pH (<6) [6]. In spite of the high contents of anaerobic and aerobic bacteria, a low diversity found by sequencing of the samples. The only taxon that was obtained as multiple clones from this mill, was closest but distantly related to *E. cecorum,* about 95–96% similar [11, 33]. This low similarity to any described species of bacteria means that these cloned sequences most likely belong to one or several novel species of *Enterecoccus* or a related genus [33]. *Enterococci* are known as lactic acid resistant, good growth in CO_2 rich environment, including fermented foods, animal gut, and reported common in sewage plants of paper mills in Canada [4, 11, 12]. Several Enterococcal species, including *E. cecorum*, are human pathogens notorious as carriers of genes for antibiotic resistance [23], but it is not meaningful to speculate on the human health significance of the paper machine colonizers before strains have been isolated and their physiology studied.

Only one sequence of *Clostridium* spp. was found expected in connection with the organoleptic problems in the end product, <95% sequence match and the humble phylogenetic distance to described species of *Clostridium* indicating as a potential new genus [33].

The 4 m^3 effluent mill yielded upon cloning not only *E. cecorum* like sequences but also *B. coagulans*, *B. thermoamylovorans*. These species indicating presence of thermo-

philic starch degrading populations [5], similar to what has been reported for more open discharge mills [7, 28]. Another finding previously described from paper mill recycling water was the presence of a sequence similar to *Sphingomonas* sp. [7, 20, 31].

Cultivation independent methods (qPCR) based of 16S rRNA gene copies do not distinguish between viable and dead microorganisms. In the present study we found that the numbers of 16S rRNA copies in the recycling waters of both closed cycled mills were close to the numbers obtained by cultivation, when the presence of two to ten gene copies are assumed per cultivable bacterial cell. A significant part of the 16S rRNA genes thus represented viable bacteria.

In this paper archaea were monitored inside paper mills for the first time. The results indicated that in the closed cycle mills archaea represented a major colonizer in the solids and deposits but not in the water phase of the water circuits. Surprisingly all clones obtained belonged to the same genus, *Methanothrix*. Thus archaean population may be even less diverse than the bacteria population. The closed cycle mill MSS water may have favored *Methanothrix* converting acetate to methane as energy source. The DNA obtained by archaeal primers from the zero effluent mill appeared non-clonable with the techniques used in the present work. Therefore it is possible that in the zero discharge mill, archaeal taxons that are hither to unknown may have enriched, and possibly also in the low effluent mill in addition to the *Methanothrix*. But even then, the present results show that low effluent paper mill may represent man made environment for archaea.

The diversity found by sequencing of open cycle mills was not found in mills studied in this paper and the bacteria belonged predominately to gram positive types, compared to the more gram negative bacteria found in more open systems [20]. The difficulties to clone the DNA could indicate a high G + C content of microbiota present, or difficulties due to high amount of DNA-bound bivalent. Therefore, it would be of high interest to use pyro-sequencing to analyze the samples to determine if the diversity is that low in these processes.

Acknowledgments This work was supported by Ashland Deutschland GmbH (L.K.Ö., S I., J.S.), Academy of Finland Center of Excellence grant Nr. 118637 (J.K., M.S.S.) and by Ph.D. scholarships from the graduate school for Applied Biosciences (ABS) (to J.P.) and EnSTe graduates school (to J.E.). We also thank Helsinki University Viikki Science Library for excellent information services, the Faculty of Agriculture and Forestry Instrument Center for technical support, and Paulina Lankinen and Tuula Suortti for many kinds of help.

References

1. Blanco A (2003) Microbiology in papermaking. Recent Res Dev Appl Microbiol Biotechnol 1:87–134
2. Blanco MA, Negro C, Gaspar I, Tijero J (1996) Slime problems in the paper and board industry. Appl Microbiol Biotechnol 46:203–208. doi:10.1007/s002530050806
3. Brochier-Armanet C, Boussau B, Gribaldo S, Forterre P (2008) Mesophilic crenarchaeota: proposal for a third archaeal phylum, the Thaumarchaeota. Nat Rev 6:245–252
4. Butaye P, Devriese LA, Haesebrouck F (1998) Effects of different test conditions on MICs of food animal growth-promoting antibacterial agents for Enterococci. J Clin Microbiol 36:1907–1911
5. Combet-Blanc Y, Ollivier B, Streicher C, Patel BKC, Dwivedi PP, Pot B et al (1995) *Bacillus thermoamylovorans* sp. nov., a Moderately Thermophilic and Amylolytic Bacterium. Int J Syst Bacteriol 45:9–16
6. Davidson PM, Taylor MT (2007) Chemical Preservatives and natural compounds. In: Doyle MP, Beuchat LT (eds) Food microbiology, fundamentals and frontiers. ASM Press (American Society for Microbiology), Washington DC, pp 713–745
7. Desjardins E, Beaulieu C (2003) Identification of bacteria contaminating pulp and paper machine in a Canadian paper mill. J Ind Microbiol Biotechnol 30:141–145
8. DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci USA 89:5685–5689. doi:10.1073/pnas.89.12.5685
9. Edwards U, Rogall T, Blocker H, Emde M, Bottger EC (1989) Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16S ribosomal RNA. Nucleic Acids Res 17:7843–7853. doi:10.1093/nar/17.19.7843
10. Ekman J, Kosonen M, Jokela S, Kolari M, Korhonen P, Salkinoja-Salonen MS (2007) Detection and quantitation of colored deposit-forming *Meiothermus* spp. in paper industry processes and end products. J Ind Microbiol Biotechnol 34:3–211. doi:10.1007/s10295-006-0187-z
11. Fortina MG, Ricci G, Mora D, Manchini PL (2004) Molcecular analysis of artisanal Italian cheesed reveals *Enterococcus italicus* sp. nov. Int J Syst Evol Microbiol 54:1717–1721. doi:10.1099/ijs.0.63190-0
12. Gautier F, Archibald F (2001) The ecology of "Fecal indicator" bacteria commonly found in pulp and paper mill water systems. Water Res 35:2207–2218. doi:10.1016/S0043-1354(00)00506-6
13. Grobe KJ, Zahler J, Stewart PS (2002) Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. J Ind Microbiol Biotechnol 29:10–15. doi:10.1038/sj.jim.7000256
14. Hamm U, Schnabel S (2007) Effluent-free papermaking: industrial experiences and latest developments in the German paper industry. Water Sci Technol 65(6):205–211. doi:10.2166/wst.2007.230
15. Johnsrud SC (2000) Paper mill micro-organisms. Invest Tecnica Papel 146:499–508
16. Kanto Öqvist L, Jörstad U, Pöntinen H, Johnsen L (2001) Deposit control in the paper industry.In: 3rd ECOPAPERTECH Conference Helsinki, June, pp 269–280, ISBN 951-97513-8-6
17. Kanto Öqvist L, Salkinoja-Salonen MS, Pelzer R (2005) Novel evaluation methods for paper machine deposits. Prof Papermaking Wochenblatt Papierfabrikation 1:36–42
18. Kolari M, Mattila K, Mikkola R, Salkinoja-Salonen MS (2001) Mechanism of biofilm formation in paper machine by *Bacillus* Species: the role of *Deinococcus geothermalis*. J Ind Microbiol Biotechnol 27:343–351. doi:10.1038/sj.jim.7000201
19. Kolari M, Nuutinen J, Rainey FA (2003) Colored moderately thermophilic bacteria in paper-machine biofilms. J Ind Microbiol Biotechnol 30:225–238
20. Lahtinen T, Kosonen M, Tiirola M, Vuento M, Oker-Blom C (2006) Diversity of bacteria contaminating paper machines. J Ind Microbiol Biotechnol 33:734–740. doi:10.1007/s10295-006-0105-4
21. Lindberg LE, Holmbom BR, Väisänen OM, Weber AM-L, Salkinoja-Salonen MS (2001) Degradation of paper mill water com-

ponents in laboratory tests with pure cultures of bacteria. Biodegradation 12:141–148. doi:10.1023/A:1013175421662
22. Maukonen J, Mättö J, Wirtanen G, Raaska L, Mattila-Sandholm T, Saarela M (2003) Methodologies for the characterization of microbes in industrial envionments: a review. J Ind Microbiol Biotechnol 30:327–356. doi:10.1007/s10295-003-0056-y
23. Mundy LM, Sahm DF, Gilmore M (2000) Relationships between enterococcal virulence and antimicrobial resistance. Clin Microbiol Rev 13:513–522
24. Philippot P, Zuilen MV, Lepot K, Thomazo C, Farqhuar J, Kranendonk JV (2007) Early Archean microorganisms preferred elemental sulfur, not sulphate. New players in an ancient cycle. Science 317:1534–1537. doi:10.1126/science.1145861
25. Roest K, Heilig HGHJ, Smidt H, de Vos WM, Stams AJM, Akkermans ADL (2005) Community analysis of a full-scale anaerobic bioreactor treating paper mill wastewater. Syst Appl Microbiol 28:175–185. doi:10.1016/j.syapm.2004.10.006
26. Rättö M, Suihko M-L, Siika-aho M (2005) Polysaccharide-producing bacteria isolated from paper machine slime deposits. J Ind Microbiol Biotechnol 32:109–114. doi:10.1007/s10295-005-0210-9
27. Schenker AP, Singelton FL, Davis CK (1998) Proceedings of EUCEPA, Chemistry in Papermaking, 12–14 Oct, pp 331–354
28. Suihko M-L, Sinkko H, Partanen L, Mattila-Sandholm T, Salkinoja-Salonen M, Raaska L (2004) Description of heterotrophic bacteria occurring in paper mills and paper products. J Appl Microbiol 97:122–1235. doi:10.1111/j.1365-2672.2004.02416.x
29. Suzuki MT, Taylor LT, DeLong EF (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 59-nuclease assays. Appl Environ Microbiol 66:4605–4614. doi:10.1128/AEM.66.11.4605-4614.2000
30. Väisänen O, Elo S, Marmo S, Salkinoja-Salonen MS (1989) Enzymatic characterization of Bacilli from food packaging paper and board machines. J Ind Microbiol N Y 4:419–428. doi:10.1007/BF01569637
31. Väisänen OM, Weber A, Bennasar A, Rainey FA, Busse H-J, Salkinoja-Salonen MS (1998) Microbial communities of printing paper machines. J Appl Microbiol 84:1069–1084. doi:10.1046/j.1365-2672.1998.00447.x
32. Väisänen OM, Nurmiaho-Lassila E-L, Marmo SA, Salkinoja-Salonen MS (1994) Structure and composition of biological slimes on paper and board machines. Appl Environ Microbiol 60:641–653
33. Wang Q, Garrity MG, Tiedje JM, Cole JR (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267. doi:10.1128/AEM.00062-07