Using novel DNA methods to achieve higher process efficiency and performance

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ABSTRACT: Uncontrolled microbiological activity is a challenge for recycled fiber (RCF) mills as it can have negative effects on production and end-product quality. The microbes that exist in these systems have been largely unknown, and the strategies employed to control microbiology have been non-specific. Understanding the specific microbial groups present in RCF mills, their properties, and where they exist, as well as having the ability to accurately measure the true troublemakers, are key to targeted control of the bad actors.

In this study, we present the results of a global survey of over 40 RCF paper machines. The same RCF-specific problem-causing bacterial groups were found on different continents, including large densities of newly identified bacteria in paper processes. Those can degrade cellulose and starch, produce acids and odorous substances, and have a significant impact on fiber strength and additive consumption. We also demonstrate how modern DNA tools can quantify the impact of biocidal countermeasures against the actual troublemakers, including bacteria found to degrade cellulose during RCF pulp storage, which may be linked to a negative impact on end-product strength. These novel DNA tools give producers updated biocide program key performance indicators (KPIs) and actionable information to more effectively design and adjust microbiological control to achieve higher process efficiency and performance.

Application: We have identified problem-causing bacteria in RCF processes and shown that they may deteriorate fiber strength properties. Analysis of the bacterial community in the process tells about process health and how much bandwidth there is for improvements.

Paper and paperboard machines using recycled fiber (RCF) raw material frequently suffer from problems caused by high bacterial activity. The RCF typically contains high amounts of starch and various impurities that enable much higher bacterial densities vs. machines using virgin fiber. Therefore, RCF machines often suffer from problems such as pH drop, high conductivity, degradation of additives such as starch, bad smells, and dangerous gases that have even caused fatal accidents in recent history [1].

Biocides are applied to RCF paper and board machines for mitigating these microbiological problems. To operate a successful microbe control program, it is essential to have measurement methods for evaluating the effect of biocide dosing. The microbiological status of paper machines has historically been monitored using various methods that provide little to no information about the types of microorganisms that exist in the process.

Use of DNA-based analytics with Next Generation Sequencing (NGS) has revolutionized microbial research in clinical, food, and environmental sectors with the ability to target unculturable bacteria and reliably identify thousands of different bacteria in one sample. It is now possible to get in-depth data from microbiomes at a reasonable cost per sample. Often, the analysis reveals groups that have never been cultivated or properly named. But even in these cases, some conclusions about their metabolism and function can be drawn based on the properties of their close relatives or where similar bacteria have been found before.

Paper machine microbiology has traditionally relied on study of cultivated isolates of troublemaker groups like, as examples, bacteria-causing pink slimes [2] or spore-forming bacteria [3]. The few existing publications with NGS methods on paper machine microbiomes have detected, for example, *Tepidimonas* and *Chryseobacterium* from a mill using virgin fiber [4]. We are not aware of any wider, let alone global, surveys of paper machine microbiomes revealing benign and more problematic communities. Bacterial species causing problems at the RCF machines are even more under-studied. Kanto-Oqvist [5] reported that two machines with zero or low effluent contained *Enterococcus* and *Bacillus* as the main bacterial contaminants. Granhall [6] showed that two machines contained bacteria belonging to phyla *Actinobacteria* and *Bacteroidetes*.

In this research paper, we present results from a global microbiological study conducted on more than 40 different paperboard machines utilizing RCF raw material. Modern DNA-based methods were used to identify and characterize the harmful bacterial groups living in the wet-end of these machines. Several new analytical methods were developed for detection and quantification of the most harm-

Measurement	Device	Standard
Basis weight	Mettler Toledo	ISO 536 "Paper and board—Determination of grammage"
Tear strength	Lorentzen & Wettre	ISO 1974 "Paper—Determination of tearing resistance—Elmendorf method"
Tensile strength	Lorentzen & Wettre	ISO 1924-3 "Paper and board—Determination of tensile properties—Part 3: Constant rate of elongation method (100 mm/min)"
Short span compression test (SCT)	Lorentzen & Wettre	ISO 9895 "Paper and board—Compressive strength—Short-span test"

I. Sheet testing devices and ISO Standard Methods used for producing paper sheets.

ful species. Finally, these methods were used to reveal the ultimate causative agents behind problems leading to process disturbances and production losses in board machines using RCF. We also show that bacterial activity in RCF processes can deteriorate fiber strength properties.

MATERIALS AND METHODS

Fiber strength in laboratory test

Preparation and treatment of pulp

A 50 g linerboard (from two different mills: 50% from a mill using ~50 % unbleached kraft pulp and ~50 % recycled fiber, and 50% from a mill using 100% recycled fiber) was repulped with process water from a third mill using recycled fiber. The final consistency of the pulp was 1.0%. The pulp suspension was divided into 12 bottles, and starch suspension (native potato starch) was added into each bottle so that the concentration of added starch was 1.5 g/L. Six of the bottles (numbers 1–6) were put into an incubator with no other treatment. The other 6 bottles (numbers 7–12) were first pasteurized (1 h, +80°C) in order to inactivate any enzymes present. After that, the bottles (7–12) were additionally treated with a biocide (50 mg/L monochloramine as active chlorine). After this, all 12 bottles were kept at +40°C with shaking for 4 days. During the incubation, 10 mg/L monochloramine was added daily into bottles 7–12. At the beginning of the incubation, the pH of the pulp was 6.0, redox was -46 mV, and conductivity was 4.8 mS/cm.

Preparation of handsheets

After 4 days, the pH, redox, bacterial amounts, and cellulase activity were measured from each bottle. One laboratory handsheet (100 g/m^2) was made from the pulp in each bottle with a Rapid-Koethen sheet former. First, each pulp sample (1% consistency) was mixed in a separate mixing vessel equipped with baffles (30 s, 1000 rpm). Then, the sample was diluted with tap water to 0.5% consistency and mixed for another 30 s (1000 rpm). Next, it was poured to the Rapid-Koethen sheet former, and water was drained out through a wire with suction. No additional chemicals were used in the sheet forming. Formed sheets were dried with a vacuum dryer (93°C, 10 min). Before testing in the laboratory, sheets were pre-conditioned for 24 h at 23°C in 50% relative humidity, according to ISO 187 "Paper, board and pulps—Standard atmosphere for conditioning and testing and procedure for monitoring the atmosphere and conditioning of samples." After the handsheets were made, the strength properties were measured according to **Table I**.

Bacterial community analysis

Sample collection and DNA extraction

Aquatic process samples, typically white water (WW) and RCF pulp suspension, were collected from RCF-utilizing paper machines. Sample temperature, pH, and redox potential were recorded at the time of the sampling. For microbial community analysis, a representative 0.5 mL process sample was mixed with a DNA preservation solution that was either commercially available or provided by a sequencing service laboratory. The DNA was extracted with a method based on efficient cell disruption by bead-beating, using either the proprietary protocol of a sequencing service laboratory or a commercially available environmental DNA extraction kit. Density of prokaryotes (bacteria and archaea) was measured with quantitative polymerase chain reaction (qPCR), and results were transformed to a cells/mL process sample.

DNA sequencing and data analysis

Community compositions were determined by means of Illumina high throughput sequencing of partial 16S rRNA gene, targeting either hypervariable regions V4-V5 or region V4. Sequencing was outsourced to commercial sequencing service laboratories; samples compared to each other in this publication were always processed with the same protocol in the same service laboratory. Sequence raw data (fastq format) was processed by removing poor quality, chimeric, and other than prokaryotic 16S rRNA sequences. The remaining at least 10000 good-quality sequences per sample were classified against the Silva V. 138 reference database for phylum, class, order, family, and genus level identification.

	Non-treated	Treated
Grammage, g/m² (n=5)	98.47 ± 2.05	99.81 ± 2.47 (+1.4%)
Ash content, %	4.00	4.35 (+8.8%)
Tensile index, Nm/g (n=20)	27.62 ± 2.07	30.92 ± 1.78 (+12%)
SCT index, Nm/g (n=20)	16.58 ± 1.1	17.16 ± 0.98 (+4.9%)
Tear index, Nm²/kg (n=20)	6.23 ± 0.38	6.84 ± 0.46 (+9.9%)
Aerobic bacteria, cfu/mL (n=6)	120 000 000 ± 17 000 000	8 700 ± 3 300
Anaerobic bacteria, cfu/mL (n=6)	37 000 000 ± 6 600 000	970 ± 2 000
Cellulase activity, mU/mL (n=6)	1.01 ± 0.15	0.44 ± 0.08
pH (n=6)	5.8 ± 0.0	6.3 ± 0.0
Redox, mV (n=6)	-459 ± 23	94 ± 10
SCT = short span compression test.		

II. Biocide treatment was successful in maintaining fiber strength properties. Recycled fiber (RCF) pulp was stored in the laboratory for 4 days with and without biocide treatment. After the storage, pulp conditions were measured and handsheets were prepared. Strength parameters of the handsheets were measured using standard methods. Standard deviations for the results were calculated using Microsoft Excel.

qPCR assay for *Thermicanus*

A specific polymerase chain reaction (PCR) assay was developed to quantify a novel group of RCF troublemaker bacteria, genus *Thermicanus*/family *Thermicanaceae*. Details of the novel assay are specified in patent application WO2021250317A1 [7].

qPCR assay for *Bacteroidales*

Another specific PCR assay was developed to quantify a novel group of RCF troublemaker bacteria, which belong to the order *Bacteroidales*. Details of the novel assay are a trade secret. Application of DNA-based analysis of *Bacteroidales* to help preservation of RCF cellulose strength is covered by patent application WO2021214385A1 [8].

RESULTS AND DISCUSSION

Bacterial activity decreases fiber strength

We studied the effect of bacterial activity on fiber strength in the laboratory. We treated some of the RCF pulp samples with biocides and some were not treated, and after storage, we prepared handsheets. **Table II** shows that the amount of both aerobic and anaerobic bacteria was much higher in the bottles without biocide present. Additionally, cellulase enzyme activity in these samples was higher, and endpoint pH and redox were lower. Strength properties were measured from handsheets prepared from the pulp. All measured values were better in the pulp that had been treated with the biocide. Tensile, tear, and short span compression test (SCT) indexes were 4.9%–12% higher in the handsheets from treated pulp vs. untreated pulp. At the same time, ash content was 8.8% higher in sheets from the treated pulp, which makes the strength difference even more significant.

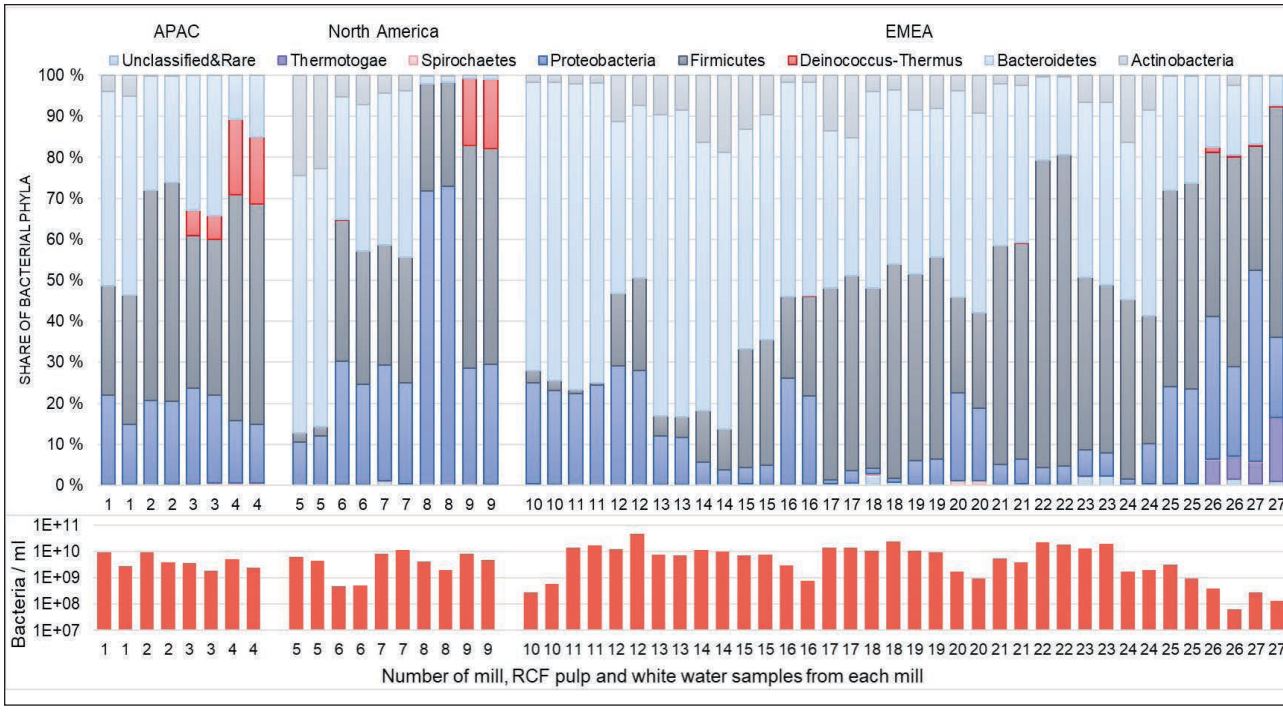
Most likely, bacteria produced cellulase enzyme in the RCF pulp with no biocide treatment, and cellulase activity resulted in the decrease of the fiber strength. Microbiological fermentation of sugars released from cellulose and starch led to a pH drop and eventually lower sheet ash content. To our knowledge, this is the first time that deterioration of cellulose fiber strength properties caused by bacterial activity in paper machine conditions has been reported.

In this case, the untreated pulp samples contained >10^8 bacterial cells/mL. In many mill cases, significantly higher bacterial numbers have been detected (e.g., **Fig. 1** and **Fig. 2**), and thus the fiber degradation can be even faster in real mill conditions. The risk for microbiological cellulose degradation is especially high in RCF, because high amounts of starch and other impurities can sustain much higher microbial density and activity than cleaner virgin fiber.

Bacterial genera present at mills using recycled fiber

We conducted a global study from board machines using recycled fiber. We collected samples from over 40 different paper machines in North America, Europe, Asia, and Australia. For most sites, we collected samples multiple times during a three-year period, and in total we analyzed bacterial communities from about 400 samples.

Microbial communities were remarkably similar in RCF pulp and WW samples from the same mill (Fig. 1). Mills with the lowest temperatures hosted typically *Bacteroidetes* and *Proteobacteria*, and at highest temperatures *Deino-*



1. Bacterial community composition on phylum level in samples from recycled fiber (RCF) mills on three different continents (part of our global survey data). The RCF machine number is shown on the x-axis, and adjacent bars represent RCF pulp and white water from the same mill. Paper machines within each region are arranged based on process temperature from lower (left) to higher (right). Red bars in the lower part of the figure show bacterial densities in the same samples based on a quantitative polymerase chain reaction (qPCR) method.

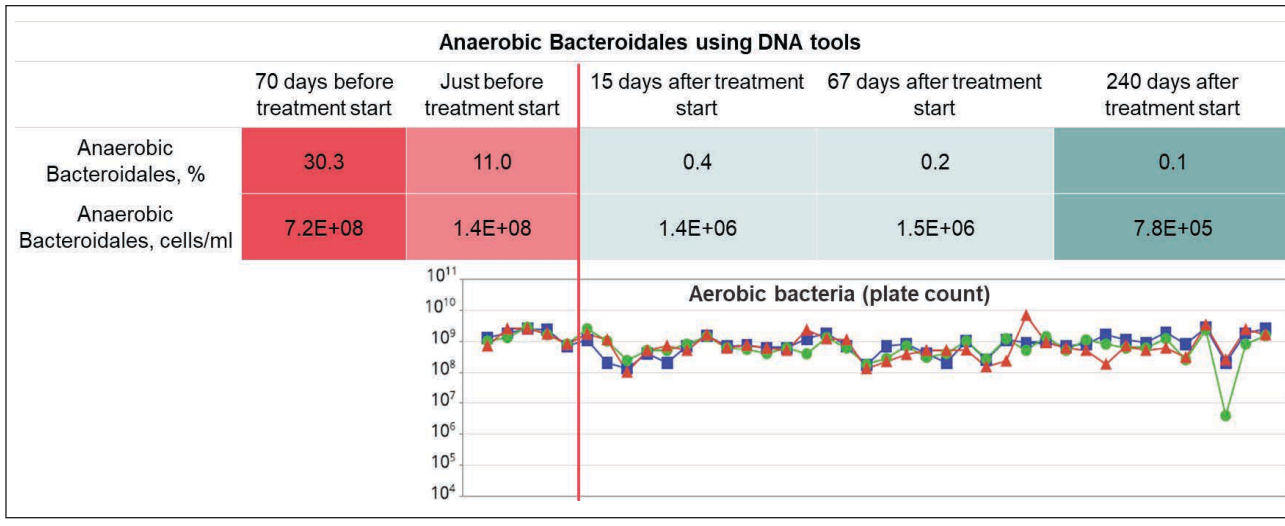
coccus-Thermus and *Thermotogae* were found. *Firmicutes* could be very abundant, especially at machines with medium to high temperatures. At lower taxonomic levels, we could find many bacteria typical of the digestive tract of ruminants and other herbivores. With few exceptions, bacterial densities in the process samples were very high at up to 1010 cells/mL. We also quantified fungi with qPCR, but these were much less abundant, typically less than 0.001% of the bacterial amount (data not shown), and they are not typically problems for the recycled fiber process. Archaea, which can be identified with the same NGS analysis as bacteria, were detected very rarely and in very small percentages.

The single most abundant bacterial genus in our global RCF machine survey was *Cloacibacterium*. As the name hints, this genus was originally found from municipal wastewater samples, but not from human feces [9]. With an optimum growth temperature lower than that of human body, *Cloacibacterium* is facultatively anaerobic and can ferment sugars it releases by hydrolyzing starch. Unlike many other RCF bacteria, *Cloacibacterium* is readily observed with normal aerobic plate counts. Interestingly, nearly or exactly the same sequence was detected at all the mills, indicating that the same "wastewater bacterium" may have spread and contaminated RCF machines mills on several different continents. According to our observations, *Cloacibacterium* is highly successful and competitive in the RCF process and can contribute to pH decrease and oxygen consumption in towers, but it is not one of the worst bacteria present at high numbers (both with cultivation and with DNA methods) and can be observed at times of good runnability. We always detected other, more anaerobic troublemakers at RCF mills at times of more severe issues with process runnability or end-product quality.

Our global survey also identified that some machines contain aerobic *Bacteroidetes*, such as *Sphingobacterium*, and aerobic Proteobacteria, such as various *Alphaproteobacteria*, *Tepidimonas*, and *Pseudoxanthomonas*. Some of these are known slime-formers in virgin fiber machines. They could potentially also cause slime issues in an RCF machine, although slimes are typically a minor issue compared to the other microbiological challenges in RCF machines.

Thermicanus*, a novel problem-causing bacterium in the paper industry*

Thermicanus bacteria were found to be very common in some mills. **Table III** shows the results from three different machines. Mill A is in the United States, and mill B is in China. We found exactly the same bacterium in both mills, demonstrating the global presence of this bacterium. In these examples, the proportion of *Thermicanus* was 21%–51% of the total bacterial community, and there were even > 109 *Thermicanus* cells/mL in the process samples. In all these cases, the amount and proportion of *Thermicanus* was higher when the process conditions (character-



2. Process follow-up using DNA tools and conventional plate counting. The amount of anaerobic Bacteroidales was analyzed using a qPCR method specifically designed for this bacterial group. The proportion of this group was calculated from total bacterial amount based on DNA quantification. This analysis was done 5 times during the follow-up period of 10 months. Total aerobic count was analyzed weekly from three sampling points using low nutrient agar plates, and the bacteria were cultivated at +37°C for 2 days. The results with DNA tools correlate with the machine runnability, unlike conventional bacterial cultivations (red line = start of microbe control treatment).

ized by redox, conductivity, and pH) were worse. For example, in Mill B at machine 2 in 2018, pH was 6.55, redox was +57 mV, and conductivity was 1575 µS/cm. At that time, the proportion of *Thermicanus* was 21.2%. Next year, at clearly worse process conditions (pH 6.23, redox -160 mV, conductivity 3020 mV), the proportion of *Thermicanus* was about double, 41.1%, and the amount of *Thermicanus* bacteria was almost three times higher than before.

Thermicanus belongs to the order *Bacillales* and only one species, *Thermicanus aegyptinus*, has been described so far. It is thermophilic (optimum growth temperature 55°C–60°C), can grow anaerobically, and produces several different organic acids [10]. It can also break down cellobiose, which is a disaccharide derived from cellulose degradation, and thus it may also play a role in cellulose degradation in paper machine processes. Recently, it was found to be a dominant bacterium when paper degradation was studied using fluid from cow rumen as a starting bacterial culture [11]. This again shows that *Thermicanus* may play a role in cellulose and pH degradation. To our knowledge, this is the first time it has been identified in the papermaking process and linked to poor process conditions.

Case study 1: DNA tools are more accurate in process follow-up vs. traditional cultivation methods

Historically, the follow-up of microbiological status in the paper industry has been done using microbiological cultivations. However, the total amount of culturable bacteria seldom correlates with the machine runnability and process health [12,13].

Machine	Mill A		Mill B PM 1		Mill B PM 2	
Sampling time	2018	2019	2018	2019	2018	2019
Redox, mV	-35	118	-140	-172	57	-160
Conductivity, µS/cm			2170	3250	1575	3020
pH	6.53	6.50	6.46	6.43	6.55	6.23
Total bacteria (cells/mL, qPCR)	4.7E+09	2.4E+09	4.9E+09	8.8E+09	3.6E+09	5.5E+09
Thermicanus, %	35.6	28.9	33.2	51.3	21.2	41.1
Thermicanus, cells/mL	1.7E+09	6.9E+08	1.6E+09	4.5E+09	7.6E+08	2.3E+09
qPCR = quantitative polymerase chain reaction.						

III. Relative and absolute abundances of* Thermicanus*, total bacteria, and other process parameters at two separate samplings on three linerboard machines.

This is especially the case in machines utilizing RCF raw materials, because these machines almost always contain high bacterial counts, both in good and poor process conditions. Figure 2 shows data from a mill case. This mill suffered from high microbial activity, visible as a rapid pH drop and conductivity increase following a system boil-out. This led to process disturbances and end-product quality issues. We started a microbe control program using organic biocide containing 2,2-dibromo-3-nitrilopropionamide (DBNPA), bronopol, and chloromethyl-methylisothiazolone (CMIT/MIT). It was added into the pulp chest just after the RCF pulper. Also, a zinc-based amylase enzyme inhibitor product was used to prevent starch degradation. After the treatment started, the conditions at the mill improved significantly. The mill could reduce planned shutdowns from three times per month to outages twice per month. Unplanned shutdowns, even during warmer summer months, were eliminated. These had been frequent during the previous summer due to high process conductivity and runnability problems.

We monitored the RCF pulp and process water microbial situation during the trial; total bacteria (both culturable and DNA-based) and a specific group of anaerobic presumed troublemakers were detected at the mill with NGS sequencing. Figure 2 shows how the microbiological situation changed when the microbe control program was started. Previously, the proportion of anaerobic *Bacteroidales* was at times up to one third of the total bacterial community, and their number was over 108 cells/mL. Microbe control treatment collapsed the amount of these bacteria, both their share and number.

Simultaneously, we did traditional plate counts for the samples. In this data, also shown in Fig. 2, no significant change was observed. Additionally, we cultivated anaerobic bacteria from the same samples (data not shown), but that analysis also showed no change during the trial. As a conclusion, the bacteria causing the problems at the mill were anaerobic and belonged to order *Bacteroidales*. Their number and share correlated very well with the observed machine runnability and process conditions, unlike the results obtained with traditional cultivation methods. It is obvious, therefore, that these true troublemakers did not grow on the agar plates.

This qPCR method for anaerobic *Bacteroidales* provides an interesting opportunity for predictive analysis about mill conditions. The proportion of the problem-causing bacteria will increase before they cause issues at the machine, and thus the results may allow predictive optimization of the biocide program before problems arise at the mill.

	Before trial		Trial, sample 1		Trial, sample 2		Trial, sample 3		3 weeks after the trial	
Bacterial genus (%)	RCF pulp	White water	RCF pulp	White water	RCF pulp	White water	RCF pulp	White water	RCF pulp	White water
Sphingobacterium	0.0	0.0	0.9	0.4	0.4	0.4	0.9	1.1	0.0	0.2
Meiothermus	0.0	0.0	1.2	0.5	1.4	0.8	0.6	0.8	0.0	0.0
Thermus	11.4	8.4	5.4	3.7	3.3	3.3	0.1	0.1	4.2	2.3
Anoxybacillus	0.4	0.3	3.0	1.9	20.6	16.0	4.0	2.9	0.0	0.6
Bacillaceae_unclassified	1.2	1.2	38.0	35.1	10.2	10.0	2.4	2.9	0.4	2.6
Bacillales_unclassified	0.5	0.5	2.5	3.5	21.5	16.4	7.0	6.4	0.9	0.5
Thermicanus	50.3	52.8	7.2	5.8	5.5	10.6	5.8	7.0	47.5	50.8
Fonticella	0.0	0.0	0.5	0.2	1.7	0.6	2.8	0.8	0.0	0.0
Thermoanaerobacterium	0.4	0.3	4.5	2.7	11.4	11.1	32.4	15.9	0.0	0.8
Veillonellaceae	0.2	0.1	0.3	0.1	0.3	0.3	1.3	0.6	0.0	0.1
Chelatococcus	1.0	0.9	0.4	0.4	0.2	0.2	0.2	0.3	1.4	1.0
Burkholderiaceae_unclassified	0.9	0.9	2.4	5.5	0.8	1.1	1.0	1.6	0.3	0.3
Tepidimonas	0.0	0.0	0.9	0.5	0.8	0.8	1.6	2.3	0.0	0.0
Hydrogenophilus	0.1	0.1	0.7	0.4	0.9	1.3	3.6	8.1	0.0	0.0
Tepidiphilus	28.0	29.2	15.0	9.1	11.7	14.6	12.4	21.9	32.3	29.3
Enterobacteriaceae_unclassified	0.0	0.0	1.3	0.6	0.4	0.4	2.4	2.8	0.2	0.2
Acinetobacter	0.0	0.0	1.2	0.7	0.3	0.3	2.8	2.7	0.0	0.1
Pseudomonas	0.0	0.0	2.3	9.7	0.4	0.5	0.4	0.6	0.0	0.0
Pseudoxanthomonas	4.9	4.9	5.6	4.6	3.4	3.4	1.6	2.7	11.6	9.6
Stenotrophomonas	0.0	0.0	1.0	0.7	0.5	0.6	2.5	3.9	0.1	0.1
Total cells/ml (DNA)	1E+09	6E+08	2E+08	5E+07	2E+08	2E+07	1E+07	6E+06	1E+09	1E+09
Thermicanus cells/ml (qPCR)	4E+08	2E+08	4E+06	1E+06	4E+06	2E+06	6E+05	3E+05	2E+08	1E+08
Thermicanus % (qPCR/total)	39 %	34 %	2 %	3 %	2 %	8 %	4 %	4 %	19 %	9 %

IV. Bacterial community in a linerboard machine during a microbe control trial. The proportions of different bacterial genera were calculated based on sequencing data. Total cell amounts were calculated based on DNA concentration. The amount of* Thermicanus *was quantified using a quantitative polymerase chain reaction (qPCR) method. Bacteria with >1 % share in at least one sample are shown, and the cells are colored using Microsoft Excel conditional formatting. Monochloramine treatment was started on this machine just after the first sampling and ended after 2.5 months, soon after the third sampling during the trial.

Case study 2: Mill suffering from high Thermicanus

A large North American mill producing linerboard and corrugating medium from RCF did not utilize any appreciable amount of biocide in its moderately closed process. The mill intended to close its water system even more in the future and investigated the use of more aggressive biocides in preparation for this. A decision was made to evaluate a chloramine-based chemistry to stabilize the process pH, reduce functional additive use, and increase production yield.

Table IV shows the microbial community composition at five different dates. The first sampling was done just before the biocide trial started, three were done during the trial, and the last sampling was about three weeks after the treatment had ended.

Thermicanus dominated the bacterial community in early spring before the trial. Based on sequencing data, it contributed >50% of the bacterial cells. The proportion with specific qPCR method was slightly lower, but still more than one-third. Otherwise, the bacterial community was very simple: there was almost 30% *Tepidimonas*, about 10 % *Thermus*, and 5 % *Pseudoxanthomonas*. All these bacteria or their close relatives are known to cause slimes in virgin fiber paper machines [4,14,15], and their presence in high amounts increases the risk of biofouling and runnability issues in an RCF process as well. In addition to these four major colonizers, only 8 other bacteria were present at low proportions.

The first samples after the biocide trial start were collected after 19 days. The total amount of bacteria had decreased about 90%, but the amount of the presumed main problem-causing bacterium, *Thermicanus*, decreased even more, about 99%, and its share from the total community was now only 2%–7%. Also, the proportions of the potential slime-forming bacteria decreased. Overall, the community was much more diverse (more species present), and the dominant bacterial species belonged to order *Bacillales*. It is notable that their amount did not increase significantly, only their proportion, because the number of other bacteria decreased so much. The results about three weeks later (Trial, sample 2) were very similar.

For the last month of the evaluation, the biocide treatment was intensified, and on the third sampling during the trial, the number of total bacteria was even about 90% lower than before. The community composition was otherwise similar to earlier sampling times, but the proportion of *Thermoanaerobacterium* had increased. Again, their amount did not increase, only their proportion. *Thermoanaerobacterium* is a spore-forming anaerobic bacterium, and it is likely that it did not really grow in the process but originated from raw materials instead.

We did the final sampling about three weeks after the biocide trial had ended. The bacterial community had returned remarkably close to the situation three months earlier. *Thermicanus* was again the most common bacterium, and many of the other species had again vanished from the community. Also, the total number of bacteria was at the same level as before the trial. This shows that certain bacteria are best adapted to the conditions prevailing on this machine, and they are common unless a microbe control program is in operation.

Machine testing reflects a similar trend to that seen in our Global Survey as to the impact of *Thermicanus* (Table III). During this trial, when the amount and proportion of *Thermicanus* decreased, an increase in pH and redox and a decrease in conductivity resulted (**Table V**). This is especially evident in the final phase of the trial (sample 3). These improvements reversed after the trial ended and the *Thermicanus* again became a larger portion of the population.

These observed paper machine changes resulted in reduced doses of several wet-end additives. The machine also reached higher production rates at given additive levels, indicating some efficiency gains due to the improved stock and water conditions. The production gains were also lost after the trial, as the pH, redox, and conductivity reverted to pre-trial levels.

We used two different methods, NGS sequencing and qPCR, for the quantification of *Thermicanus*. Based on qPCR results, the proportion of *Thermicanus* was slightly lower than that based on sequencing, but the difference

	Before Trial		Trial, Sample 1		Trial, Sample 2		Trial, Sample 3		3 Weeks After the Trial	
	RCF Pulp	White Water	RCF Pulp	White Water	RCF Pulp	White Water	RCF Pulp	White Water	RCF Pulp	White Water
pH	6.2	6.8	6.6	6.9	6.7	6.9	7.1	7.2	6.55	6.47
Redox, mV	10	-58	-106	40	-18	42	10	108	8	63
Conductivity, µS/cm	3605	3537	3930	3906	4389	4303	3371	3327	3992	3927
Temp, °C	49.3	54.7	52.4	55.2	52.9	56.2	50.7	55.6	53.7	57.0

V. Measured parameters in a linerboard machine during a microbe control trial.

Bacterial Group	Harmful Properties	Growth Temperature in Paper Machines
Anaerobic *Bacteroidales* (e.g., *Prevotellaceae, Dysgonomonas*)	Starch and cellulose degradation, acid production, growth only in anaerobic conditions	<45°C
Anaerobic *Firmicutes* (e.g., *Clostridium, Ethanoligenens, Fonticella, Ruminococcus, Thermoanaerobacterium, Veillonellaceae*)	Starch and cellulose degradation, acid production, dangerous gases and bad smells, growth only in anaerobic conditions	>35°C
Thermicanus	Starch and cellulose degradation, acid production	>45°C
Lactic acid bacteria	Starch and cellulose degradation, acid production	<45°C
Facultative *Bacillales*	Starch degradation and acid production, end-product spores	>30°C

VI. Harmful bacterial groups identified for RCF.

was not big and the same conclusions can be made based on both methods. This confirms the reliability of both methods. These methods can be used in slightly different ways and for different purposes. The sequencing method reveals the total community in the process and proportions of each member. Once the most problematic bacteria have been identified, they can be monitored using specific qPCR assay. The qPCR method is much faster than sequencing; the lead times for results after sample arrival to the laboratory are 1–2 days for the qPCR method and about 2–3 weeks for the sequencing method. In addition, sequencing analysis has significantly higher costs.

Like the survey results in Fig. 1, the bacterial community in this case was at each sampling time very similar in both sample points. It seems that the whole process is occupied with the same bacterial community, and its members can prevent the growth of other bacteria entering the process from the environment. In other paper machines, we have seen that the community can remain stable even for several months or years (data not shown).

Harmful bacterial groups on machines using RCF require new methods

Based on the extensive analysis of the machine samples, we identified bacteria that have potential to be harmful to RCF processes. These groups were present in the machine processes during events of poor runnability or other process failures and were absent, or at least scarce, during good process/performance conditions. **Table VI** lists five of the most harmful groups identified.

Anaerobic *Bacteroidales* is a group of bacteria that had not been previously linked to problems on paper machines. They were especially common in RCF machines at lower temperatures, with a maximum at +45°C. They are also common inhabitants in the digestive tracts of warm-blooded animals, including humans [16]. These bacteria get most of their energy from the fermentation of sugars and simultaneously produce organic acids, which cause a pH drop. Additionally, they most likely degrade cellulose fibers in RCF towers like they do in the herbivore digestive tracts. *Bacteroidales* bacteria cannot be detected with common laboratory cultivation media.

Anaerobic *Firmicutes* is another group of obligate anaerobic bacteria that can be common in problematic situations. In addition to starch and cellulose degradation and acid production, it is known that these bacteria can produce malodorous substances such as butyric acid or dangerous gases such as hydrogen [17-19]. Some of these grow on anaerobic cultivation media, but many of those common in RCF systems do not.

Thermicanus is also a novel bacterial genus for the paper industry. In fact, very little is known about these bacteria from any environment, and there are only a few scientific publications about them. Botta [11] reported that *Thermicanus* dominated bacterial communities in office paper fermenting lysimeters at 55°C, degraded cellulose/cellobiose, and produced methanol and volatile fatty acids. *Thermicanus* requires high temperatures (>45°C) and typically needs other bacterial partners. They too are difficult to grow in the laboratory.

Lactic acid bacteria are mostly fermentative, and they can produce many different organic acids as side products during their growth. Thus, they can cause a pH drop on the paper machine. Many species can degrade starch and some also degrade cellulose [20], leading to possible strength issues. On some machines, more than 50% of the bacteria have been lactic acid bacteria. In our study, genus *Enterococcus* was globally the most abundant RCF lactic acid bacterium; other genera were detected only sporadically.

Facultative *Bacillales* is the only bacterial group from these five that has been formerly known to be common in the paper industry. Previously, they have been linked to end-product hygiene issues in machines producing food packaging board or papers [3]. In paper machines using

recycled fiber, they can cause a pH drop, while some species are fast growing and efficient starch degraders. According to our study, they seem to be more common in paper machines with high temperatures. *Bacillales*, like some anaerobic *Firmicutes*, can form heat-resistant spores that end up in the end-product. This is a highly effective way for bacteria to spread around the world with RCF raw material.

CONCLUSIONS

It is well established that uncontrolled microbiological activity in a recycled fiber process can have negative impacts on process stability, production efficiency, and end-product quality. Historically, detailed awareness of the bacteria that are troublemakers in RCF processes was unknown. Therefore, in order to attain this knowledge, we developed and implemented novel DNA methods in a global survey. Interestingly, but maybe not surprisingly based on the challenges that many RCF users face, many of the bacteria identified during this study were starch-degrading and acid-producing bacteria. However, it was enlightening that, in some RCF processes, cellulose-degrading bacteria were present in significant numbers. Now we better understand that bacteria in RCF processes not only impact system pH and conductivity, but they can also impact strength of board.

While having the ability to identify specific bacteria in edge and utilizing these methods for the benefit of the packaging board industry.

ABOUT THIS PAPER

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About this journal

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We studied this topic because we saw severe microbiological problems in paper and paperboard machines using recycled fiber, but there was no information available about the microbes causing these problems. While problem-causing bacteria on fine paper machines (e.g., biofilm formers) were already known, we concentrated on recycled fiber (RCF) processes in this study. This is also the first time that bacterial community analysis with Next Generation Sequencing (NGS) has been used extensively in the paper industry.

The most difficult aspect of this study was obtaining good quality samples from paper machines around the world. This issue was solved by developing easy sampling kits and through the help of Kemira's global sales organization.

Through this study, we discovered totally new bacterial communities existing in the paper industry. The community compositions were in line with the machine conditions in that the analysis showed harmful bacteria can make conditions unfavorable for papermaking. Of particular interest was that there were high amounts of cellulose-degrading bacteria previously known from animal digestive tracts that may be able to deteriorate fiber strength.

For mills, bacterial community analysis reveals the status of the process. Is there a need for improved microbe control? With regular follow-up, we can predict future problems before they occur and proactively adjust the microbe control program.

Our next step is to analyze samples from more and more mills, simultaneously building up knowl-



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a process is nice, the real benefits of having a method to accurately enumerate and identify potential troublemakers in RCF processes are significant. Not only can it be an effective tool for root cause analysis of problems being experienced, but it also will assist producers in improving their process to minimize the potential harmful effects. The studies suggest that RCF processes have a "buffer" capacity with respect to bacterial populations and that it does not change daily in a significant way. Sequential analyses with new DNA tools can provide valuable insights on process health, potential risk factors, and even potential process improvement bandwidth.

Finally, the new methods presented have the potential to be the next generation of microbiological monitoring within a process as we move to the future. Over time, as DNA methods and analysis are refined and the equipment and processing costs are reduced, it is expected that DNA will become the new standard of monitoring and controlling microbiology in paper mill processes to attain higher process performance, more effective control strategies, and optimum chemical consumption. **TJ**

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