Bacterial Community Characterization in Paper Mill White Water

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The paper production process is significantly affected by direct and indirect effects of microorganism proliferation. Microorganisms can be introduced in different steps. Some microorganisms find optimum growth conditions and proliferate along the production process, affecting both the end product quality and the production efficiency. The increasing need to reduce water consumption for economic and environmental reasons has led most paper mills to reuse water through increasingly closed cycles, thus exacerbating the bacterial proliferation problem. In this work, microbial communities in a paper mill located in Italy were characterized using both culture-dependent and independent methods. Fingerprinting molecular analysis and 16S rRNA library construction coupled with bacterial isolation were performed. Results highlighted that the bacterial community composition was spatially homogeneous along the whole process, while it was slightly variable over time. The culture-independent approach confirmed the presence of the main bacterial phyla detected with plate counting, coherently with earlier cultivation studies (*Proteobacteria, Bacteroidetes*, and *Firmicutes*), but with a higher genus diversification than previously observed. Some minor bacterial groups, not detectable by cultivation, were also detected in the aqueous phase. Overall, the population dynamics observed with the double approach led us to hypothesize a possible role of suspended bacteria in the re-formation mechanisms of resistant biofilms.

Keywords: Paper mill; 16S rRNA; Isolation; Library; White water

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INTRODUCTION

Paper production is a very important industrial sector worldwide. World paper production has reached 183.8 million tonnes in 2011, and the European paper industry produces 25.4% of the total amount. Italy is the eighth exporter in the World and the fourth producer in Europe (CEPI 2012).

Paper production is among the most water-demanding of industrial processes, and the reduction of water consumption is among the main measures to improve environmental protection and cost efficiency. This need has led most paper mills to recycle process water by sophisticated internal loops that are being constantly improved and perfected to strongly reduce wastewater discharge.

The enhancement of microorganism proliferation is one of the most threatening consequences of improved water recycling. Although many studies consider fresh water

entering the process as the main cause of contamination (Väisänen *et al.* 1998; Kanto Öqvist *et al.* 2001; Blanco *et al.* 2004; Kanto Öqvist *et al.* 2005), microorganisms are also introduced through raw materials and chemicals used in paper production (*e.g.* fiber, starch, carbonates, *etc*.) (Kanto Öqvist 2008; Blanco *et al.* 1996; Väisänen *et al.* 1998; Ekman 2011; Peltola 2011). Once microorganisms enter the process, some of them find optimal conditions to proliferate: temperatures ranging from 37 °C to 60 °C, pH ranging from 5 to 10 (Ekman 2011), and the presence of cellulose and organic compounds, which provide abundant sources of nutrients (Blanco *et al.* 1996, Salzburger 1996, Väisänen *et al.* 1998, Raaska *et al.* 2002, Kolari *et al.* 2003, Kolari 2007; Kanto Öqvist *et al.* 2008). In this context, the paper mill becomes an effective incubator, and recycled water becomes a source of bacterial contaminants as important as external sources are. The microbial colonization of papermaking machines can threaten the manufacturing process by causing more frequent halts for maintenance and cleaning, and the occurrence of end-product faults such as web breaks, specks and holes, spots and color alterations, with more than 50% of the problems of medium or high severity (Pauly 2002). Biofilm-forming bacteria are among the main reasons of concern in paper industry, as they can obstruct filters, valves and sprays, thus reducing the water flux in pipes, leading to reduced machine lifetime for the enhanced corrosion.

The slimy biological deposits can also affect the quality of the end product because they can be detached from surfaces and can cause paper web breaks, or colored spots in the produced paper (Sanborn 1965; Väisänen *et al.* 1994; Blanco *et al*. 1996; Chaundhary *et al.* 1997; Lindberg *et al.* 2001; Pauly, 2002; Desjardins and Beaulieu 2003; Kolari 2003; Rättö *et al.* 2005). Earlier studies have demonstrated that more than 60% of end-product defects are caused by holes or deposits on the paper surface, in which bacterial DNA has been detected (Haapala *et al.* 2010). The presence of bacterial metabolites can even damage both raw materials and chemical compounds used within the process (Borenstein 1994).

To control bacterial proliferation in paper mills, the preferred strategy involves the use of chemical biocides, despite their high costs (Blanco *et al.* 1996; Lahtinen *et al.* 2006) and environmental impact. Biocides can be divided in oxidative and non-oxidative, and both types are used (Pauly 2002; Kiuru *et al.* 2010a, 2010b). Depending on specific applications, traditional biocides, such as chlorine compounds, are being replaced with lower-impact alternatives (European Commission 2001). The need for regular physical cleanings of machines with dispersants/detergents and physical treatments (Pauly 2002) is also related to bacteria proliferation, as the most resistant deposits on surfaces and membranes are linked to biofouling (Peltola 2011). Some non-chemical methods to control bacterial proliferation in paper mills have been tested as well, such as electric fields (Matsunaga *et al.* 1998) and ultrasonic treatments (Lambert *et al.* 2010), but their use is still at an experimental stage, and only limited real-scale applications have been documented.

The detection and characterization of paper-mill harbored bacteria can help to deeply understand the dynamics of proliferation and to find new methods to control bacterial growth and development, such as selective biocides with lower environmental impact, or alternative operation/maintenance measures. Economic advantages are also conceivable, as biocide dosages, as well as maintenance operations requiring process halts, could be optimized for a selective effect on identified bacteria. Few papers on the characterization of bacterial communities in paper mills are available (*e.g.* Rasimus *et al.* 2011; Suihko *et al.* 2009; Maukonen *et al.* 2006), and most of them apply only culture-dependent approaches (*e.g.*, Kashama *et al.* 2009).

The exclusive use of cultivation attempts is not sufficient to provide a complete description of all the microorganisms present in both natural and industrial environments. This is because the largest portion of microorganisms (generally more that 99%) cannot be cultivated under standard laboratory conditions (Amann *et al*. 1995). Well studied environments that display viable but not culturable microorganisms include soil (Torsvik *et al.* 1990); the activated-sludge process for waste-water treatment (Wagner *et al*. 1993); clinical samples exhibiting mixed communities of biofilm-forming bacteria resistant to antibiotics (Rogers *et al.* 2009); and foods and beverages (Mamlouk *et al*. 2009). For these reasons, a combination of culture-dependent and independent methods can maximize the estimation of microbial richness in complex ecosystems, as well as the detection of non-cultivable but functional microbes.

The aim of this research was to improve the knowledge of bacterial community composition and dynamics in paper mills using i) a fingerprinting molecular approach (Terminal Restriction Fragment Length Polymorphism, T-RFLP) along the whole process water circuit of the paper mill during a three-month period, and ii) a snapshot analysis of microbial composition in a significant point of the circuit, using both a DNA-based approach and a cultivation attempt.

MATERIALS AND METHODS

Paper Production Process and Water Circuits

The paper mill under study is owned and operated by the Sofidel Group located in central Italy (Porcari, Lucca). It produces paper tissue reels (110,808 tonnes produced in 2010, Sofidel 2011) starting from pure virgin cellulose fibers. The paper production cycle includes a mechanical pulping stage at 52 °C, a pulp mixture preparation and purification stage, and a continuous papermaking stage including a head box, a wire section, a press section, a drying section at 120 °C, and a reeler. The use of pure virgin cellulose avoids the need for any pH correction along the whole process (pH 6.5 to 7.5). The water cycle is fed by pumped fresh groundwater and includes a complex set of internal recycling loops, as briefly schematized in Fig. 1.

The main process-water recycling circuits are constituted by the fiber-rich wire water, which is recycled for stock dilution (white water 1), and excess water from sheet-forming, suction and press, as well as cleaning water, which are circulated in a secondary circuit (white water 2 long circulation) after polydisk filtering at 38 °C. The extensive water recycle scheme allows the plant to keep its water consumption to as low as 7.12 L per kg of produced paper (Sofidel 2011), which is significantly lower than the 10 to 15 L/kg range reported in the current reference document on best available techniques (European Commission, 2001) for paper tissue production from virgin fibres, with a plan for further reduction in the near future. The specific wasted water amounted to 3.98 L/kg in 2011. The adopted strategy for bacteria control is mainly based on non-oxidative (pulping, freshwater treatment, papermaking) and oxidative (papermaking) biocides.

Collection and Storage of Samples

Samples from different sites and compartments of the paper mill were collected three times, in November 2011, January 2012, and February 2012 (Table 1), using sterile 50 ml falcon tubes. These samples were used to assess the homogeneity of distribution of bacterial communities inside the whole paper mill with a fingerprinting molecular analysis by T-RFLP.

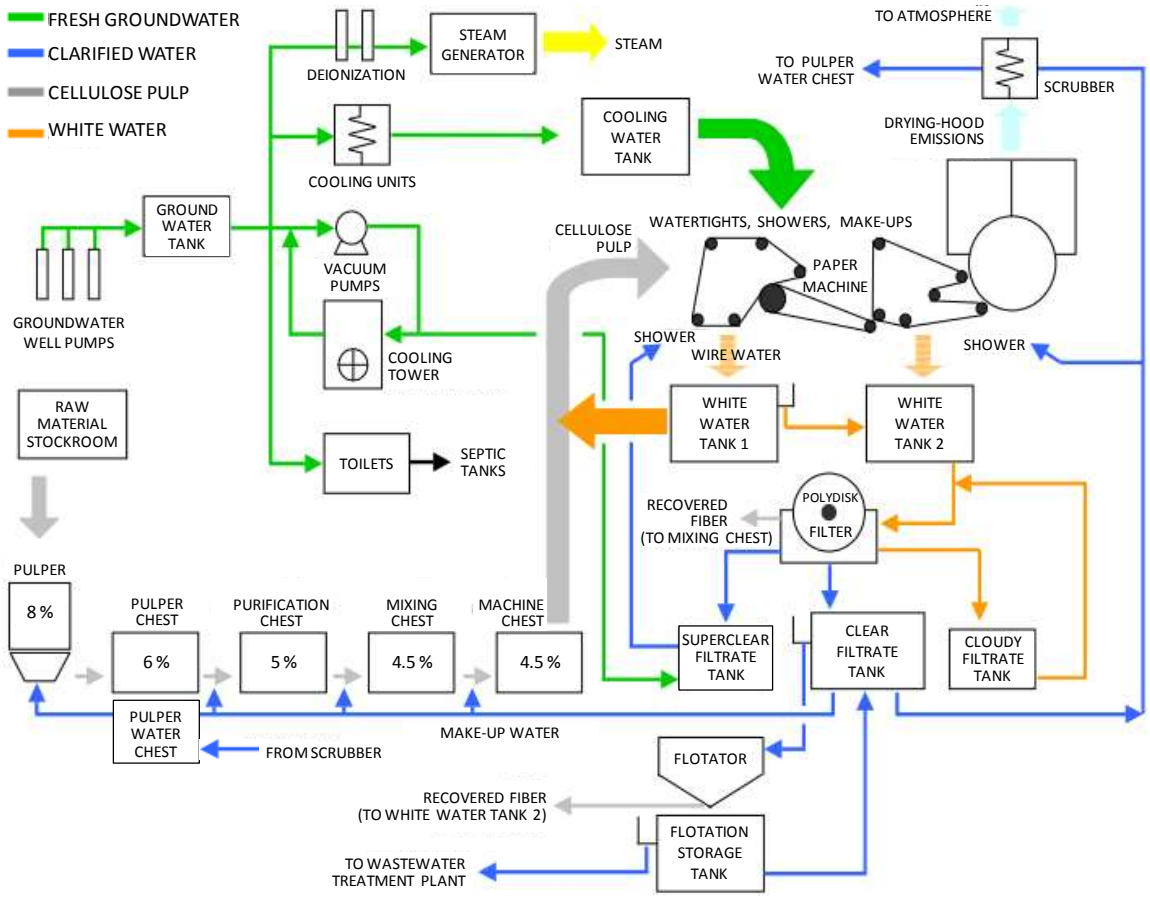

Fig. 1. Water-cycle schematics of Sofidel's paper mill in Porcari (Sofidel 2011, adapted).

All samples were in an aqueous phase and showed different amounts of cellulose fiber for different collection sites. After collection, all samples were immediately brought to the laboratory for storage of subsamples. For each falcon tube, 10 subsamples were prepared, and stored at -20 °C until use.

Table 1. List of Samples Collected in Different Compartment of Sofidel Paper Mill in November 2011, January 2012 and February 2012

Sample name	Description	Process temperature	Sample number
T11V	Pulper chest	48°C	1
T16V	Mixing chest	47°C	2
T17V	Machine chest	46°C	3
T20 P	Disc filter recovered fiber outlet	38°C	4
Disc_G	Disc filter biofilm	38°C	5
Disc_V	Disc filter main tank	38°C	6
SCWWv	Superclear filtrate tank	37°C	7
CWWv	Clear filtrate tank	37°C	8
TWv	Cloudy filtrate tank	37°C	9
H_2OI	White water tank 1 (wire water)	37°C	10
H_2OII	White water tank 2	40°C	11
H_2O_FlotV	Flotation tank recovered water	35°C	12

For the snapshot analysis, an aqueous sample was collected in May 2012 from the pulper water chest using a sterile 50 mL falcon tube, and immediately brought to the laboratory for bacterial plating and isolation of colonies.

Bacterial Plating and Analysis

R2A medium (Reasoner and Geldreich 1985) was used at final pH 7.2 ± 0.2 (at 25 °C) for bacterial plating and isolation of colonies, according to the serial dilution method described in Koch (2007). The sample collected in May 2012 was plated in triplicate. The R2A plates were aerobically incubated at 37 °C for 4 days to allow also slow-growing bacteria to develop. After incubation and counting, colonies having different morphology were picked up from each plate, transferred in R2A broth and incubated overnight at 37 °C in aerobic conditions.

Genomic DNA extraction from liquid cultures was performed using GeneElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Germany). gDNA from each colony was used for 16S rRNA gene amplification. PCR was performed as described in Chiellini *et al.* (2013b). RFLP was performed using BsuRI restriction endonuclease (Fermentas, Canada). Digested fragments, obtained after 3 h 30 min of incubation at 37 °C, were visualized by electrophoresis on 2% agarose gel using ethidium bromide staining. 16S rRNA gene sequencing was performed on organisms providing unique RFLP electrophoresis pattern by Macrogen, Inc. sequencing service (South Korea).

DNA Extraction, T-RFLP, Construction of 16S rRNA Gene Library, Sequencing

Total DNA extraction was performed on all samples described in Table 1 using a SoilMaster™ DNA extraction kit (Epicentre Biotechnologies, WI U.S.A.) according to the manufacturer's instructions. The T-RFLP analysis was performed on the samples listed in Table 1. 16S rRNA genes were directly amplified from extracted DNA using the following program: initial denaturation at 94 °C for 10 min, followed by 30 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min with a final extension step at 72 °C for 10 min. Thermocycling was performed using a C1000 Touch™ thermal cycler (Biorad, U.S.A.). Universal bacterial primers were used: 8F (5'-AGA GTT TGA TYM TGG CTC AG-3') and reverse 1492R (5'-GGN WAC CTT GTT ACG ACT T-3') (Lane *et al*. 1991). Primer 8F was labeled with two different dyes (Applied Biosystems, CA, U.S.A.): 6-FAM and NED. In the first case, the template was digested with restriction endo-nuclease BsuRI (GG^CC, 0.2 u/µL, Fermentas, Canada) and in the second case with restriction endonuclease RsaI (GT^AC, 0.2 u/µL, Fermentas). The use of more than one restriction endonuclease in T-RFLP approach is recommended for a better resolution of the experiment (Liu *et al.* 1997). After restriction, DNA was precipitated with cold 100% ethanol to eliminate salts in a 5424 R refrigerated centrifuge (Eppendorf, Italy) at 4°C and 10,000 RCF. For each reaction, a mix with 0.5 µL of loading buffer (GeneScan™ 600 LIZ, Applied Biosystems), a maximum of 5.5µL of sample (the volume of samples was calculated on the basis of its final concentration after cold ethanol precipitation) and 14 µL of deionized formamide (Applichem, Germany) was prepared. A Non Metric Multi-Dimensional Scaling (NMDS) analysis was performed on T-RFLP data matrix as described in Chiellini *et al.* (2013a and 2014). For the library construction (Amann *et al.* 1995) of the snapshot sample, 16S rRNA genes were directly amplified from extracted DNA of white water samples collected in May 2012, using universal bacterial primers, 8F (5'-AGA GTT TGA TYM TGG CTC AG-3') and reverse 1492R (5'-GGN WAC CTT GTT ACG ACT T-3') (Lane *et al.* 1991), with the same conditions adopted for the

amplification of the T-RFLP analysis. The amplification product was cloned in a plasmid vector (pCR®2.1-TOPO, TOPO TA Cloning® Kit, Invitrogen, UK) and inserted in chemically competent cells (One Shot TOP10, Invitrogen, UK). A representative number of inserted fragments were subsequently amplified by control PCR with primers M13F and M13R, provided with the TOPO® TA Cloning Kit. Digestion of the 16S rRNA gene sized fragments was performed with restriction endonuclease BsuRI (0.2 u/lL, Fermentas®, Canada). Further details on the cloning protocol are described by Chiellini *et al.* (2012). The 16S rRNA gene-sized fragments were digested with restriction endonuclease BsuRI (0.2 u/μl, Fermentas®, Canada). Digested fragments were visualized by electrophoresis on 2% agarose gel and subsequent ethidium bromide staining. Fragments showing an identical electrophoresis pattern were grouped together by T-RFLP analysis, and each group was characterized by sequencing an appropriate number of cloned inserts. Plasmid DNA was extracted with EuroGold Plasmid miniprep kit® (EuroClone®, Italy), and sequenced with primers M13R and M13F by the Macrogen, Inc. sequencing service (South Korea).

Detection of Chimeric Sequences and Sequence Annotation

All the retrieved sequences were scanned for chimera detection using Bellerophon server (Hugenholtz and Huber 2003). Chimeric sequences were analyzed by cutting them in proximity of the supposed recombination site and treating them as separate sequences according to Chiellini *et al.* (2013c).

NCBI BLAST analysis (Altschul *et al*. 1997) was used to determine the affiliation of clone sequences. Afterwards, sequences were inserted in SILVA 111 database (Quast *et al*. 2013) and aligned using the appropriate tool from ARB program package (Ludwig *et al*. 2004).

RESULTS

T-RFLP Results

The results of Non Metric Multi-Dimensional Scaling (NMDS) analysis on T-RFLP data matrix of samples collected in November 2011, January 2012, and February 2012 are shown in Fig. 2. A minor differentiation is evidenced among the three groups of samples, which appear slightly separated in the plot. Altogether, within each group of samples it was not possible to recognize any differentiation on the basis of samples collected in different paper mill compartments.

Construction of 16S rRNA Gene Library

A 16S rRNA library was constructed by screening a total of 106 recombinant clones; 43 clones, representative of 25 T-RFLP digestion patterns, were sequenced. Four of the 43 sequenced clones (9.3%) were identified as being chimeric sequences. The recombination site of each chimeric sequence was detected; sequences were cut at the recombination site and the partial sequences obtained after chimera cutting were independently included in the analysis as representatives of different microorganisms. After chimera detection, the number of obtained sequences (Table 2) increased to 47 (39 non-chimeric sequences and 8 chimeric partial sequences). Sequences were deposited into the EMBL (European Molecular Biology Laboratory) database with accession numbers from HF912287 to HF912333.

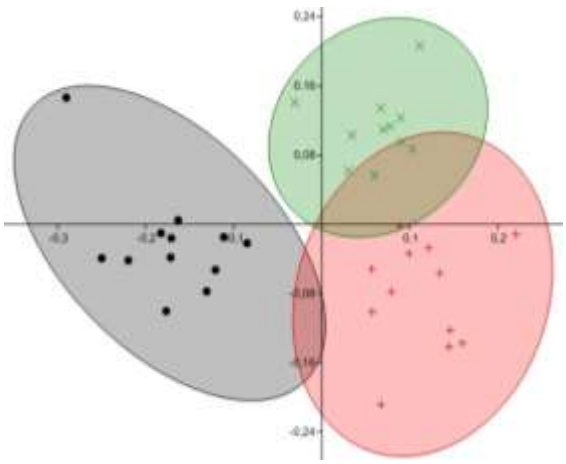

Fig. 2. Non Metric Multidimensional Scaling plot obtained with T-RFLP data from samples collected in November 2011 (black circles), January 2012 (red crosses), and February 2012 (green crosses). Stress value = 0.1892. Ellipses indicate the 95% confidence intervals.

Table 2. Analysis of Sequences Obtained from the Construction of 16S rRNA Gene Library

Sequenced clone	Absolute abundance in clone library	Similarity	Affiliation	Taxonomic classification
H_2OII_ 55	1	88% AB244313	Uncultured bacterium	*Spirochaetes Spirochaetia Spirochaetales*
H_2OII_ 3 H_2OII_ 28 H_2OII_ 8 H_2OII_ 41	23	99% NR042187	*Cloacibacterium normanense*	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae*
H_2OII_ 104_3P	1	97% NR_042160	*Chryseobacterium molle*	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae; Chryseobacterium*
H_2OII_ 60 H_2OII_ 36	3	87% NR040905	*Crocinitomix catalasitica*	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Cryomorphaceae; Crocinitomix*
H_2OII_ 23 H_2OII_34 H_2OII_ 62	9	92% AB682148)	*Filimonas lacunae*	*Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Filimonas*
H_2OII_ 18	4	93% AB682416	*Niabella aurantiaca*	*Bacteroidetes; Sphingobacteriia; Sphingobacteriales; Chitinophagaceae; Niabella*

Sequenced clone	Absolute abundance in clone library	Similarity	Affiliation	Taxonomic classification
H_2OII_101	1	99% CP000359	*Deinococcus geothermalis*	*Deinococcus-Thermus; Deinococci; Deinococcales; Deinococcaceae; Deinococcus*
H_2OII_77	2	96% AB680850	*Bacillus* sp.	*Firmicutes; Bacillales; Bacillaceae; Bacillus*
H_2OII_90	2	98% JQ435699	*Bacillus* sp.	*Firmicutes; Bacillales; Bacillaceae; Bacillus*
H_2OII_51	1	99% AB681220	*Enterococcus columbae*	*Firmicutes; Lactobacillales; Enterococcaceae; Enterococcus*
H_2OII_2 H_2OII_1 H_2OII_70	5	98% NR042844	*Paenibacillus timonensis*	*Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus*
H_2OII_64	2	95% AB362821	*Paenibacillus* sp	*Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus*
H_2OII_30	1	99% AM283040	*Paenibacillus* sp.	*Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus*
$H_2OII\_109\_5P$	1	92% JQ419678	*Paenibacillus* sp.	*Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus*
H_2OII_100 H_2OII_27	10	98% GQ922067	*Balneimonas* sp. (*Microvirga* sp.)	*Proteobacteria; Alphaproteobacteria; Rhizobiales; Methylobacteriaceae; Microvirga*
H_2OII_11	1	96% BX842648	*Bdellovibrio bacteriovorus*	*Proteobacteria; Deltaproteobacteria Bdellovibrionales; Bdellovibrionaceae; Bdellovibrio*
H_2OII_48	1	96% EF219370	*Georgfuchsia toluolica*	*Proteobacteria; Betaproteobacteria; Rhodocyclales; Rhodocyclaceae; Georgfuchsia*
H_2OII_31	2	97% AB682368	*Microvirga aerilata*	*Proteobacteria; Alphaproteobacteria; Rhizobiales;*

Sequenced clone	Absolute abundance in clone library	Similarity	Affiliation	Taxonomic classification
				Methylobacteriaceae; Microvirga
H_2OII_ 33 H_2OII_ 92	6	99% FR774558	*Pseudoxanthomonas* sp.	*Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas*
H_2OII_ 65_3P H_2OII_ 43	3	99%FR774580	*Pseudoxanthomonas sp.*	*Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas.*
H_2OII_ 97	1	97% AB017799	*Rhodobacter* sp.	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter*
H_2OII_ 32 H_2OII_ 25	4	80% CP003304	*Rickettsia canadensis*	*Proteobacteria; Alphaproteobacteria; Rickettsiales; Rickettsiaceae; Rickettsieae; Rickettsia*
H_2OII_ 19_5P	1	99% AY538706	*Schlegelella* sp.	*Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Schlegelella*
H_2OII_ 13 H_2OII_ 24 H_2OII_ 22 H_2OII_ 37 H_2OII_ 40 H_2OII_ 102 H_2OII_ 6	15	99% AY594193	*Tepidimonas arfidensis*	*Proteobacteria; Betaproteobacteria; Burkholderiales; Tepidimonas*
H_2OII_ 19_3P	1	99% JN713899	*Tepidimonas* sp.	*Proteobacteria; Betaproteobacteria; Burkholderiales; Tepidimonas*
H_2OII_ 104_5P	1	99% HE817886	*Tepidimonas* sp.	*Proteobacteria; Betaproteobacteria; Burkholderiales; Tepidimonas*
H_2OII_ 109_3P H_2OII_ 74 H_2OII_ 65_5P	4	99% NR_025265	*Thermomonas hydrothermalis*	*Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Thermomonas*

Results from the 106 analyzed sequence fragments retrieved after chimera analysis on the screened clones highlighted the dominance of the *Proteobacteria* phylum (46%) in all white water samples and, in particular, the presence of *Alpha*, *Beta*, *Gamma*, and *Delta* subclasses. 38% of the retrieved sequences belonged to *Bacteroidetes* related bacteria, and 11% to the *Firmicutes* phylum. Other minor fractions were attributed to bacteria belonging to *Deinococcus-Thermus* (1%) and *Spirochaetes* (1%) phyla. 4% of screened clones were associated to bacteria belonging to other minor groups (Fig. 3).

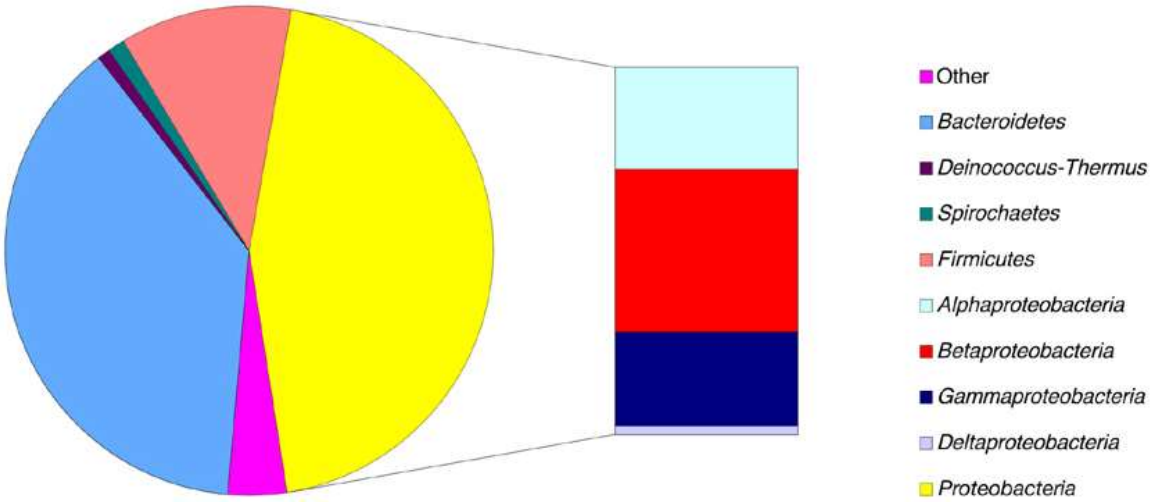

Fig. 3. Phyla attribution obtained from 16S rRNA library construction of process white water sample

Culture-Dependent Analysis

Bacterial plate counts performed on white water subsamples highlighted the presence of 10^7 to 10^8 living bacterial cells (Colony Forming Unit, CFU) per mL of sample. A total of 19 bacterial colonies were isolated (Table 3) and subjected to 16S rRNA-RFLP screening. Digestion pattern analysis evidenced the presence of six different patterns; consequently, a representative of each pattern was sequenced. The retrieved sequences were deposited into the EMBL database with accession numbers from HF912281 to HF912286.

Figure 4 represents the results obtained from the analysis of all colonies. The majority of detected cultivable bacteria (58%) were attributed to the *Proteobacteria* phylum, and in particular to the *Beta* (21%) and *Gamma* (37%) subclasses. 37% of the isolated colonies belonged to the *Bacteroidetes* phylum, and the remaining 5% were attributed to the *Firmicutes* phylum.

All sequences, coming from both the culture-dependent and independent approaches, were analyzed not only at the phylum level but also at the genus level; the genera detected with the two different methods are compared in Fig. 5.

The genus *Cloacibacterium* (labeled in orange in Fig. 5) is highly represented both in isolates from plates (36.8%) and in library clones (24.8%) (Fig. 5, first and second column on the left, respectively), as well as the *Paenibacillus* genus (5.3% and 10.5%, respectively) and *Tepidimonas* genus (15.8% and 16.2%, respectively). Some genera are more represented among isolate bacteria than in library construction, as for example the *Thermomonas* genus (31.6% in the isolates vs. 2.9% in the library) and the *Schlegelella* genus (5.3% vs. 1% respectively).

Table 3. Analysis of Sequences Obtained from Isolation of Colonies

Sequenced clone	N° clones	Similarity	Affiliation	Taxonomic classification
1_54H_2OII	6	99% NR_025265	*Thermomonas hydrothermalis*	*Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Thermomonas*
2_37H_2OII	7	99% FJ544401	*Cloacibacterium normanense*	*Bacteroidetes; Flavobacteriia; Flavobacteriales; Flavobacteriaceae.*
4_54H_2OII	3	99% AY594193	*Tepidimonas arfidensis*	*Proteobacteria; Betaproteobacteria; Burkholderiales; Tepidimonas*
10_37H_2OII	1	99% AY538706	*Schlegelella* sp.	*Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae; Schlegelella*
11_54H_2OII	1	99% AB681369	*Pseudoxanthomonas taiwanensis*	*Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae; Pseudoxanthomonas*
12_37H_2OII	1	95% EU977789	*Paenibacillus* sp.	*Firmicutes; Bacillales; Paenibacillaceae; Paenibacillus*

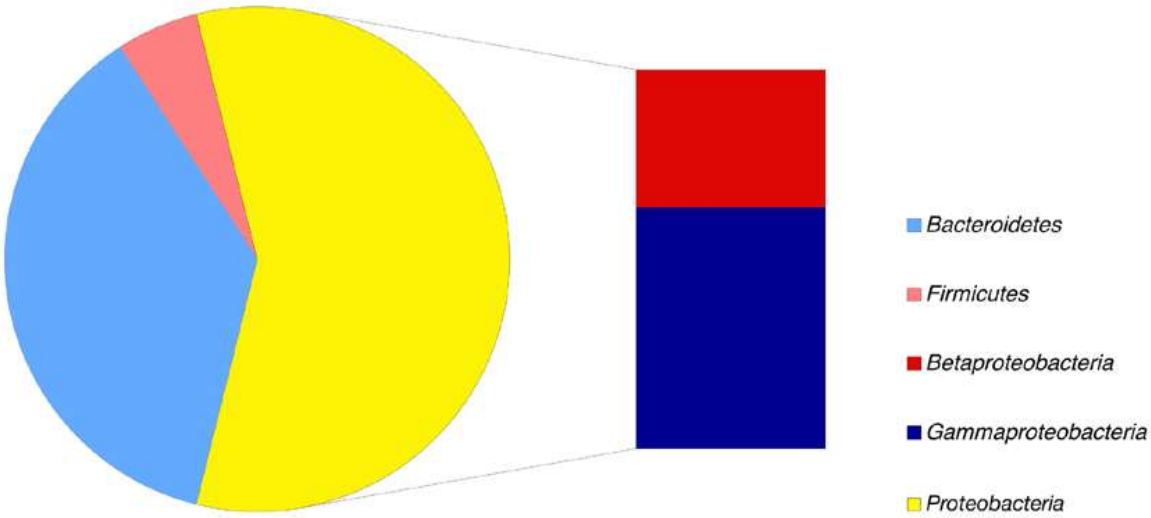

Fig. 4. Phyla attribution obtained from sequencing the bacterial isolates of white water sample

Other bacteria genera were only detected by library screening, as for example *Filimonas*, *Bergeyella*, *Deinococcus*, *Balneimonas*, *Spirochaetes*, *Niabella*, *Rhodobacter*, *Enterococcus* and *Bdellovibrio*; these genera represented 14% of the screened clones in the library.

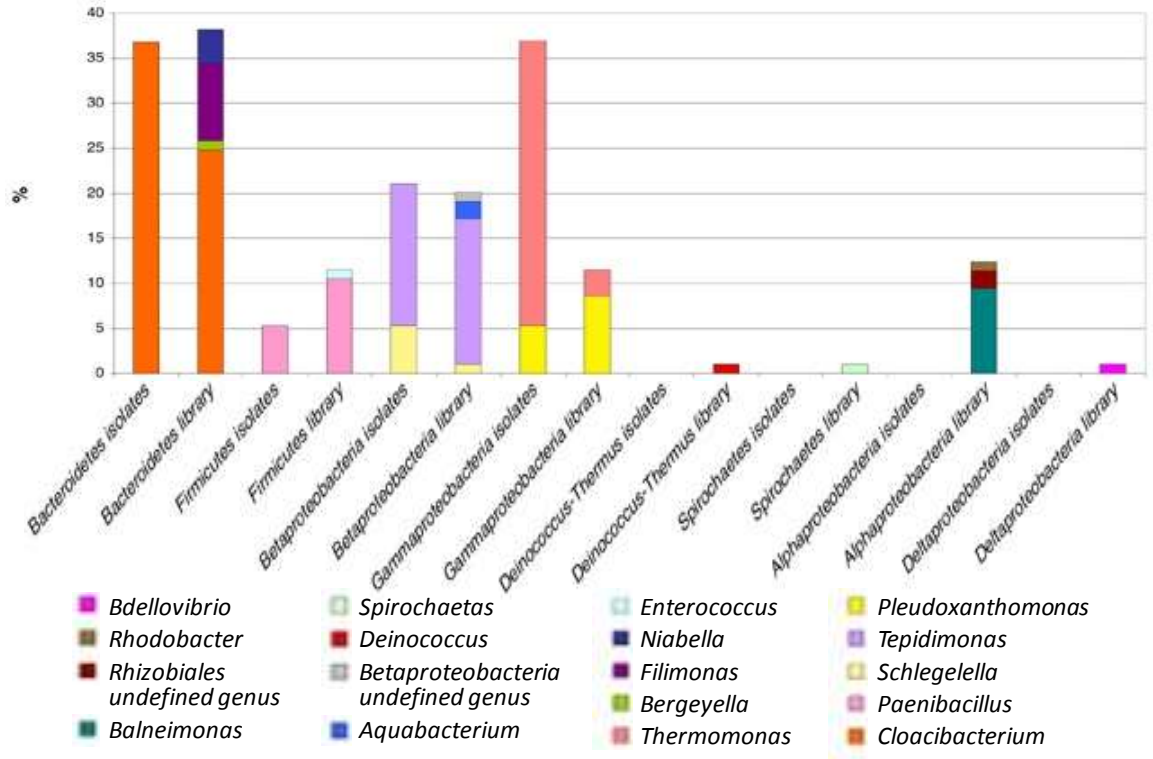

Fig. 5. Analysis of sequenced clones and isolates from white water samples at genus level

DISCUSSION

Scientific literature on the analysis of bacterial communities within the different compartments of a paper mill is nowadays scanty, especially when considering culture-independent approaches (Tiirola *et al.* 2009, Lahtinen *et al.* 2006, Granhall *et al.* 2010, Rasimus *et al.* 2011). The majority of available studies have adopted culture-dependent techniques (Desjardins and Beaulieu 2003, Kolari 2003, Kolari *et al.* 2003, Suihko *et al.* 2004, Suihko and Skyttä 2009), thus underestimating the microbial diversity of the original sample. In this study, both culture-dependent and independent approaches were applied in order to i) maximize the amount of information that could be retrieved on bacterial community composition, and ii) gain criteria on which of the two approaches should be chosen to obtain the best insights into bacterial community dynamics in this specific industrial environment.

T-RFLP Analysis on Bacterial Communities in the Paper Mill

The preliminary analysis of T-RFLP data from Table 1 samples highlighted an almost homogeneous distribution of bacterial communities in the different compartments of the paper mill at the same sampling time. Slight fluctuations in bacterial community composition were evidenced at different times (Fig. 2). These variations could be determined by differences in the input raw materials (origin, kind of wood, *etc.*), as well as by seasonal variations of room temperature. Also the adopted countermeasures against microbial proliferation, made necessary by its severe repercussions on end-product quality and production efficiency, contributed to the selection of bacteria able to grow in this specific environment. The spatial homogeneity of the aqueous phase microbial community, combined with its temporal variability, led us to hypothesize a possible

spreading role of suspended bacteria (which are easily transported along the process-water cycle) in the re-formation mechanisms of biocide-resistant biofilms, which represent the most severe threat to end-product quality and process efficiency.

The homogeneous distribution of bacteria, shown by the T-RFLP results along the whole water cycle, led us to deepen the examination by a snapshot analysis of a single significant point, in order to obtain a detailed representation of the local microbiota, which would well enough represent the whole bacterial community of the paper mill.

Snapshot Analysis of White Water

The comparison of isolates and library assays performed during the snapshot analysis of the examined sample showed that all cultivated bacteria were represented in the 16S rRNA gene library, where they represented about 25% of the microbial diversity retrieved within the library (6 bacteria out of 25 RFLP patterns). Furthermore, these 6 bacterial isolates represented about 60% (63/105) of the 16S rRNA library bacterial clones. These data showed that, in this specific environment, the dominant fraction of bacterial population was represented by cultivable bacteria. This finding, which somehow contrasts with the generally assumed concept that only a small fraction of environmental bacteria are cultivable (Amann *et al.* 1995), is possibly due to the continuous use of biocides, physical treatments and cleanings, which favors the establishment of a cultivable bacterial community. As a consequence, in this specific environment, bacterial plating seems able to provide an adequate estimation of the dominant bacteria. However, the culture-independent approach highlighted also the microbial diversity not retrieved by bacterial plating. Although the further detected bacteria represented a minor fraction of the overall population, they can play a role in the biofilm formation mechanisms. A possible further investigation of this clue would come from the comparison of data retrieved from aqueous and biofilm samplings.

The 16S rRNA library construction highlighted a greater diversification of *Bacteroidetes* and *Firmicutes* phyla at genus level than the cultivation approaches did. The phylum *Bacteroidetes* was only represented by the genus *Cloacibacterium* in the plate isolates, while it was represented by four different genera in the library (*Cloacibacterium*, *Niabella*, *Bergeyella* and *Filimonas*). *Cloacibacterium* showed roughly comparable abundance percentages in both library (24.8%) and isolates (36.8%). Strains of the species *Cloacibacterium normanense* were previously isolated from biofilms of paper mill machines using both culture-dependent (Suihko and Skyttä 2009) and culture-independent (Tiirola *et al.* 2009) approaches. Bacteria belonging to this genus are characterized by a facultative anaerobic metabolism and they produce colored pigments ranging from yellow to orange. Their optimum growth temperature is around 30 °C (Krieg *et al.* 2011), and this characteristic is in agreement with the average temperature measured inside most of the compartments of the paper mill (ranging from 33 °C to 45 °C). Within the *Bacteroidetes* phylum, three more genera were detected in the library: *Niabella*, *Bergeyella*, and *Filimonas* for a total fraction of 14% for the three genera.

The genus *Niabella* includes strictly aerobic and mesophilic members that were first detected in greenhouse soil (Kim *et al.* 2007). *Bergeyella* genus, the single species of which *Bergeyella zoohelcum* was isolated from clinical specimens, grows at 37 °C but not at 42 °C (Vandamme *et al.* 1994). Conversely, *Filimonas* genus includes the strictly aerobic, filamentous, and viscous exopolymer-producing species *Filimonas lacunae*, which has a typical growth temperature range of 10 to 35 °C (Shirator*i et al.* 2009).

This is the first time that members of these genera have been detected in paper mills. These evidences highlighted the importance of culture-independent approaches to retrieve more complete data at the taxonomic diversity level (Amann *et al.* 1995) and to detect less abundant but functional bacteria in this specific environment.

Considering the *Firmicutes* phylum, results highlighted the presence of the *Paenibacillus* genus in the plate isolates, while the library also evidenced the presence of the *Enterococcus* genus. Therefore, the biodiversity retrieved with a cultivation-independent method was higher also in this case, although *Paenibacillus* showed roughly comparable percentages in the library (10.5%) and in the isolates (5.3%).

Paenibacillus includes facultative anaerobic species, growing in a temperature range of 28 to 50 °C, that inhabit several niches such as soil, water, clinical samples, and insect larvae. Recently, the species *Paenibacillus chartarius* newly isolated from a paper mill was described (Kämpfer *et al*. 2012). Strains of this species are aerobic and grow at temperatures between 15 °C and 45 °C (Kämpfer *et al*. 2012). Moreover, both members of the genera *Paenibacillus* and *Enterococcus* were detected among the bacterial community of activated sludge only by cultivation-independent methods (Heylen *et al.* 2006).

In contrast with previously obtained results, Beta and Gamma subclasses of *Proteobacteria* showed the same number of genera with both approaches (three genera for *Betaproteobacteria* and two genera for *Gammaproteobacteria*). For the *Gammaproteo-bacteria* group, the two genera (*Pseudoxanthomonas* and *Thermomonas*) were detected with both approaches, while for *Betaproteobacteria* only 2 out of 3 genera (*Tepidimonas* and *Schlegelella*) were detected with both approaches. These results suggest that most microorganisms belonging to *Gammaproteobacteria* and *Betaproteobacteria* subclasses, that are present in paper making, could be cultivable. Many of them have also been previously isolated from paper machines with culture-dependent techniques (Desjardins & Beaulieu 2003, Kolari 2003, Kolari *et al.* 2003, Suihko *et al.* 2004, Suihko and Skyttä 2009). Some bacterial groups were only detected by library construction (phyla *Deinococcus-Thermus* and *Spirocheates*; subclasses *Deltaproteobacteria* and *Alphapro-teobacteria*) representing just the 14% of total screened clones. Although numerically less relevant, it is interesting to note the occurrence of bacteria from the phylum *Deinococcus–Thermus*, which are known for their resistance to extreme environmental stresses and for the production of thermostable enzymes. In particular, the species *Deinococcus geothermalis*, also detected in this study, is tolerant to high level of toxic solvent stress at high temperature (Kongpol *et al.* 2008).

CONCLUSIONS

1. The bacterial community of the examined paper mill was observed as being spatially homogeneous along the white water cycle, and slightly variable in time from November 2011 to February 2012. The variations can be due to differences in input stock, water contamination, or ambient temperature, while the countermeasures adopted against microbial proliferation were judged to be responsible for the selection of a restricted bacterial community. The bacterial diversity was rather limited when compared to other environments, and the dominant phyla (β and γ *Proteobacteria*, *Bacteroidetes* and *Firmicutes* in order of descending presence) were represented by easily cultivable bacteria.

2. As a consequence, in this environment, cultivation methods can provide an adequate estimation of the dominant bacteria.
3. However, the culture-independent approach detected a higher number of genera, especially within the *Bacteroidetes* and *Firmicutes* phyla, thus exhibiting a higher diversity than that estimated by simple plating. These results highlighted the impact of culture-independent testing in detecting less abundant but functional bacteria.
4. The detection of filamentous genera, together with the spatial homogeneity and temporal variability of the aqueous microbial community, suggested a possible spreading role of transported bacteria in the re-formation mechanisms of the biofilms that severely threaten end-product quality and process efficiency. Hence, the characterization of bacterial communities could lead to a greater comprehension of the biofilm formation dynamics, and to possible solutions for this widespread problem that afflicts almost all paper mills.

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