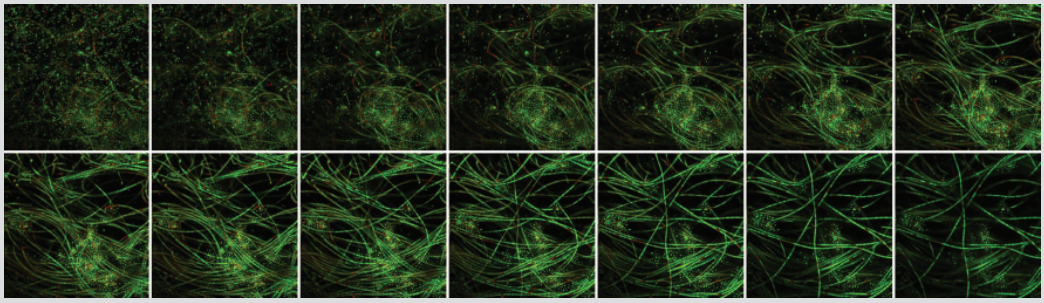

Microbial Life and Deposits in Paper Machine Circuits

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Front cover: CLSM from deposit on coupon placed in biological treated water sent back as makeup water to the paper machines.

To my dear husband and son

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II **Kanto** Öqvist L. Pelzer R., Salkinoja-Salonen M. S., Grüner G., Nature and Measurement of Deposit Layers in Paper Machine Loops (2007), Proceedings of the Tappi Papermakers and PIMA international Leadership Conference, March 11-15, Jacksonville FL, Session MC-3
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III Kanto Öqvist C., Kurola J., Pakarinen J., Ekman J., Ikävalko S., Simell J., Salkinoja-Salonen M. S., Prokaryotic microbiota of recycled paper mills with low or zero effluent, Journal of Industrial Microbiology and Biotechnology, under review, JIMB-D-08-00120

IV Peltola M., Kanto-Öqvist C., Ekman J., Kosonen M., Jokela S., Kolari M., Korhonen P. and Salkinoja-Salonen M. S., Quantitative contributions of bacteria and *Deinococcus geothermalis* to deposit and slimes in paper industry, Submitted

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THE AUTHOR'S CONTRIBUTION

Paper I: Charlotta Kanto Öqvist interpreted the results and wrote the paper. She planned the experiments, and carried out most of the experimental work, with support from colleagues at the company where she works or at an external institute.

Paper II: Charlotta Kanto Öqvist interpreted the results and wrote the paper. She planned the experiments, and contributed with ideas and knowledge how to develop the online equipment. Analyzes were either done collectively by colleagues at the company where she works, or at an external institute.

Paper III: Charlotta Kanto Öqvist interpreted the results and wrote the paper. She planned the experiments, and carried out most of the experimental work. The sequences were acquired as commercial service.

Paper IV: Charlotta Kanto Öqvist contributed to the paper with the experimental design and analysis of *Deinococcus* deposits *in situ* in machine circuits, including the electron microscopy. She also contributed by writing part of the paper.

ABBREVIATIONS

AHL	*N*-acylhomoserine-lactone
AOX	absorbable organic halogens
ATP	adenosine triphosphate
ATR-EW	Attenuated total internal reflection with evanescent waves
ATR-SPR	Attenuated total internal reflection with surface plasmon Resonance
ATTC	American Type Culture Collection
BCDMH	1-Bromo-3-chloro-5,5-dimethylhydantoin
CFU	colony forming unit
CLSM	confocal laser scanning microscopy
COD	chemical oxygen demand
CMC	carboxy methyl cellulose
CTC	5-cyano-2,3-ditolyl tetrazolium chloride
DCM	dichloromethane
DMH	dimethylhydantoin
DNA	deoxyribonucleic acid
EDX	energy dispersive X-ray
EPS	extracellular-polymeric substances
EtBr	ethidium bromide
EU	European Union
FISH	fluorescence *in situ* hybridization
FTIR	Fourier transform infrared spectroscopy
HPLC	high pressure liquid chromatography
kb	kilobasepairs
LC-MS	liquid chromatography mass spectrometry
LH-PCR	length heterogeneity PCR
MIC	microbially influenced corrosion
MTBE	methyl *tert*-butyl ether
NCBI	National Center for Biotechnology Information
PCR	polymerase chain reaction
PM	paper machine
PVA	poly vinyl acrylate
PVOH	poly vinyl alcohol
Py-GC-MS	pyrolysis-gas chromatography-mass spectrometry
qRT-PCR	quantitative real time PCR
RNA	ribonucleic acid
rRNA	ribosomal RNA
SEM	scanning electron microscopy
TOC	total organic carbon
THF	tetrahydrofuran
VFA	volatile fatty acids

GLOSSARY FOR NON PAPERMAKERS

additives	Products, fillers added to the fiber suspension
AKD	alkyl keten dimmer, sizing aid
ASA	alkenyl succinic anhydride, sizing aid
broke	re-pulped paper from own production
Chemical pulp	fibers separated with chemicals, almost no lignin left
chest	smaller tanks in the paper machine circulations
DCS	dissolved and colloidal substances
DLVO	theory that describes the force between charged surfaces interacting through a liquid medium (named after Derjaguin, Landau, Verwey and Overbeek)
Felts (press felts)	in the press section after wire, to squeeze out water before drying section
filler	added inorganic substances to the fiber suspension
GCC	ground calcium carbonate, filler
headbox	spreads the fiber suspension over the wire
mechanical pulp	fibers separated by “grinding” and heat, contains a lot of lignin and other substances
OBA=FWA	optical brighteners, fluorescent whitening agents
oxidative bleaching	fiber bleaching with peroxide or chlorine compounds
PCC	precipitated calcium carbonate, filler
Pitch	resin from the wood
reductive bleaching	fiber bleaching with dithionite or hydrosulfite addition
runnability	paper machine running without any stops
showers	spray nozzles that clean the wires and felts around the machine
sizing, size	makes the paper hydrophobic
Tappi	Technical association for the pulp and paper industry
TMP	thermo-mechanical pulp, fibers separated by “grinding” and heat, contains a lot of lignin and other substances
Wet end	machine system until the paper has dried
white water	water that are drained directly under the wire
wire	first step where fiber suspension dewater to be paper

ABSTRACT

This thesis deals with the roles of microorganisms and different chemicals in the formation of deposits and biofilms at paper and board machines. “Deposit” in this thesis means solid matter that accumulates and immobilizes on machine areas or interfaces meant for unhindered flow of slurries, liquids or air. The deposit is a “biofilm” when microorganisms, or substances produced by them, are its major or otherwise significant building block.

The work in this thesis builds on the hypothesis that i.) knowledge on the biotic and abiotic components of the deposit, and ii.) understanding their roles in the build-up, architecture, biological, physico-chemical and technical properties of the deposit will guide the researcher towards preventing or reversing the formation of unwanted deposits in a sustainable way. Multiple analytical tools were used for documenting the buildup of the deposit, including electron microscopy, confocal laser scanning microscopy (CLSM), energy dispersive X-ray (EDX) analysis, pyrolysis gas chromatography - mass spectrometry (Py-GCMS), ion exchange chromatography, gas liquid chromatography and microbiological analyses. I took actively part in developing innovative tools, based on back-scattered light sensoring that can be used for on-line measurement of biofilm in the water loops and containers of paper machines.

In the thesis work it was discovered that many of the paper making chemicals interacted forming organic, adhesive layers on steel surfaces in machine circuits. Structures were found, by light microscopy originally judged as microbes, but electron microscopy revealed that they were alum that precipitated as aluminum hydroxide at pH 6.8 in the white water of recycled fiber using machines. Alum is still used as a fixative today among many paper makers, even if the process conditions have changed from acid to neutral pH. It is considered to be the “aspirin” for paper makers; the risk of this was clearly seen in this thesis work. Organic deposits were found, soaps (calcium soaps) of different compounds, like pitch, at the base of the deposit of many paper/board machines.

Bacteria, morphologically resembling *Deinococcus geothermalis*, were demonstrated to grow as colonies firmly attached to the surface of clean stainless steel coupons, immersed in circulation waters at paper machines. Such deinococcal colonies could function as a pedestal, adhesion aid, for later massive attachment of other microorganisms, explaining why deposits frequently contain deinococci as a small, but never as the major building block of paper machine deposits.

For assessing the quality of water (raw water, warm water or bio-water) at the paper machines, the measurement method is important. The correlation between cultivation methods and possible contamination of bacteria seen on the coupon surfaces was low, especially when filamentous bacteria were part of the contamination.

Environmental concerns have forced paper and board machines to close their circuits.

The circulation and reuse of the process waters lead to higher processing temperatures and increase of the colloidal and dissolved material in the circulations. The chemical composition of three different mills was studied, with different final discharges of, 0 m^3, 0.5 m^3 and 4 m^3 waste water per ton product produced, one with reuse of bio-water. The zero discharge mill accumulated high amounts of organic carbon in the circulation waters (> 10 g L^{-1}), including volatile acids (lactic, acetic, propionic and butyric). Contents of sulfate, chloride, sodium and calcium were also high, > 1 g L^{-1} of each. The major part (40 %) of all identifiable bacterial 16S rRNA gene sequences were closest but yet distantly related (<96 %) to *Enterococcus cecorum.* In the 4 m^3 per ton product discharging mill, additionally *Bacillus thermoamylovorans* and *Bacillus coagulans* were found. Slimes and deposits contained high amounts, $\geq 10^8$ g^{-1}, of archaea, but only one genus, *Methanothrix* had a sequence match close enough for identification. The results showed that closing the water circuits strongly limited the diversity of the mill microbiota but allowed efficient mineralization of the dissolved and suspended matter.

TIIVISTELMÄ

Väitöskirjani käsittele mikrobien ja erilaisten kemikaalien rooleja saostumien ja biofilmien muodostumisessa paperi- ja kartonkikoneilla. ”Saostuma” tässä työssä tarkoittaa kiinteän aineen kertymää konepinnoille tai rajapinnoille konekierroissa, jotka on tarkoitettu massasulppujen, lietteiden, vesien tai ilman kuljetukseen. Saostumasta tulee ”biofilmi” silloin kun sen oleellinen rakennekomponentti on mikrobisolut tai niiden tuotteet.

Väitöstyöni työhypoteesina oli, että i. tietämys saostumien koostumuksesta, sekä ii. niiden rakenteesta, biologisista, fysikaalis-kemiallisista ja teknisistä ominaisuuksista ohjaavat tutkijaa löytämään ympäristöä säästäviä keinoja estää epätoivottujen saostumien muodostus tai purkaa jo muodostuneita saostumia. Selvittääkseni saostumien koostumista ja rakennetta käytin monia erilaisia analytiikan työkaluja, kuten elektronimikroskopiaa, konfokaali-laser mikroskopiaa (CLSM), energiadispersiivistä röntgenanalyysiä (EDX), pyrolyysi kaasukromatografiaa yhdistettynä massaspektrometriaan (Py-GCMS), joninvaihtokromatografiaa, kaasukromatografiaa ja mikrobiologisia analyysejä. Osallistuin aktiivisesti innovatiivisen, valon takaisinsirontaan perustuvan sensorin kehittämistyöhön, käytettäväksi biofilmin kasvun mittaukseen suoraan koneen vesikierroista ja säiliöistä.

Työni osoitti, että monet paperinvalmistuksessa käytetyistä kemikaaleista reagoivat keskenään tuottaen orgaanisia tahmakerroksia konekiertojen teräspinnoille. Löysin myös kerrostumia, jotka valomikroskooppisessa tarkastelussa oli tulkittu mikrobeiksi, mutta jotka elektronimikroskopia paljasti alunasta syntyneiksi, alumiinihydroksidiksi joka saostui pH:ssa 6,8 kiertokuitua käyttävän koneen viiravesistä. Monet paperintekijät käyttävät vieläkin alunaa kiinnitysaineena vaikka prosessiolot ovat muuttuneet happamista neutraaleiksi. Sitä pidetään paperitekijän ”aspiriinina”, mutta väitöstutkimukseni osoitti sen riskit. Orgaanisten saostumien alkuperä oli aineiden, kuten pihkan, saippuoituminen (kalsium saippuat) niin että muodostui tahmankasvua ylläpitävä alusta monilla paperi- ja kartonkikoneilla.

Näin solumuodoiltaan *Deinococcus geothermalis*ta muistuttavia bakteereita kasvamassa lujasti teräskoepalojen pintaan kiinnittyneinä pesäkkeinä, kun koepaloja upotettiin paperikoneiden vesikiertoihin. Nämä deinokokkimaiset pesäkkeet voivat toimia jalustana, tarttumisalustana muiden mikrobien massoille, joka selittäisi miksi saostumat yleisesti sisältävät deinokokkeja pienenä, muttei koskaan pääasiallisena rakenneosana.

Kun paperikoneiden käyttämien vesien (raakavedet, lämminvesi, biologisesti puhdistettu jätevesi) laatua tutkitaan, mittausmenetelmällä on suuri merkitys. Koepalan upotusmenetelmällä todettu biofilmikasvu ja viljelmenetelmällä mitattu bakteerisaastuneisuus korreloivat toisiinsa huonosti etenkin silloin kun likaantumisessa oli mukana rihmamaiseti kasvavia bakteereja.

Huoli ympäristöstä on pakottanut paperi- ja kartonkikoneiden vesikiertojen sulkemiseen. Vesien kierrätys ja prosessivesien uudelleenkäyttö nostavat prosessilämpötilaa ja lisäävät koneella kiertävien kolloidisten ja liuenneiden aineiden määriä. Kiertovesien pitoisuuksia tutkittiin kolmessa eriasteisesti suljetussa tehtaassa, joiden jätevesipäästöt olivat 0 m^3, 0,5 m^3 ja 4 m^3 tuotetonnia kohden, joka yhdessä tehtaassa perustui puhdistetun jäteveden uudelleen käyttöön. Nollapäästöisellä tehtaalla kiertovesiin kertyi paljon orgaanisesti sidottua hiiltä (> 10 g L^{-1}), etenkin haihtuvina happoina (maito-, etikka-, propioni- ja voi-). Myös sulfaatteja, klorideja, natriumia ja kalsiumia kertyi paljon, > 1 g L^{-1} kutakin. Pääosa (>40%) kaikista bakteereista oli 16S rRNA geenisekvenssianalyysien tulosten perusteella sukua, joskin etäistä (< 96%) ainoastaan *Enterococcus cecorum* bakteerille. 4 m^3 päästävältä tehtaalta löytyi lisäksi *Bacillus thermoamylovorans* ja *Bacillus coagulans*. Tehtaiden saostumat sisälsivät arkkeja suurina pitoisuuksina, $\geq 10^8$ g^{-1}, mutta tunnistukseen riittävää sekvenssisamanlaisuutta löytyi vain yhteen arkkisukuun, *Methanothrix*. Tutkimustulokset osoittivat että tehtaan vesikiertojen sulkeminen vähensi rajusti mikrobiston monimuotoisuutta, muttei estänyt liuenneen aineen ja kiintoaineen mineralisoitumista.

1. BACKGROUND

Problems caused by deposits (and slimes) in papermaking are well known, and they may lead to impaired quality, reduced runnability and increased costs for paper making. Deposits formed can be divided into non-biological (stickies, pitch and scale) and biological (slime) [Sanborn, 1965; Blanco *et al.* 1996] or into organic (organic substances and microbes) and inorganic material. The latter makes it easier to understand the mechanism of the deposit formation in the paper industry [Kanto Öqvist *et al.* 2001]. Nevertheless, most of the deposits found contain all these basic types, which have been included one after the other to form a matrix [Simons *et al*; 2003]. The first layer is considered to be crucial to the formation mechanism [Schenker *et al.* 1998; Kolari *et al.* 2001].

2. REVIEW OF THE LITERATURE

2.1. Some current and historical aspects of deposit and slime control in the pulp and paper industry

In the worldwide paper industry the tendency due to environmental restrictions goes towards lower specific water consumption per ton paper or board produced [Hamm *et al.* 2007; Flemming *et al.* 1998]. This creates problems with higher process temperatures, increase in concentrations of suspended solids, and an increase in colloidal and dissolved materials in the process circulation [Blanco, 2003]. Increases in the contents of dissolved nutrients, may favor microbial growth [Johnsrud, 2000]. The question is how this will influence the microbial population inside the paper mill and thereby the process and the product qualities.

A well-known cause of quality and runnability problems in the papermaking process is formation of deposit and slime [Sanborn, 1965; Blanco *et al.* 1996; Lindberg *et al.* 2001; Väisänen *et al.* 1994; Desjardins and Beaulieu, 2003; Rättö *et al.* 2005; Chaudhary *et al.* 1997]. However, real cases indicate that deposits are not the dominating problem for a closed mill, but odor problems in the product and in the surroundings.

Many deposit control programs, including treatments of slime, are designed based on measurements done in the water loops. However several studies indicate that also in paper machines the efficacy of biocides on planktonic bacteria is not a reliable indicator of the performance of a treatment program to treat deposits and slime formation [Grobe *et al.* 2002; Kolari *et al.* 2003; Blanco *et al.* 1996].

Already in 1965, Sanborn published a book regarding slime control in the pulp and paper industry, where he described problems and actions that should be taken the same way today. He stated that deposits in the industry encountered are mixtures of biological and non-biological deposits (fibers, pitch-like and chemical accumulations). He described the importance to understand what the problem is, to successfully control the causal agent, and the importance of “good house-keeping”. Both microscopical analysis and chemical analysis should be performed from spots in the paper and deposits in the machine, accepting this is sometimes difficult for papermakers. Sanborn also excels in explaining possible effects of seasonal variations on deposit and slime formation.

The shortcomings of the bacterial plate count as an indicator of mill problems were also brought up by Sanborn. Whereas only trained workers can make this type of evaluation meaningful, he suggested that the deposits should be scraped away to be analyzed.

He also pointed out that “in spite of similarities in the paper making process, no two mills are the same”, and the newer types of materials used in the machine systems would decrease the ability for deposition (from wood chests etc.). He described three ways of growth inhibition:

- Increased processing temperatures to about 63-82 ºC
- Installation of equipment surfaces that discourage attachment and promote free flow.
- Control of microbiological growth through chemical treatment.

Tappi press has also published a book "Microbiology of Pulp and Paper" [Appling *et al.* 1955]; however this book considers all problems with deposits and slime control in the paper industry to be of microbial origin.

2.2. The paper machine loops

This part introduces the different paper machine loops and interfaces in the machine systems to make the aims and the results of this thesis understandable.

Due to the higher mill closure, one has started to look at the paper making production systems in three different main circulations; schematically shown in Figure 1.

Environmental protection leads to lower water consumption to decrease the fresh water input to the mill and partial or total water circuit closure. It leads to increased stability,

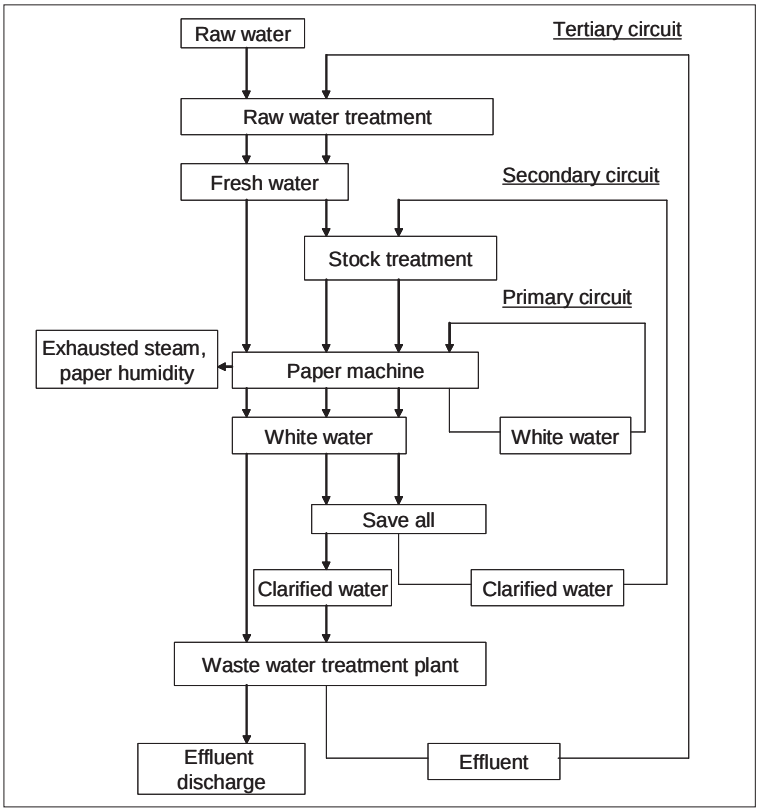


Figure 1: The circuits in the paper machine systems adopted from Blanco (2003), containing three main circulations for the water.

Table 1: Concentrations of VFA and other volatile organic compounds of a zero discharge mill and a mill discharging 20 L kg^{-1} paper produced. Adopted from Geller (1984).

Mill closure, specific water discharge L kg^{-1}	0	20
Lactic acid mg L^{-1}	730	1500
Acetic acid mg L^{-1}	2650	190
Propionic acid mg L^{-1}	690	200
Succinic acid mg L-1	30	<1
Butyric acid mg L^{-1}	450	110
Oxalic acid mg L^{-1}	4	<1
Acetoin mg L^{-1}	5	<1
Ethanol mg L^{-1}	70	20
Butanol mg L^{-1}	13	<1
Iso-propanol mg L^{-1}	80	<1
Propanol mg L^{-1}	20	<1

reduction in losses of fines, fibers, loads and additives, and enhanced dewatering, due to higher temperatures. Microbial life will be affected by the higher temperatures in the process water, by the increase in suspended solids, and increase in colloidal and dissolved material [Blanco, 2003]. If the mill only closes their circuits the COD values reach far above 10 000 mg L^{-1} [Geller, 1984], which influences the runnability and paper/board quality. Table 1 shows an example of the concentration of volatile organics in a zero discharge mill and an open paper mill.

Several paper and board mills have solved this problem caused by the organic volatiles when closing the water circuits by installing a tertiary circuit for the water, where the water is taken back from the sewage plant after clarification and microbiological treatment (Figure 1). Table 2 shows a chemical analysis of the white water before and after installation of a tertiary circuit with both anaerobic and aerobic treatment steps for circulation water in a board mill discharging zero m^3 water per ton board produced [Hamm and Schabel, 2007].

To be able to use calcium carbonate as fillers in the paper/board, most of the paper mills today are running at neutral or alkaline pH, the opposite of what Harju-Jeanty and Väätänen recommended in paper machine circuits in their paper 1984 for control of microorganisms. They suggested that all paper machines should run at acid pH for a natural way to control microorganisms in the paper machine circuits.

2.3. Paper making

2.3.1. Paper grades

The different paper grades and thereby the different raw materials make it difficult to generalize when it comes to deposit formation in the paper industry. Many different paper grades are produced, and each type has its own chemical composition and raw material mix. Even within one brand of the so-called graphical papers, the differences can be seen in Figure 2.

Table 2: Chemical analysis of the white water in a zero discharge mill before and after installation of a tertiary circuit including anaerobic and aerobic treatment of circulation water [Hamm and Schabel, 2007]

		Tertiary circuit
	Without	**With**
pH	6.3	7.2
Conductivity mS cm^{-1}	9.0	5.0
COD mg L^{-1}	32 800	6 400
Hardness dH	375	70
Calcium mg L^{-1}	2 650	505
Sulfate mg L^{-1}	1 350	375
Chloride mg L^{-1}	430	485
Acetic acid mg L^{-1}	6 300	890
Propionic acid mg L^{-1}	600	355
n-Butyric acid mg L^{-1}	350	<20

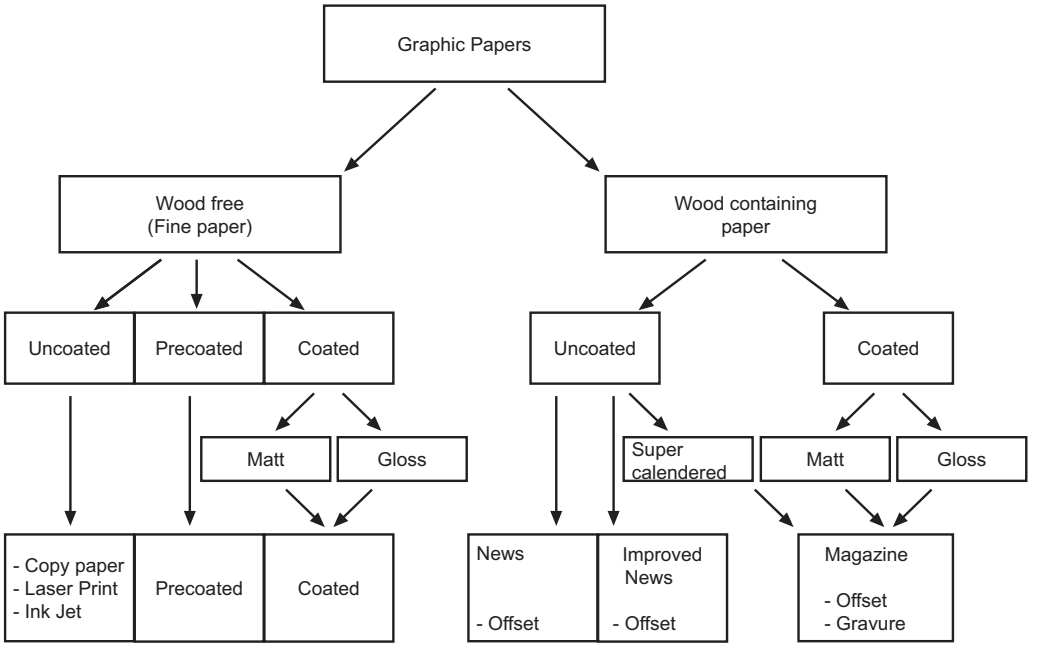


Figure 2: Different types of graphic papers [adopted from Persson, 2004].

One essential difference between the paper grades is in the fiber material. “Wood free” means based on chemical pulp and “wood containing” means based on mechanical pulp [Biermann, 1993]. In addition, within each branch, the additives and chemicals added to achieve the right paper properties vary. The additives are described under the section 2.4.3. The graphical paper grades account for about 30% of the world paper and board production [Alén, 2007].

In the stiffer brands of paper the sheet is mostly built up of several layers, and is called board. Folding boxboard is the common name for a board used for the production of boxes. Chipboard is the common name for board containing recycled fibers in one or several layers. Kraft liner is the name of board if it is made of more than 70% virgin fiber. If the board contains up to 100% recycled fiber pulp it is called test liner [Persson, 2004]. Corrugated or medium board is made from semi-chemical pulp and/or recycled fibers, forming the corrugated structure for boxes [Persson, 2004; Alén, 2007; Biermann, 1993]. Board is also produced in several bleached qualities i.e. in liquid board or coated board for white surfaces with good printability.

Tissue is the name for papers like toilet or kitchen paper, and is often made from bleached kraft or sulfite pulp [Biermann, 1993], and nowadays also from recycled fibers. Other more specialized paper types available in the market are e.g. greaseproof paper, bond paper, construction board, egg cartons and other molded products, which all contain their own special mixture of pulp and additives [Biermann, 1993].

2.3.2. Sources of recycled fibers for paper making

Recycled paper fiber contains many items affecting the deposit formation and microbiology in the mills and is therefore separately explained. Recycled fiber is an important raw material for the paper industry; the total production of recovered paper was about 145 million tons in 2003 [Alén, 2007]. In many parts of the world the collection of used paper is organized by independent companies, which sell it to the paper mills.

Recycled fiber can be used in many different paper grades, and the use is increasing year by year. The quality demands of the raw material differ between the application fields and therefore the recovered paper must be sorted before processing. Textile fabric, pieces of wood, glass, plastic and other foreign materials are mainly removed when sorting [Persson, 2004]. Recovered paper can be divided into different quality grades. To facilitate handling and transport the paper is normally pressed into bales, see Figure 3.

Figure 3: Typical handling of recycled magazines and papers [quoted from Persson, 2004].

The demands on re-use are getting greater in many countries. World consumption of recovered fiber reached 160 million tons in 2002 and by 2010 analysts estimate that about half of the fibers used in papermaking worldwide will be recycled fibers [Blanco *et al.* 2004]. The disinclination for burning waste products increases, which makes it difficult to find suitable dumping grounds and, it is considered to be a good material to recycle. The greatest admixture is made in tissue paper, but the percentage in other grades increases constantly. Kitchen roll and toilet paper are examples of tissue products that can be produced entirely from recycled fiber [Persson, 2004].

2.3.3. Papermaking additives

Today it is almost impossible to achieve the optimum properties required for the different paper qualities without the use of additives [Alén, 2007; Persson, 2004; Biermann, 1993]. The additives can be divided into two groups: 1. functional additives (the most important ones listed in Table 3), which are chemicals added to give the paper new properties, and 2. processing aids (the most important are listed in Table 4) to make the functional additives attach to the fibers, or to affect the runnability of the machine.

Table 3: Functional additives for paper making, summarized according to data from Alén (2007), Persson (2004) and Biermann (1993).

Dry strength chemicals	starch chemically modified starches surface sizing starch gums (galactomannans) chitosan carboxy methyl cellulose (CMC) synthetic dry-strength additives
Wet strength chemicals	wet strength resins (e.g. urea-formaldehyde (UF), melamine-formaldehyde (MF), polyaminoamide-epichlorohydrin (PAAE)
Sizes (internal and surface)	neutral sizing e.g AKD, ASA
Dyes	acid sizing e.g. rosin size direct dyes, basic dyes, pigment dyes, acid dyes
Optical brightening agents (OBA)	disulpho type, tetrasulpho type, hexasulpho type
Coating color additives	insoluble latex binders: styrene-butadiene (SB), styrene-acrylate (SA), and polyvinyl acetate (PVAc) soluble binders: starches, proteins, cellulose derivatives, CMC, PVA, PVOH
Fillers	clay (kaolin), calcium carbonate (chalk or limestone,ground calcium carbonate, (GCC), precipitated calcium carbonate(PCC), talc, titanium dioxide
Other coating additives	dispersants, pH control agents, dyes and OBA, lubricants (e.g. calcium stearate, paraffin waxes, polyethylene waxes soy lecithin/oleic acid blends), crosslinkers (e.g.UF and MF, glyoxal, imidazoline derivatives, ammonium zirconium carbonate (AZC)

Table 4: Process chemicals or aids summarized according to data from Alén (2007), Persson (2004), Biermann (1993).

Fixatives and retention and drainage polymers	inorganic fixatives: alum, sodium aluminate, polyaluminium chloride (PAC), polyaluminium nitrate (AN), polyaluminium nitrate sulfate (ANS)
	organic fixatives and retention aids: polyaminoamide-epichlorohydrine (PAMAM), polyethylene imine (PEI), dicyandiamide formaldehyde (DSD), starch based compound (St), polydiallyldimethyl-ammonium chloride (PDADMAC), polyacrylamide (PAM), polyethylene oxide (PEO), polyvinyl amine (PVAm)
Adsorbents	bentonite, micronised talc
Nano- and microparticle retention and drainage systems	PAM, cationic St or guar combined with an anionic particle like colloidal silica, alumina or anionic organic polymer (PAM, cellulose or lignisulfonate)
Multicomponent system	nano- and microparticle retention and drainage systems combined with additional fixative and micropolymers
Defoaming and deaeration agents	oil-based emulsions (oil and solid particles like N,N'-etylenebis(stearamide)(EBS) wax or silica), silicones with silicone oil and hydrophobised silica particles or EBS particle emulsions, surfactants containing fatty alcohol derivatives like esters and ethoxylated or propoxylated derivatives and emulsions containing fatty alcohol-based wax emulsions and additives
Biocides and detergent-related agents	biocides (i.e., microbicides or slimicides), detergents (and/or detackifiers)

Important is that many of the paper making additives contribute to deposit formation, and must thereby be taken into consideration when analysis and treatments are done to improve paper quality and runnability of the paper/board and paper machines [Kanto Öqvist *et al.* 2001]. Fillers added are less expensive than the fiber and improve the paper properties. Without chemical additives it would, in most cases, be difficult to reach the desired paper properties exactly, and the chemicals can improve the production economy. Examples of additive functions are [Alén, 2007; Persson, 2004; Biermann, 1993; Eklund and Lindström, 1990]:

- prevent the paper from absorbing water [size, hydrophobation agent]
- give strong fiber bonds (dry strength agent)
- give water proof bonds (wet strength agent)
- bind fine material, increase production (retention and drainage agent)
- increase opacity (fillers)
- increase brightness (OBA)

Table 5 summarizes the different additives that are used to differentiate paper grades.

Table 5: Examples of additives function and use in different paper grades [adopted from Persson, 2004].

Type of paper product **Additive function**

	Retention/ Dewatering agent	*Dry strength agent*	*Wet strength agent*	*Hydro-phobic agent*	*Filler*
NEWSPRINT/STANDARD	*X*				*(X)*
NEWSPRINT/IMPROVED	*X*	*X*			*X*
MAGAZINE PAPER, WOOD CONTAINING	*X*	*X*			*X*
FINE PAPER, WOOD-FREE	*X*	*X*		*X*	*X*
SACK PAPER	*X*	*X*	*X*	*X*	
KRAFT PAPER	*X*	*X*	*X*	*X*	*(X)*
LINER, BROWN	*X*	*X*		*X*	
LINER , WHITE SURFACE LAYER	*X*	*X*		*X*	*X*
FLUTING, SEMI-CHEMICAL					
FLUTING, RECYCLED FIBRE	*X*	*X*			
FOLDING BOXBOARD	*X*	*X*		*X*	*X*
BLEACHED SOLID BOARD	*X*	*X*		*X*	*X*
LIQUID CARTON BOARD	*X*	*X*		*X*	
TISSUE	*X*	*(X)*	*X*		

2.4. Microbiological problems of papermaking

Microbes are present in every paper machine, and their presence in the process is inevitable. The circulation water contains biodegradable dissolved substances, and the pH and temperature are favorable for microbial life [Alén, 2007; Blanco *et al.* 1996], but compared to many other industries paper machine waters and wastewaters contain insufficient nitrogen and phosphorus to satisfy bacterial growth requirement [Slade, 2004]. Measurements of the C:N:P (carbon : nitrogen : phosphorous) ratio done by Väisänen *et al.* 1994 are shown in Table 6.

Table 6: C: N : P ratios calculated from the data presented by Väisänen *et al*, 1994 (deviation 20%).

Machine	**C : N : P**
1	100 : 2 : 0.2
2	100 : 2 : 0.5
3	100 : 1.5 : 0.3
5	100 : 1.2 : 0.7
6	100 : 2.2 : 0.7

2.4.1. Biofilm and microbially induced spots or holes in paper product

In 1984 by the consensus of the leaders in biofilm research, a biofilm was defined as a collection of microorganisms, predominantly bacteria, enmeshed within a three-dimension gelatinous matrix of exo-cellular polymers secreted by microorganisms [Bryers, 2000]. Biofilms can occur at solid–liquid, solid–air, liquid-liquid and liquid–air interfaces [Bryers, 2000]. Locations of these interfaces found on a paper machine are shown in Figure 4 where the cylinder represent the pulp and water chests, the cube represent the primary circuit, the blue part and forward represent the wire, press and drying section of a paper machine. Most microorganisms can form biofilms and more than 99% of all microorganisms on earth live in such aggregates [Costerton *et al.* 1987].

A feature all biofilms have in common is that the organisms are embedded in a matrix of microbial origin, consisting of extracellular polymeric substances (EPS). The EPS comprise mainly polysaccharides and proteins, which form hydro gel matrices [Wingender *et al.* 1999]. Fossilized biofilms are the first records of life on Earth, dating back 3.5 billion years [Schopf *et al.* 1983]. Biofilms represent by far the most successful form of life, colonizing soils, sediments, mineral and plant surfaces in nature, including extreme environments, such as pore systems in glaciers, hot vents, electrodes and even highly irradiated areas of nuclear power plants [Satpathy, 1999]. Biofilms are involved in the biogeochemical pathways of carbon, nitrogen, hydrogen, sulfur, phosphorus and most metals, and the biofilm organisms perform self-purification processes in nature. For example, the biotechnologies in drinking and wastewater treatment, based on biofilms. Life embedded in the EPS matrix offers important advantages for biofilm organisms. They can maintain stable arrangements of synergistic micro-consortia, and due to that, manage the degradation of complex substrates [Wimpenny, 2000]. The matrix absorbs nutrients from the environment and is, thus, part of a general microbial strategy for survival under oligotrophic conditions [Decho, 1990 and 2000]. Flemming (2002) lists some of the ecological advantages, which can be seen in Table 7.

An excess of biofilms formed in the machine circuits leads to paper defects (spots and holes) or causes web breaks when slime lumps are sloughing off [Alén, 2007; Kolari, 2003; Rättö, 2005; Väisänen *et al.* 1998]. Several different types of bacteria have been isolated from spots in the paper products and deposits at the paper machine. Table 8 shows examples of bacteria detected in deposits and paper spots with molecular biology methods or cultivation methods with molecular methods as a complement [Ekman *et al.*

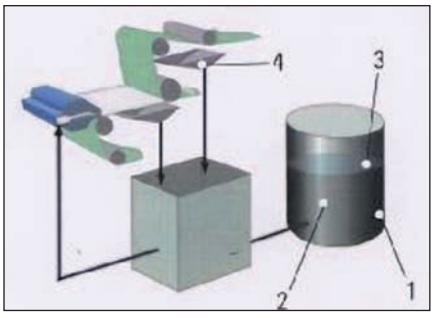


Figure 4: Schematic drawing of a paper machine showing interfaces, where biofilm can build up at a paper machine: 1. solid-liquid, deep down in chests and in pipes 2. liquid-liquid, agglomeration in the water phase 3. liquid-air, on the aerobic interface where water levels are continuously changing 4. air-solid, on the machine parts where no liquid water are flowing (own picture).

2007; Kolari *et al.* 2001 and 2003; Lahtinen *et al.* 2006; Denner *et al* 2006; Desjardins and Beaulieu, 2003; Busse *et al.* 2002; Oppong *et al.* 2003; Väisänen *et al.* 1998].

The most common contaminant is the genus *Meiothermus*, found at 18 out of 24 machines investigated (four countries). Almost one fifth of the deposits contained $\geq 10^9$ *Meiothermus* 16S rRNA gene copies [Ekman *et al.* 2007]. A novel genus and species detected from colored paper machines biofilms was, *Rubellimicrobium thermophilum* [Denner *et al.* 2006].

Difficulties of bacteria to attach to clean steel surfaces have been studied by Kolari *et al* (2001), using bacterial strains from the paper industry. *Deinococcus geothermalis* was found to be the primary attacher in the formation of deposits at the paper machines [Kolari *et al.* 2002]. Deposits in the paper industry contain a complex combination of all substances circulating in the process (fillers, fibers and fines, resins, sizing agents, binders etc.) [Schenker, 1997]. Sanborn's (1965) and Alén's (2007) comments on the complexity of the deposit formation in the paper industry and the difficulties to draw an exact line between microbial deposits and chemical deposits, show the importance

Table 7: Ecological advantages of the biofilm mode of growth, adopted from Flemming (2002).

Function	Relevance
Adhesion to surfaces	Primary biofilms and microcolonies Prerequisite for further biofilm development
Aggregation of cells, formation of flocs and biofilms	Immobilisation of cells High cell density possible
EPS as a structural element of biofilms	Mechanical stability Development of microconsortia Concentration gradients Retention of extracellular enzymes Polysaccharide–exoenzyme interactions Prevention of loss of lysed cell components Convective mass transport through channels Pool of genes, easy horizontal gene transfer Matrix for exchange of signalling molecules Light transmission into biofilm depth
Protective barrier	Tolerance against biocides, metals, toxins Protection against phagocytosis Protection of exoenzymes by complexation Protection against some predator species
Sorption properties	Accumulation of nutrients Water retention, protection against desiccation Accumulation of pollutants in sludge

Table 8: Examples of bacteria present in paper machine deposits/slimes and recently analyzed by partial sequencing of the 16S rRNA gene or cultivation methods since 1998 [Ekman *et al.* 2007; Kolari *et al.* 2001 and 2003; Lahtinen *et al.* 2006; Denner *et al.* 2006; Desjardins and Beaulieu, 2003; Busse *et al.* 2002, Oppong *et al.* 2003; Väisänen *et al.* 1998].

Bacteria in paper machine ciculation waters and deposits	**Reference**
Acinetobacter radioresistens	Väisänen et al.1998
Acidovorax	Desjardins and Beaulieu, 2003
Aeromonas	Desjardins and Beaulieu, 2003
Allorhizobium	Desjardins and Beaulieu, 2003
Azorhizobium	Lahtinen et al. 2007
Azorhizophilus	Desjardins and Beaulieu, 2003
Azospirillium	Desjardins and Beaulieu, 2003
Aureobacterium	Väisänen et al.1998
Bacillus	Väisänen et al.1998; Kolari et al. 2001, 2003; Oppong et al. 2003; Desjardins and Beaulieu, 2003
Bacillus cereus	Väisänen et al.1998; Kolari et al. 2001
Bacillus coagulanns	Väisänen et al.1998
Blastobacter	Desjardins and Beaulieu, 2003
Brevibacillus	Desjardins and Beaulieu, 2003
Brevibacterium	Väisänen et al.1998
Burkholderia cepasia	Väisänen et al.1998
Deinococcus geothermalis	Väisänen et al.1998; Kolari et al. 2001, 2002, 2003
Enterobacter	Desjardins and Beaulieu, 2003
Flectobacillus	Oppong et al. 2003
Hydrogenophaga	Desjardins and Beaulieu, 2003
Leptothrix	Desjardins and Beaulieu, 2003
Meiothermus ruber	Ekman et al. 2007
Meiothermus silvanus	Kolari et al. 2003; Ekman et al. 2007
Methylobacterium mesophilicum	Väisänen et al.1998
Microbacterium	Desjardins and Beaulieu, 2003
Nocardiopsis alba	Oppong et al. 2003
Paenibacillus	Kolari et al. 2003; Oppong et al. 2003
Pantoea agglomerans	Väisänen et al.1998
Pseudomonas	Desjardins and Beaulieu, 2003;
Pseudomonas stutzeri	Väisänen et al.1998; Kolari et al. 2003
Pseudoxanthomonas	Desjardins and Beaulieu, 2003
Ralstonia pickettii	Väisänen et al.1998
Ralstonia solanacearum	Väisänen et al.1998
Rubellimicrobium thermophilum	Denner et al. 2006
Rhizobium	Lahtinen et al. 2007; Desjardins and Beaulieu, 2003
Sinorhizobium	Lahtinen et al. 2007
Sphingomonas	Väisänen et al.1998; Desjardins and Beaulieu, 2003; Lahtinen et al. 2006
Streptomyces	Oppong et al. 2003
Thermomonas haemolytica	Busse et al. 2002; Kolari et al. 2001, 2003
Xanthobacter	Desjardins and Beaulieu, 2003

of understanding the mechanisms leading to their formation. The paper industry spends about 200 million euros annually for so called slime control [Alén, 2007].

There are several publications indicating the microorganisms having a sessile and a planktonic phase, and that almost all of them are capable of producing biofilms [Flemming, 2002; Costerton, 1987; Ghannoum and O'Toole, 2004]. Practical experience shows however that no simple correlation between the cell number in the water phase and biofilm formation exists. Paper machines with a very high count of colony-forming units in the water circulations have been described operating without any problems, and other machines below 1000 colony forming units in the water circuits which exhibit severe biofilm formations at the machine [Alén, 2007; Kanto Öqvist *et al.* 2001].

2.4.2. Microbiological growth in cellulose or additives

All types of wood are colonized by bacteria. In the winter the most dominant species found in the Norway spruce (*Picea abies*) were identified as spore forming *Bacillus sp.* [Hallaksela *et al.* 1991]. In the pulping steps some bacterial growth can occur when mechanical or semi-mechanical pulping is done, due to the content of bacteria in the wood as well as in the water used for grinding. In production of thermo-mechanical pulp the temperature is about 80-100° C, which can reduce the amount of live microorganisms present. Grinding of wood releases carbohydrates that can be utilized as nutrients [Biermann, 1993; Lindberg, 2004]. Chemical pulping is done at temperatures higher than 140° C, pH 12 and of high pressure. This process will destroy all microorganisms [Biermann, 1993].

Throughout the papermaking process there are almost always conditions that favor microbiological growth, from storage tanks and towers, to mixing chests and headboxes and these places are therefore normally rich in microbes [Väisänen *et al.* 1998].

Inorganic material, such as talc, various types of clays and calcium carbonates used for pitch control, in the retention programs, or as fillers are either from natural mines and thereby loaded with microorganisms, or synthetically produced like PCC which then are normally contaminated from storage tanks and the make up water [Väisänen *et al.* 1998]. When the storage time is long, this often creates opportunities for microbiological growth. These products and tanks, therefore, are often treated with biocides. Other additives like sizes and wet strength agents do not normally contain high loads of bacteria due to preservation by the suppliers [Väisänen *et al.* 1998].

Starches are normally used as dry strength aids in the wet end or as surface sizing. The consistency of the slurry is about 40 % dry weight and the consistency of the cooked starch is about 8 % dry weight. Even if the cooking step reaches 130° C, the residual time at this high temperature is <10 seconds and allows the heat resistant spores from the contaminated slurry to regrow in the machine tank [Väisänen *et al.* 1998; Suominen, 2004]. Therefore, biocide treatments are an important tool for decreasing contamination of the machine, and to reduce the break down of the starch molecules which strongly affects the performance of the starch [Alén, 2007]. Treatment of starches with nisin has been evaluated and looked promising [Pirttijärvi *et al.* 2001].

High microbiological activity for example in the broke tower can lower the redox potential and the pH to such an extent that it will affect the whole wet end. This will lead to unstable wet-end conditions and thereby to a risk for chemical deposition due to unstable process conditions [Alén, 2007].

Tanks and vessels may receive little cleaning, and can thereby be contaminated with deposits, which constantly feed the additives with microorganisms [Blanco *et al.* 2004].

2.4.3. Odor problems

Paper mills can have bacterially caused smell problems by H_2S or organic acids. H_2S production caused by bacteria is a known phenomenon in paper machine circuits where reductive bleach is used. When a paper mill closes their water loops, the amount of volatile organic acids increase in the process, which increases the amount of compounds with very pungent smells [Blanco, 2004], both in the paper/board and in the surroundings of the mills. The extreme environment of the mill can be decreased when the water undergoes aerobic and anaerobic microbiological treatment before bringing this water back into the process [Hamm and Schabel, 2007]. The same conditions can also occur in chests and storage towers having long turnover time, creating favorable conditions for anaerobic growth [Alén, 2007].

2.4.4. Hygienic quality of the paper products

Bacteria enter the papermaking process from many sources and are not only capable of disrupting the process by slime formation, but can survive in the finished paper or board. Paperboard used for food packaging should be free of any microbes endangering food safety [Pirttijärvi *et al.* 1996]. Examples are some aerobic bacteria (*Bacillus*, *Brevibacillus*, and *Paenibacillus*) capable of forming heat-resistant spores. The production of high-hygiene products such as liquid-packaging board or other food-packaging grades requires the amount of spores to be low. There are indications that the concentration of bacterial spores can be as much as 100-200 times higher at the interface between the polyethylene excrution and the board, than in the cellulose matrix [Suominen *et al.* 1997] Bacterial diversity in the wet end is typically greater containing predominantly Gram-negative bacteria [Väisänen, 1991], than in the end products, in which the contaminants seem to be limited to the spores that survive the heat of the drying section [Alén, 2007]. Bendt (1985) writes that the only way to get rid of the endospores is to use oxidizing chemicals, as can be seen under 2.7.1, the non-oxidizing biocide glutaraldehyde is also considered efficient against endospores.

2.4.5. Microbially influenced corrosion (MIC) in the paper industry

Corrosion can occur below biofilms on the machine surfaces. Uneven biofilm coverage creates neighboring liquid cells with different redox potentials, oxygen, and chloride concentrations on the metal surface, and this is a driving force for the corrosion. Other microbiological products like acids and hydrogen sulfides further speed up corrosion [Alén 2007; Blanco *et al.* 1996; Heitz *et al.* 1996]. MIC imposes industry with major economic problems due to its effects on the machinery [Blanco *et al.* 2004].

2.5. Chemistry of deposits on paper machines

The conditioning film is considered to be crucial to the formation mechanism of a deposit [Schenker *et al.* 1998; Kolari *et al.* 2001]. An understanding of the origin of that layer as well as the building of the bulk deposit is important for designing an efficient treatment program [Kanto Öqvist *et al.* 2001]. The particle size has an important influence on the deposit formation. Figure 5 demonstrates the classification of the constituents present in the paper machine circulation water by their particle sizes. A major part of the substances involved in deposits are colloidal substances [Klein and Grossmann, 1995].

2.5.1. Organic deposits of papermaking

The organic papermaking additives AKD and ASA are commonly associated with the fouling of paper machines, both in the wet end and the dryer section [Knubb and Zetter, 2002; Koskela *et al.* 2003; Nguyen, 1998; Petander *et al.* 1998]. The strong affinity of sizing agents towards PCC is also known [Petander, 1998]. Also tacky organic materials are known to be included in organic deposition, typically coming from virgin pulp, recycled pulp, broke, additives, adhesives and waxes [Alén, 2007; Negro *et al.* 1999]. A large amount of these compounds comes from the wood, and are called extractives because they are either soluble in different nonpolar solvents, e.g. diethyl ether, dichloromethane (DCM) or methyl *tert*-butyl ether (MTBE), acetone, ethanol, hexane, toluene, tetrahydrofuran (THF), or are soluble in water [Alén, 2007]. Most of the time though, extractives and resins are both used as collective names for all lipophilic components, which can be extracted from wood by nonpolar organic solvents. Different types of extractives are necessary to maintain the diversified biological functions of the tree i.e. fats constitute the energy source of the wood cells, whereas lower terpenoids, resin acids and phenolic substances protect the wood against

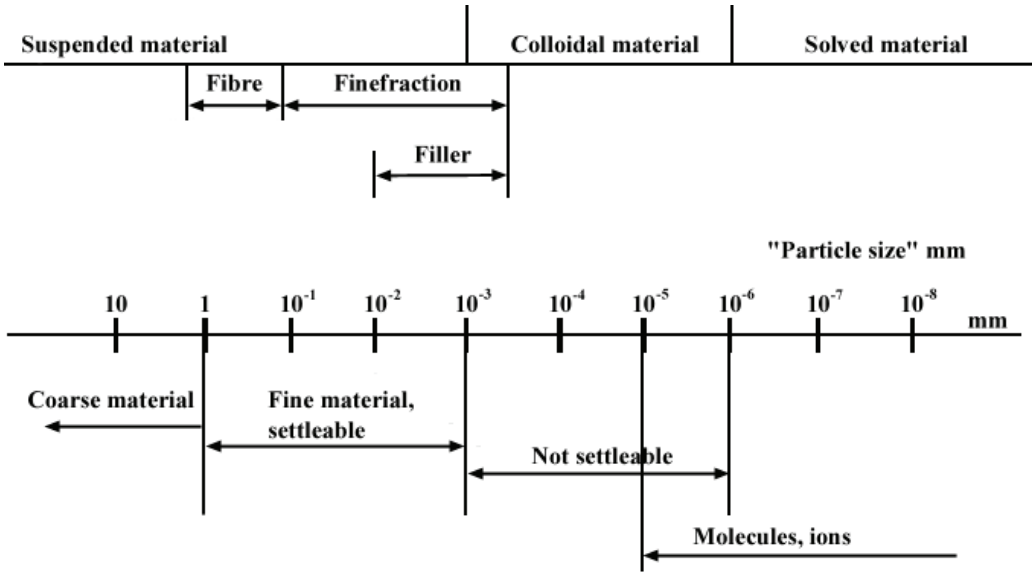


Figure 5: Sizes of particles and constituents present in the water circulations of paper machines [adopted from Persson, 2004]

microbiological damage or insect attacks [Sjöström, 1992]. The deposits on paper machines usually contain a mixture of several of these substances [Hubbe *et al.* 2006]. Tacky materials are hydrophobic and insoluble in water. Their deposition behavior is controlled by temperature, hydrodynamic and mechanical forces, and changes in chemical environment (pH, ion concentration) [Allen, 1980; Back and Allen, 2000; Carré *et al.* 1998; Dreisbach and Michalopoulos, 1989; Klein and Grossmann, 1995; Monte *et al.* 2004]. There are big differences in the amount and type of extractives coming from chemical pulping, mechanical pulping and recycled fiber [Alén, 2007]. The classical coagulation theory of organic colloids is DLVO and considers two kinds of interactions between similar dispersed particles: attractive van der Waals´ and repulsive electrostatic interactions [Kallio *et al.* 2004; Alén, 2007]. The problems caused by these materials are worse when the water systems are closed [Monte *et al.* 2004] due to the accumulation of contaminants.

2.5.2. Inorganic components in the deposits of paper machines

As a result of changes in concentrations, pH, temperature and process conditions, dissolved compounds (such as polyelectrolytes, surfactants or ions) may form insoluble salts or complexes. They can occur as stable colloids or as macroscopic phases (e.g. gels [Merta, 2001]) that form particles that are larger than colloidal size, depending on pH, temperature, gas content, concentration of substances, ionic strength etc. For example, soluble surfactants form insoluble salts (soaps) with di- or multivalent metal ions [Holmberg, 1999; Allen, 1988]. The formation of inorganic salts from ions is a relatively common fouling mechanism, since the processing waters are very often saturated with ions. Hydrophilic interfaces are commonly assumed to be the best sites for nucleation of inorganic salts [Brecevic and Kralj, 2000]. Solubility product constants (K_{sp}) for inorganic compounds frequently found in the paper industry can be seen in Table 9, and it is calculated according to the formula $A_aB_b(s) \leftrightarrow aA + bB$ which gives $K_{sp} = [A]^a [B]^b$ [Biermann, 1993], The solubility product is equal to the product of the molar concentrations of the ions involved in the equilibrium (A and B), each raised to the power of its coefficient in the equilibrium equation (*a* and *b*). The K_{sp} constant do not have a unit and a low value of the constant means that the compound has a very low solubility in water.

2.5.3. Biological deposits on nonliving surfaces

Bacteria in natural aquatic populations have a marked tendency to interact with surfaces, and biofilms are defined as communities of microorganisms attached to a surface [O´Toole *et al.* 2000]. Bacteria adhere to nonliving surfaces, initially in reversible association and eventually in an irreversible adhesion [Marshall *et al.* 1971; Costerton *et al.* 1987; Watnick *et al.* 2000; O´Toole *et al.* 2000] and initiate the development of adherent bacterial biofilms. The surface quality is important for the attachment, and is dependent on hydrophobicity and hydrophilicity of the surfaces [Costerton *et al.* 1987]. Costerton *et al* (1987) explained the mechanism of adhesion to be glycocalyx polymers, but this was revised in recent studies [Bryers, 2000; Kolari *et al.* 2002; Saarimaa *et al.* 2006; Parkar, 2001; Ghannoum and O´Toole, 2004; Peltola *et al.* 2008]. These indicate also that glycoproteins may be involved in the initial attachment, followed by aggregation and microcolony formation, possibly ending in biofilm formation by

Table 9: Solubility product constants at 25º C. Data taken from Biermann (1993).

Substance	Formula	Ksp
Aluminum hydroxide	$Al_2(OH)_3$	1×10^{-33}
Barium carbonate	$BaCO_3$	5×10^{-9}
Barium sulfate	$BaSO_4$	1.1×10^{-10}
Calcium carbonate	$CaCO_3$	4×10^{-9}
Calcium hydroxide	$Ca(OH)_2$	5.5×10^{-6}
Calcium sulfate	$CaSO_4$	1×10^{-5}
Calcium sulfite	$CaSO_3$	7×10^{-8}
Iron(II) hydroxide	$Fe(OH)_2$	1×10^{-14}
Iron(II) sulfide	FeS	6.3×10^{-18}
Iron(III) hydroxide	$Fe(OH)_2$	3×10^{-39}
Magnesium carbonate	$MgCO_3$	1×10^{-5}
Magnesium hydroxide	$Mg(OH)_2$	1.5×10^{-11}
Magnesium sulfite	$MgSO_3$	3.2×10^{-3}

secretion of extracellular polymeric substances (EPS) [Ghannoum and O´Toole, 2004]. Biofilm acts as a protective layer against potentially harmful agents as well as a carbon and energy source at times of nutrient deprivation, resistance to UV light and increased rates of genetic exchange [O´Toole *et al.* 2000].

Recent studies show that there are big differences in biofilm-forming capacity of bacteria [Lindberg *et al.* 2001], and although some general concepts can be applied, many species-specific behaviors exist that reflect the unique needs of each microorganism [O´Toole *et al.* 2000].

Not only the paper industry needs to understand the mechanism of biofilm formation but it is also important to understand how to prevent biofilm problems associated with medical devices, and how they cause infections [Mack *et al.* 2006]. Spores may also attach to stainless steel in greater amounts than vegetative cells [Parkar, 2001]. Parkar (2001) did his studies in relation to the dairy industry, but the result could also be considered important for the production of food grade papers.

Most of the studies on the attachment and biofilm formation mechanism have been done with *Pseudomonas aeruginosa* and *Staphylococcus epidermis*. The mechanisms seem to be species specific and depending on the surface type, as described by Raulio *et al* (2008). The authors introduced an adhesion mechanism for *Pseudoxanthomonas taiwanensis* involving cell ghosts on which the biofilm of live cells grew, and studies done on *Deinococcus geothermalis* showed the an adhesion mechanism mediated by adhesion threads [Kolari *et al.* 2002; Peltola *et al.* 2008; Raulio *et al.* 2008]. The research results on attachment mechanisms and biofilm formation done on *Pseudomonas aeruginosa* and *Staphylococcus epidermis* species should therefore perhaps be looked upon as species specific and not as a general description of the mechanism.

2.6. Deposit treatments in the paper industry

2.6.1. Biocides

In the 1920s and 1930s the most common compound used in the paper machine circulations for bacterial control was chlorine, with an addition of copper sulfate to control fungal growth dripped into the white water chest [Sanborn, 1965]. In the 1950s to 1960s organic mercury compounds like phenylmercuric acetate and ethylmercury salts were introduced to kill microbes in the deposits [Sanborn, 1965; Appling *et al.* 1955], however, due to the severe environmental impact these compounds were prohibited or abandoned by the industry [Swapan and Farr, 1997; McCoy, 1980]. They were often used in combination with chlorophenates [Sanborn, 1965]. Several new organosulfur compounds were also introduced in the 1960s to be used in combination with the chlorophenates. Already in the 1960s, chlorine dioxide was described as a new treatment of machine circuits, with the comment that it cannot be used for more than two weeks due to its bad fungicidal effect [Sanborn, 1965]. The quaternary ammonium compounds and other surface active so called germicides, at that time, were also introduced and found to keep the machine surfaces cleaner than other substances [Sanborn 1965]. Organic tin compounds like distannoxane, bis (tri-*n*-butyltin) oxide in combination with quaternary ammonium compounds were also used but created problems by being extremely toxic for fish and other aquatic life. Chlorophenols have been banned due to the high fish toxicity [McCoy, 1980].

The function of antimicrobial agents can be divided in six lethal effects that control the growth of microorganisms, listed by McCoy, 1980.

1. Coagulation of protein
2. Inactivation of enzymes
3. Disruption of cellular lipids
4. Damage to genetic apparatus
5. Destruction of ribonucleic acid
6. Damage to cell walls

Protein is coagulated by phenols and heavy metal compounds and ions (mercury compounds precipitate especially enzymes containing thiol groups). Methylene bisthiocyanate and similar compounds inactivate iron in cytochrome containing respiratory enzymes. Chlorine dioxide specifically halts protein synthesis, perhaps by attacking ribosomes in the cytoplasm, or else by inactivating ribonucleic acid. Dodecylguanidine hydrochloride lyses the mucopeptide layer in the cell wall, causing leakage of the contents of the cell and death of the organism.

There are two fundamentally different classes of biocides; oxidizing and non-oxidizing. Oxidizing biocides can be further divided in halogenated and non-halogenated active substances (Table 10). Both types oxidize and thereby damage non-specifically any organic substances including microorganisms in the processing water [McCoy, 1980; Schrijver and Wirth 2007; Block 2001; Kitis 2004; Simons and da Silva, 2005].

Table 10: Oxidizing biocides used today in paper industry [McCoy, 1980; Schrijver and Wirth, 2007; Paulus, 1993; Block, 2001; Kitis, 2004; Simons and da Silva, 2005].

Synonym	Constituent(s)	Mode of action	Property	Main application
		Non-halogenated		
Peracetic acid	Peracetic acid	More effective than hydrogen peroxide. Broad spectrum including spores. Generate organic radicals (CH3CO·) that react equal to hydroxyl radicals, but longer stable. Sulfhydryl and sulfur bonds are oxidized. May inactivate catalase.	Solution	Fresh and white water
Hydrogen peroxide	Hydrogen peroxide	Generate hydroxyl radicals (HO·) which is highly reactive and responsible for the antimicrobial action. It can attack membrane lipids, DNA, and other cell components. Catalase and peroxidase are enzymes produced in respiring cells to protect the cells from damage by steady-state levels of metabolically generated hydrogen peroxide. Effective between pH 2-10. Active against spores.	Solution	Recirculation water
		Halogenated		
Halogenated alkylhaydantoin	BCDMH, DMH	Effective against filamentous bacteria. The bromine compound is immediately released as free hypobromous acid. In general these compounds releases hypochlorous acid slower than other halogenated compounds.	Solid or in-situ generation with NaOCl	Fresh water, White water, Recirculation water
Ammonium bromide, Ammonium sulfate	NH4Br, (NH4)2SO4	Creates mono-haloamines reacting like other chlorine compounds, but more slowly and will therefore not destroy i.e. additives	In-situ generation with NaOCl	Stock circulation White water
Sodium hypochlorite	NaOCl	At low dosage, only inhibitory against biofilm (0.5-5 ppm available chlorine). A higher dosage (50 ppm available chlorine) results in reduction of biofilm.	Solution	Fresh water
Sodium bromide	NaBr	Mixed with NaOCl and added to the water circulation hypobromous and hypochlorous acids are formed. The presence of hypobromous acid increases the efficiency against biofilm, and make the added chemicals (NaOCl) less sensitive at pH above 7.	Solid or in-situ generation with NaOCl	Fresh water
Chlorine gas	Cl2	Combines readily with protoplasm, forming stable nitrogen-chlorine bonds with proteins. Effectiveness increases at low pH, high temperature, and high concentration. Chlorine destroys spores, but Desulfovibrio desulfuricans develops a resistance to chlorine.	Gas	Fresh water
Chlorine dioxide	ClO2	More lethal than chlorine to vegetative cells and spores. Effectiveness not markedly affected by changes in pH, although its toxicity is enhanced at higher pH. The antimicrobial activity lies in its high oxidation potential, which completely disrupts the synthesis of protein.	Gas, in-situ generation	Cooling water, Fresh water

Table 11: Non-oxidizing biocides used in the industry today [McCoy, 1980; Heitz *et al.* 1996; Schrijver *et al.* 2007; Paulus, 1993; Block, 2001].

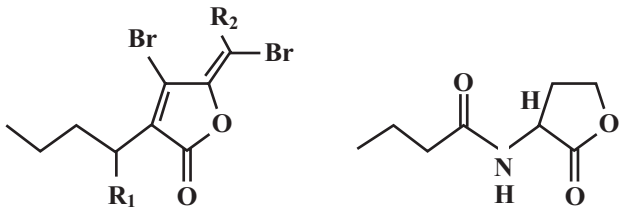
Synonym	Constituent(s)	Mode of function	Main application
		Quaternary ammonium compounds	
PHMB	Poly(hexamethylene biguanide) hydrochloride	Membrane active	Fresh water treatment
QAC	N,N-Dimethyl-N,N-didecyl ammonium chloride, benzalkonium chloride	Surface active on negatively charged receptor groups on the cell wall and cytoplasmic membrane. Dissolves large areas of the cell wall and lysis occurs. Practically without effect on spores. Effective between pH 6-8.	Fresh and white water
		Nitriles	
DBNPA	2,2-Dibromo-3-nitrilopropionamide	Effective against slime forming organisms. Not persistent slimicide, which does not cause waste water problems. Theory about the function is attachment to cell surface, inhibition of ß-Galactoside permeas.	Water circuits and preservation
MBT	Methylene bisthiocyanate	Disrupts the function of cytochrome dehydrogenase by blocking the electron transport forming a weak salt (Fe(CNS)3. Hydrolyses rapidly above pH8. Inactivated by ferric ions in the treated water.	Preservation
		Thiazol	
CMIT	5-Chloro-2-methyl-4-isothiazolin-3-on	CMIT is higher effective than MIT, but they are often used in a mixture. Broad spectrum microbistat. Microbicidal effect only slowly (after 24 h). Hydrolyze > pH 8. Is active on cellular level, reacts with cell nucleophiles.	Preservation
MIT	2-Methyl-4-isothiazolin-3-on	See CMIT	Preservation
BIT	1,2-Benzisothiazolin-3-on	Heat and pH stable (3-11), reaction mechanism equal to CMIT	Preservation
		Carbamates	
	Dimethyldithiocarbamate	Chelating agents that combine with metal ions in enzymes essential to the metabolism of microorganisms. Stable between pH 7-13	Preservation
		Aldehydes	
GDA	Glutardialdehyde	Good sporicidal efficacy. Works by interactions with the cell constituents, e.g. thiol groups of proteins. pH is the most important factor in regulating the activity of the agent. Killing time is faster at higher pH, five times faster at pH 8.5 than pH 5. In acid state, the effectiveness of the substance is lower.	Water circuits and preservation
THPS	Tetra-(hydroxymethyl)-phosphonium sulfate	A quaternary phosphonium salt which releases formaldehyde.	Water circuits
Bronopol	2-Bromo-2-nitropropane-1,3-diol	Efficacy over a wide pH range (5-9) Combined action due to two toxophoric groups (activated halogen and methylol), which may react with the cell's nucleophilic centers, e.g. with SH-group-carrying enzymes.	Water circuits

Non-oxidizing biocides work by interfering with areas of a cell's structure specifically concerned with its survival [McCoy, 1980; Heitz *et al.* 1996; Schrijver *et al.* 2007; Block, 2001]. Products presently used in the industry are shown in Table 11.

Some new natural substances have also been found like signaling molecules that display anti-colonization activity isolated from Australian seaweed (Figure 6). These molecules have been identified to repel bacteria from the surface of the weed and are patented for use as anti-foulants. They are thought to function by blocking the communication (quorum sensing) between the bacterial cells, their molecular make-up being equal to the signaling molecule sent out by the bacteria, and thereby capable to inhibit the communication [Ghannoum and O´Toole, 2004]. Quorum sensing is the regulation of gene expression in response to fluctuations of the cell-density within cell populations. Fluctuations are sensed by chemical signal molecules (autoinducers) like N-acylhomeserine lactone (AHL) that increase in concentration as a function of cell density. Quorum sensing as a phenomenon was discovered over 30 years ago [Miller and Blasser, 2001].

Studies of the communication between bacteria causing problems in the paper industry, and the possibilities of treatments targeting signaling pathways have started and should continue [Kolari, 2003; Martinelli *et al.* 2002]. The studies by Kolari (2003) and Martinelli *et al.* (2002) come to different conclusions regarding the communication of bacteria in the paper machine biofilms. Kolaris results suggested that AHLs do not play any role contradictive to Martinellis positive results from paper machine biofilms. An application of these compounds or their antagonists is problematic, because they are scarcely available and they are difficult to apply to a surface. Apart from that, before their use in European paper or board mills they will have to undergo evaluation under the European Union's biocide guideline (Biocidal Products Directive (BPD) 98/8/EC) procedure, which has a high assessment cost estimated at about 5 million Euros per substance [Flemming, 2002].

It is assumed that some kind of initiation layer is formed [Schenker *et al.* 1998] to make it easier for the microbes to attach to the surface [Costerton *et al.* 1987]. Many of the presently used deposit control programs (including treatments of slime) are designed based on measurements done in the water loops, and it has been proven that microorganisms in a biofilm are more resistant to biocide or antibiotics treatment



Furanome **N-butanoyl-L-homoserine lactone (BHL)**

Figure 6: Structures of furanones from the red alga *Delisea pulchra* and the bacterial signal molecule *N*-butyl-L-homoserine lactone

[Costerton *et al.* 1999; Costerton, 2007]. Two mechanisms by which microorganisms can acquire a resistance to biocides are described; firstly by avoiding the effect through a physical barrier or by the secretion of enzymes; and secondly an acquired resistance due to an alteration in the microorganism, mutation or a change in the genetic makeup. The latter is considered to be less common in the paper machine systems [Palcic and Teodorescu, 2002].

2.6.2. Non-biocidal tools for deposit control

Several non-biocidal types of products and methods are used in the paper industry to reduce the influence of biofilms together with biocides or alone. In the paper industry they are often called Bio-dispersants, or just deposit control agents. Non-ionic dispersants reduce the formation of the microbiological deposits, resulting in a deposit of less thickness and consistency [Blanco *et al.* 1996]. In Table 12 the main substances used are listed [Schrijver and Wirth, 2007].

These substances are often blended together to achieve complex formulations.

Several enzymes, alone and in combination, have been evaluated for the biofilm control in the paper industry with varying success, due to the variation of the EPS composition from species to species, and machine to machine [Schenker, 1997].

Electric fields have been used both to prevent microbial adhesion and to inhibit biofilm growth [Matsunaga *et al.* 1998; Kerr *et al.* 1999] in marine environment. Practical observation, however, has shown that all kinds of electrodes immersed in water can be colonized and fouled by biofilm [Flemming, 2002].

Ultrasonic deposit control systems are also under development, no official references are available so far.

2.7. Methods used for the analysis of biofouling

2.7.1. Off-line methods

Physical, chemical, biological and optical measurement methods used in the paper industry for biofouling have been described (Table 13). According to Hilbert *et al.* (2001) the only specified method, which attempts to quantitatively determine biofilm growth is

Table 12: Deployment and type of bio-dispersants in the paper industry [Schrijver and Wirth, 2007].

Class	Synonym	Chemical basis	Main application
Surfactants	Glycols	Alcohols	White water Head box
	Amides	Alkylamides	
	Terpenes	Terpenoids	
	Lignosulfonates	Salts of ligninsulfonic acid	
	Nonionic, Anionic, Cationic, Hybrid ionic	Alcohol, Fatty acids	

the slime measuring board. The performance of the slime measuring board is more or less dependent on several factors, like the design of the measuring cell, the selected surface of the measuring board and the method of evaluation [Hilbert *et al.* 2001].

Kolari *et al* (2003) describe a promising method how a microtiter plate assay can be used in studying biofilm growth tendency, and biocide influence on the biofilm in laboratory scale for the paper industry.

Álvarez-Barrientos et *al* (2000) reported about the positive features of using the CLSM (confocal laser scanning microscope) to analyze biofilms in the paper machine loops, and how complex the matrix can be, but failed to explain how the sampling should be done. Flow cytometry and the use of fluorescence *in situ* hybridization (FISH) is also described, but still under development for the paper industry [Nohynek *et al.* 2003; Torres, 2008].

Several different positive and negative facts are described with respect to different microscopic methods used for biofilm analysis [Wolf *et al.* 2002], however in this thesis the advantages of the microscopical methods for analysis of deposits from the paper industry are explained.

2.7.2. On-line methods for measuring biofilm development

To prevent financial losses associated with runnability and quality problems, a method supplying continuous online information of the actual tendency of the deposit formation is very important to be able to optimize and extend the amount of counter measure [Flemming, 2002; Flemming, 1998; Klahre *et al.* 1998]. Only if the formation of such deposits is detected at an early stage, efficient and economic counteractive measures can be triggered. On the market one can find several types of deposit monitors based on light measurements sent through a glass surface [Swapan and Farr, 1997]. Exceptions from the glass surfaces are the by-pass units with steel plates i.e. described by Korhonen and

Table 13: Overview of methods for measuring the outcome (endpoints) of biofouling [Hilbert *et al.* 2001].

Physical methods	Chemical methods	Genetical methods	Biological methods
Oxygen depletion	Ninhydrin test	FISH test	Cell count determination
ATP measurement	Resazurin test	Polymerase chain reaction (PCR)	Luminescent bacteria test
Slime measuring board	Dehydrogenase activity	Immunofluorescence marking	Minimal inhibitory concentration
Microcalorimeter	Fluorescent redox stain (CTC)		Hemmhof test
pH and redox measurement			Bacteria identification
Volatile fatty acids			
Sulfide measurement			

Väätänen (2002) and Mattila *et al* (2002). The BioDeposit Control online measuring devise is based on measurement direct in the process. It is the only one, as far as it is known, which is not using a by pass and includes the traditional slime board originally made of wood, popular in Germany [Klahre *et al.* 1998; Hilbert *et al.* 2001].

All online biofilm monitoring techniques are based on some kind of signal obtained from the biofilm under investigation. The biofilm leaves its characteristic footprint by signal features that may be modified include and were summarized as follows by Janknecht and Melo (2003):

- intensity of light (Differential Turbidity)
- intensity of sound (Ultrasonic Frequency-Domain Reflectometry)
- color/wavelengths (Bioluminescence, Fluorometry, Spectroscopy)
- mechanical resonance frequencies (Quartz Crystal Microbalance [Lie *et al.* 2002; Pauly, 2001; Kallio, 2004])
- electrical capacitance (Dielectric Sensor)
- electrical conductivity (Electrochemical Electrodes)
- light refraction indices (Surface Plasmon Resonance)
- friction (Pressure Drop)
- thermal resistance (Heat Transfer Coefficient)
- optical input signals that are being modified into acoustic output signals (Photo Acoustic Spectroscopy)

Practically all the mentioned techniques allow continuous monitoring of biofilm formation and automatic data collection is easy to implement. This permits the set up of real-time, on-line methods for preventing biofilms with measures connected to dosage of different deposit control products and biocides. Table14 summarizes the individual methods' characteristics.

In addition to all these methods the possible use of a FTIR flow cell is also suggested, however, it cannot operate truly continuously and requires a FTIR spectrometer [Flemming, 1998], and the optical fouling monitor has been introduced to the paper industry, based on light measurements performed through plastic disc installed in a by-pass [Flemming, 2001]. Most of the methods described have to be used as by pass units.

Table 14: On-line measurement methods and their differences modified from Janknecht and Melo (2003).

	Reported detedtion limit	Industrial application	Biological activity	Physical structure	Heat resistance	Friction	Chemical composition	Biological composition	Thickness/ mass/density/ concentration
Differential turbidity (DTM)	0.1 mm	x							(x)
Fiber optic device (FOS)	10^5 cells cm^{-2}	x							(x)
Heat transfer	_	x			x				
Preassure drop	_	x				x			
Metabolic products	_	x	x					x	
Computerized image analysis	Individual cells			x				x	
Bioluminescence	_		x				x		
Fluorometry	_		x				x		
Spectroscopy	0.05 mm						x		
Attenuated total internal reflection (ATR-EW)	_						x		
Attenuated total internal reflection (ATR-SPR)	_								(x)
Nuclear magnetic resonance (NMR) spectroscopy	_			x			x		
Photoacustic spectroscopy (PAS)	10 m resolution			x			x		
Electrochemical techniques (MIC-electrodes)	_	x	x						
Impedance measurement (EIS)	0.2 mm	x							x
Capacitance measurements	108 cells cm-2								x
Vibration sensor in solid surface	3 x 10^5 cells cm^{-2}								x
Vibration sensor in liquid phase	_								x

3. AIMS OF THIS STUDY

To understand:

a) How to study paper/board machine deposits in an informative and realistic way, both as analytical tool and in real time.

b) The interactions that function as seed for the deposition in the paper/board industry.

c) The build up of deposit layers on the surfaces, and therefore the interactions between the inorganic, organic and microbial phases on individual paper machines.

d) How the microbial population of the paper machine changes, when the machine circuits close, and to explore this with cultivation dependent and independent methods.

e) And use this understanding for the design of rational and environmental sustainable strategies to minimize the chemically/microbiologically caused product deficiency and runnability problems in the paper/board machine circulations.

4. MATERIALS AND METHODS

4.1. Coupons immersed *in situ* and microscopic methods for deposit analysis

To monitor the tendency for deposit formation, on-site coupons were immersed in different positions in paper and board machines, *in situ*, not in a by-pass or in a side stream. After exposure, the coupons were taken out, fixed on site for SEM and subsequently analyzed by SEM-EDX using the methods described by Väisänen *et al* (1998), and by CLSM as described by Kolari *et al* (1998). Immersion times ranged from 1 hour to 14 days. Deposit components were analyzed for inorganic, organic and microbiological constituents. Stains used for epifluorescence or CLSM are ethidium bromide (EtBr; 40 mg/l; Sigma E1510, Sigma-Aldrich Finland Oy, Helsinki), Live/Dead stain, and FluoSpheres® (Molecular Probes Europe, Leiden The Netherlands) [Kolari *et al.* 2003]. Unbound stain was removed by repeated washing using municipal water.

Before use, the coupons were polished with waterproof silicon carbide paper, FEPA P1000 (by Struers, Espoo, Finland), washed with a detergent, and cleaned with acetone. Cleaned and polished steel coupons AISI 316L, 2 mm thick, round with a diameter of 15 mm were immersed in the paper or board machine at humid or submersed sites, and at sites known for deposit problems. Stainless steel thread was used to hang the coupons directly at the different sites, sometimes weighted to keep them submerged in turbulent chests. After the chosen time, usually after 1, 3 or 7 days, the coupons were removed and prepared for the analysis by SEM with EDX or CLSM.

4.2. Culture media

The culture media used were:
Tryptic soy agar (Becton, Dickinson and company, Sparks, MD, USA) for the generation of pure cultures. R2A for the enumeration of aerobic bacteria from water. This has been designed for bacteria originating from nutritionally poor conditions [Reasoner and Geldreich, 1985]. Chromocult® Coliform agar (Merck KGaA, Darmstadt, Germany) a selective agar for the simultaneous detection of total coliforms and *E.coli*. *Pseudomonas* CFC Selective Agar (Merck KGaA, Darmstadt, Germany) with 10 mg L^{-1} cetrimide, 10 mg L^{-1} fucidin and 50 mg L^{-1} cephalosporin, for the detection and enumeration of *Pseudomonas sp*. Sabouraud 4 % dextrose agar with 100 mg L^{-1} chloramphenicol and 100 mg L^{-1} tetracycline (Merck KGaA, Darmstadt, Germany), an acidic pH medium for the isolation of yeast and other fungi. Iron-Sulphite agar (Oxoid Ltd., Basingstoke, Hampshire, England), for the detection of thermophilic anaerobic organisms. Organisms reducing sulfite to sulfide are seen as black colonies.

The R2A plates were incubated at 45°C for 2-4 days and the other agar plates at 32°C for 1-4 days.

4.3. Organic content of the deposits

The organic content of deposits was characterized by using pyrolysis-gas chromatography/ mass spectrometry technique (Py-GC/MS). The resulting pyrogram shows the intensity of the pyrolysis products (total ion current, TIC) against the retention time. Two mass spectra (MS) per second were recorded, which enables an interpretation of the structural types of the products. Many polar products, especially acids, have difficulties passing the non-polar GC column. They were made more volatile by methylation directly on the pyrolysis foil using tetramethylammonium hydroxide (TMAH) as described by Hardell and Nilvebrant (1999).

4.4. Microscopic analysis of slimes

Light microscopy analysis was performed using a phase contrast microscope. The samples were colored using methylene blue stain and were photographed after analysis.

4.5. Sampling of waters and deposits from the mills

Water samples were taken out in sterile 50 mL bottles. Slimes/deposits were sampled as indicated in the respective papers I-IV from clarified water chest, white water chest, re-circulated bio-treated water, polymer tank, warm water tank, shower water tank and floor channels where water circulates. The samples for the DNA extractions were immediately frozen. For cultivation, the samples were sent per 24 hours delivery to the laboratory.

4.6. Chemical characterization of the 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, AOX, COD, 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 by means of duplicate samples. Deviations between the duplicates were $\leq$ 20%. The samples were filtered using Whatman® GF/A filters if indicated in the text.

4.7. Extraction and purification of DNA from slimes, deposits and process waters

The extractions were initially performed with phenol-chloroform, but then changed to the FastDNA® SPIN Kit for soil as this gave a 30% higher yield with the mill samples. The samples were incubated in FastPrep® Matrix E tubes (Qbiogene, Irvine CA, USA). For the standard extraction, a Lysis buffer with Proteinase K from MagNa® Pure LC

DNA Isolation Kit III Bacteria & Fungi (Roche diagnostics, Penzberg, Germany) was used [Ekman *et al.* 2007]. For the FastDNA® Spin Kit a special Sodium Phosphate Buffer and MT Buffer were used in the FastPrep® Instrument. The samples were then extracted according to the recommendations of the manufacturer. 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. Some modifications were done for water samples when using the FastDNA® SPIN Kit. When 1ml samples were used for extraction, the amount of sodium phosphate buffer was reduced from 978 µL to 450 µL, and the MT Buffer was increased from 122 µL to 125 µL.

4.8. Quantitative real-time PCR for the quantification of Bacteria and Archaea

The LightCycler Quantitative real-time PCR machine (Roche Diagnostic Penzberg, Germany) was used for the amplifications. The total reaction volume was 20 µl/ capillary, including 2 µl of template and 0.3 µM of each primer for Bacteria, and 0.4 µM of each primer for the Archaea. For the bacteria the primers pE and pF´ were used (product size 167 bp), and for Archaea primers 1369F and 1541R (product size 172 bp). In the reaction mix for both the Eubacteria and the Archaea 10 µl SYBR® Premix Ex Taq were added (Takara Bio Inc., Shiga, Japan) and to the Archaean analysis 0.4 µl BSA buffer (10 µg mL^{-1}). Temperature program used for the Eubacteria: 30 s at 95°C, 40 cycles of 5 s 94°C followed by annealing and extension 20 s 60°C [Ekman *et al.* 2007], and for the Archaea: 30 s at 95°C, 40 cycles of 5s at 95°C, and annealing and extension 10 s at 58°C. Melting curve analysis: from 65°- 95°C, 0.1°C s^{-1} temperature change. Fluorescence signal density was measured at the end of each amplification step continuously during melting curve analysis.

4.9. Preparation of DNA for cloning and sequencing

DNA extracted from the long circuit (clarified water) and from the solid and slime deposits were amplified using the Eppendorf Master cycler. For Bacteria the primer pair pA and pH´ [Ekman *et al.* 2007], and for Archaea primer pair 20F and 958R were used. Both primer pairs targeted conserved regions in the 16S rRNA gene of the respective domains. The reaction solution, a total volume of 50 µL, contained 2 µL template for the Bacteria, and 1 µL template for the Archaea. In both cases 5 µl of PCR buffer (with or without tenside), 1 µL of dNTP (equals 0.2 mM), and 1 µL of DyNAzyme II Taq polymerase (Finnzymes, Espoo, Finland).
MasterCycler (Eppendorf) program for Eubacteria: T=94.0°C for 10 min, 35 cycles of T=94.0°C for 1 min, T=55.0°C for 1 min, T=72.0°C for 1 min, and after that extension and annealing 10 min at T=72.0°C.
MasterCycler program for Archaea: T=94.0°C for 2.30 min, 27 cycles of T=94.0°C for 30 s, T=60.0°C for 1 min, T=72.0°C for 1 min and after that extension and annealing 10 min at T=72.0°C. After the amplification, the PCR products were checked with an agarose gel containing ethidium bromide and a DNA ladder for quality, before cloning and subsequent sequencing.

Molecular cloning and DNA sequencing was carried out with robotized instruments at the University of Helsinki, 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), inserts re-amplified with universal vector primers and purified with MultiScreen 384PCR-plates. Sequencing was done using the primer pD' [Edwards *et al.* 1989], BigDye Terminator cycle sequencing kit and ABI 3730 sequencer (Applied Biosystems, Foster City, CA, USA).

4.10. Computer analysis

The sequences were analyzed as described by Ekman *et al* (2007). The aligned sequences were analyzed by the Ribosomal Data base project II at http://rdp.cme.msu.edu/ using the Seqmatch, Classifier [Wang *et al.* 2007] and the Tree builder. Sequences $\geq$ 97% identical were considered to represent the same species [Suihko *et al.* 2004].

5. RESULTS AND DISCUSSION

5.1. New methods to create information on deposit formation in the paper industry

Many parameters affect the deposition in a machine circuit. Many have stated that the first layer is of importance for the further development of the biofilms and deposits [Schenker *et al.* 1998]. Steel is the most common material where deposition occurs in a paper/board machine circuit. On steel, the initiating passive film can change according to the environment [Olsson and Landolt, 2003]. As described under paragraph 2.3 (page 4) the paper making processes can look very different and it is therefore most likely that the mechanism of deposition differs from machine to machine. The deposits formed in the machine circuit are built from substances originating from aerosols and raw materials e. g. fresh water, wood supply, filler and wet-end chemicals.

A method to understand the mechanism of the formation of the initial and later build up of the deposits had to be developed. Any by-pass device was abandoned because the data achieved in the by-pass most likely differ from the real situation due to the variations that occur at a paper machine.

Following demands of the method had to be fulfilled:
- Flexibility, to be used in all positions at the machine
- Steel surface equal to the machine materials
- *in situ*, to be realistic
- First attachment analysis accessibility
- Matrix information of the mature deposit
- to deliver scientific data for future research work

The strategy chosen was based on deposits formed *in situ* on metal coupons placed at different sites in the paper or board machines, e.g. water chests, additive tanks, and spray water zones, basically anywhere where humidity or liquid were present. The coupons were taken out for analysis after 1 hour to 1 day to give the information on the first layer built, and coupons taken out after 1 to 14 days were used to analyze the subsequent layers. This method can also be used to understand the efficacy of different biocides on slime formation as well as other types of deposit control agents on organic deposit formations (Paper I, Figure 6 a-f, 7 a-b). A picture of the coupon can be found in Figure 8 in Paper I, which also includes details of the method. Results and implementations are shown and discussed in the following paragraphs.

The second strategy to collect more information was to develop an on-line, real-time monitoring system, for deposition on a steel surface as the basis for relevant decisions before any disturbances of the process occur.

Following demands of the real-time device had to be fulfilled:
- Flexibility, to be used in all positions at the machine
- Steel surface equal to the machine materials
- *in situ*, preferably built directly into the machine system

- Multi-parameter measurements, not only the thickness of the deposit
- Easy to handle and use

A high-grade acid resistance steel surface sensor was created that can be placed flat as part of the container wall so that the deposits on the measuring mark cannot be falsified by differences of the liquid flow or the sensor material. The sensor measures backscattered light, which triggers an electrical response from the diode array. The sensor itself is made with a small gap, for the outgoing and incoming light from a laser beam with a width of 50µm, covered by a transparent resin. Growing deposit layers have been analyzed to easily bridge this gap (coupons analysis, coupons made with resin gap, immersed in processing waters and analyzed as described above, data not shown). In this case by-pass device was built because the papermakers usually do not want to drill a hole in the chest walls before they can see the benefit of the measuring device. The device and measurement principles are described in Paper II, Figure 6 a-c and 7 a. The software package displays information on thickness, speed of growth and layer density

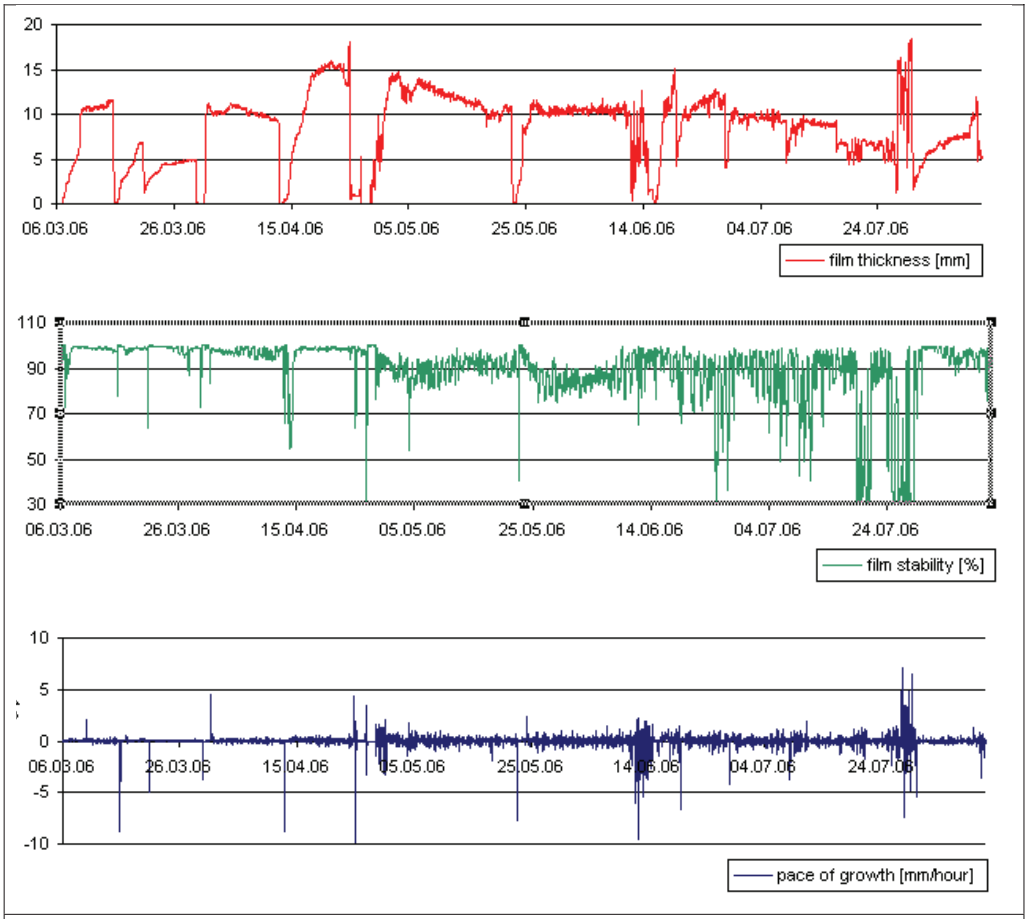


Figure 7: Signals from the on-line monitor placed in the shower water showing the differences in deposit structure when the machine treatment changes from an organic to an oxidizing biocide treatment system (from Paper II).

on a screen, directly in the control room or on an attached computer. Using the by-pass chamber, the unit can be easily included in the water system.

An example can be seen in Figure 7, where the microbiological treatment of the process water changed from a system based on the addition of organic biocides to a system based on oxidizing biocides (beginning of May). The results from the two different treatment programs can clearly be seen, where the oxidizing biocide system creates a deposit that is less stable, and much slimier. This is also demonstrated by complementary coupon analysis (Figure 9, Paper II). More examples of the possibilities to evaluate machine parameters with the on-line device are given in Paper II.

5.2. Studies of deposit formation in the paper industry

5.2.1. Deposit formation at different interfaces around the paper machine

The interfaces and the different conditions in and around a paper machine were described in Figure 4. Coupons were immersed in several paper machine systems at places representing the different interfaces, except liquid-liquid, which cannot be measured using coupons placed in the water circuits.

Representative deposits for each interface are presented in Figure 2a-f, Paper I. Coupons were placed in the process for the determination of the first layer attaching (1 day) and the following mechanisms of deposition (6-12 days). The analysis shows big differences in the build up mechanism between the different types of interfaces, except the liquid-liquid interface, which cannot be analyzed with coupons. As a result of these measurements further analyses were carefully planned, so that coupons were immersed in the right place to understand the deposition mechanism in the different positions around the paper machines.

5.2.2. First attachment, conditioning layer in the paper machine circuits

The first layer formed may vary from machine to machine due to different raw materials, additives and the process conditions.

Bacterial attachments

In Figure 8 a coupon immersed in the primary (white water) circuit in a board mill, fixed and analyzed by SEM-EDX is shown.

The first attachment morphologically looks like *Deinococcus* cells. The paper mill had runnability and quality problems, and used an oxidative, halogen-releasing system as deposit/slime treatment. The attachment mechanism of *Deinococcus geothermalis* is by adhesion threads between the microbial cells and between cells and the non living surfaces, as described by Saarimaa *et al* (2006), Peltola *et al* (2008), and Raulio *et al* (2008), (Figure 8). The adhesion molecules of *Deinococcus geothermalis* contain carbohydrates and have features connecting them with archaeal/eukaryal homologues, which could be explained by horizontal gene transfer [Olendzenski *et al.* 2000]. Kolari *et al* (2003) described in his study, that the warm paper machine temperatures offer a

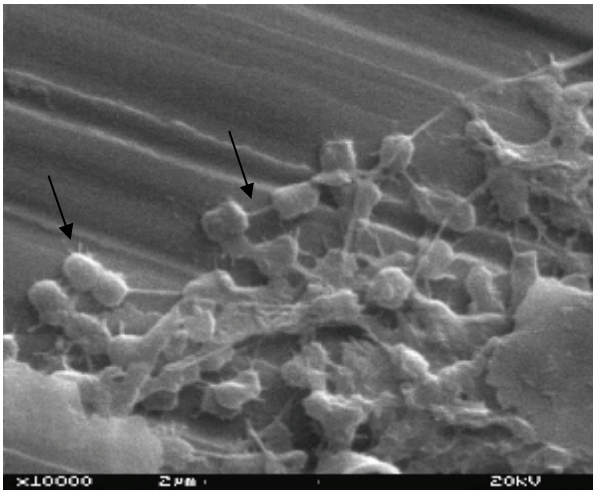


Figure 8: *Deinococcus* type bacteria on a coupon surface immersed in the white water channel for one day (board mill using recycled fiber) (SEM-micrograph, magnification x10 000) Arrows are pointing at attachment threads between cells and to the surface.

manmade ecological niche for many bacteria one would normally find in hot springs, like *Deinococcus geothermalis*. This is also suggested by studies done at a Canadian paper mill [Desjardins and Beaulieu, 2003].

Further pictures from bacteria acting as primary attacher can be found in Paper I, Figure 2 e, and 4 c, (rod shaped bacteria), and Paper II, Figure 2 a. However from the experiments performed in the paper and board mills around the world, the first attachment was only rarely reported to be bacterial, but more often to be organic.

Attachment of organic material in paper machine circulations

In Paper I, Figure 4e an example is shown of a coupon immersed for 1 day in the outlet of a dilution headbox (finepaper mill). There is nothing that morphologically resembles bacteria on the surface. No inorganic material was detected by EDX analysis*;* Py-GC/MS analysis showed that the deposits contained a high amount of the sizing aids AKD and ASA. It is known that when using AKD, the circulation temperature should not be close to the melting point of the AKD [Knubb and Zetter, 2002], because it can cause deposition in the wet end. The deposition ability of ASA is particle size dependent, and when the ASA emulsion contains overly large particles the tendency to form deposits increases [Koskela *et al.* 2003]. ASA and AKD sizing both have a high affinity to deposition in the system. With the help of the coupon analysis in this mill, it was understood that spots and breaks at the machine were caused by the sizing agents, and could be treated accordingly. In Paper II, Figure 1a-d SEM micrographs from another finepaper are shown demonstrating that the first attachment is facilitated by AKD sizing aid and the fillers (in this case PCC) actively take part in the deposition (mechanism explained in the next paragraph). Bacteria attach on top of the layer.

Figure 9: Organic layer deposited within one day on a coupon immersed in the white water channel in a paper mill using recycled fiber. A manual scratch (arrow) uncovered the clean steel surface (SEM-micrograph, magnification x5000).

In Figure 9, a case is shown where the first attachment is a thin organic layer very often found in all types of paper mills. Whenever the organic layer can be analyzed it often contains different calcium soaps of organic substances circulating in the systems (data not shown). This mill did not use any sizing agents.

Attachment of inorganic material in the paper machine circulations

Figure 10 shows an example of a coupon immersed in the white water channel in a paper mill where Alum is used as a fixative at a pH of 6.8.

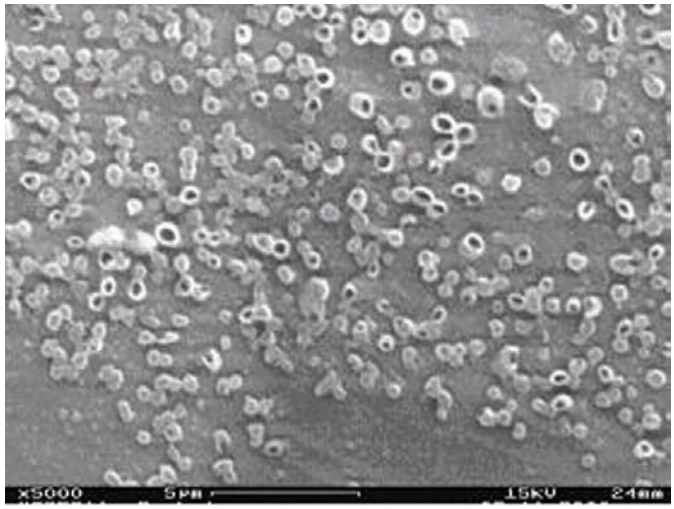


Figure 10: Inorganic material attaching to the coupon surface after 1 day of immersion in the white water channel in a board mill using recycled fiber (SEM-micrograph, magnification x5000).

An EDX analysis of the very hard layer is shown in Paper I Figure 4b.It contained mainly aluminum and oxygen. It was most likely aluminum hydroxide, which is likely to form at this pH (the aluminum chemistry at different pH values is described in Figure 11). It is not very common but may occur more and more as the mills close their water circuits. Alum is a very good coagulant/fixative for colloidal material, but has its best effect at acid pH. At neutral pH crystallization occurs, that can deposit on surfaces [Eklund and Lindström, 1990; Biermann, 1993].

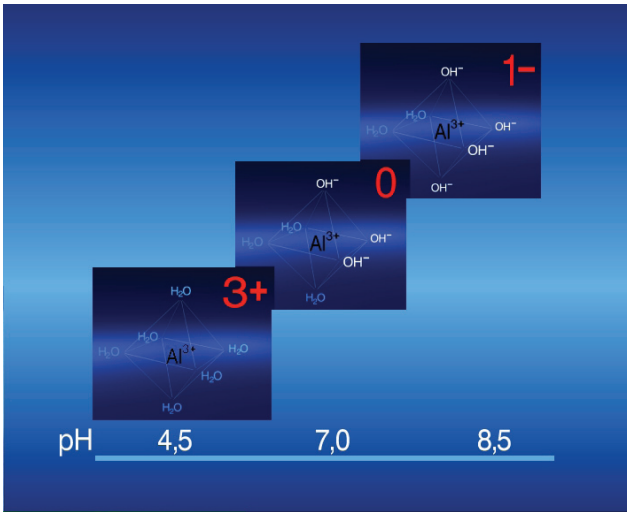


Figure 11: Chemical orientations of aluminum at different pH, showing that Alum creates aluminum hydroxide at pH 7 [adopted from Persson, 2004]

Inorganic ions (e.g. calcium) are also heavily involved in the increased deposition of pitch, which cause it to destabilize and form agglomerates and deposits [Otero *et al.* 2000]. In Table 9 (page 17), several of the inorganic substances that can take part in deposition in the paper industry are listed.

Figure 12 a and b shows an example where calcium sulfate crystals have formed on the coupons surface immersed in white water in a finepaper mill for 9 days (Figure 12 a, yellow arrows). PCC was added as filler to the process and can clearly be seen to due to its most common crystal shape from the SEM-micrograph (Figure 12 a, red arrow). This finepaper mill only discharged 4 m^3 water per ton paper produced, which increase the ion concentration in the system (see paragraph 5.3, page 41), which could be the reason for the massive crystal formation [Merta, 2001].

5.2.3. Bulk of deposits and interactions with EPS material

A typical mixed deposit containing exopolymeric substances (EPS), organic and inorganic material is shown in Figure 3 a, Paper I. The coupons were immersed for 6 days. This example represents a typical view of a mature deposit in the circuits of the paper industry. All different parts, inorganic as well as organic, are involved in forming the matrix, just like Sanborn explained already in 1965.

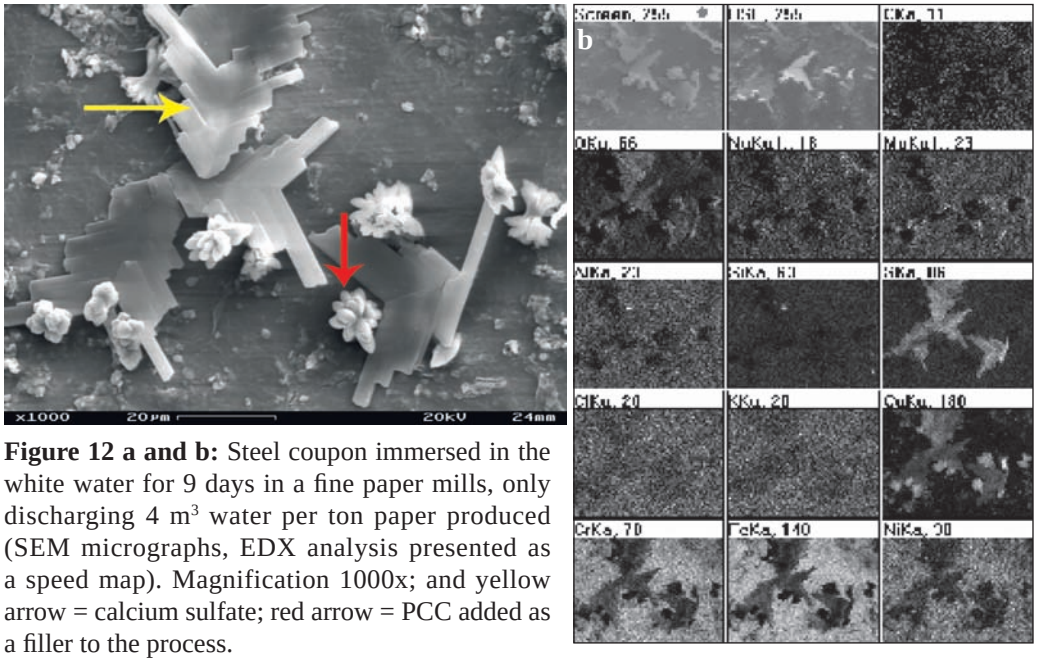


Figure 12 a and b: Steel coupon immersed in the white water for 9 days in a fine paper mills, only discharging 4 m^3 water per ton paper produced (SEM micrographs, EDX analysis presented as a speed map). Magnification 1000x; and yellow arrow = calcium sulfate; red arrow = PCC added as a filler to the process.

Very slimy deposits like in Paper I, Figure 3 b, are rarely found. It appears that the organic and inorganic materials in the deposits are entrapped in the EPS matrix.

Figure 13 a-b shows a coupon covered with bacterial deposits, containing almost no EPS material. The colony resembles those of *Deinococcus* spp [Peltola *et al.* 2008]. *Deinococcus* is known as a genus forming what a papermaker calls "pink slime" [Kolari *et al.* 2002]. The ultra structural analysis done by Raulio *et al* (2006), and Peltola *et al* (2008), gave no indication for the involvement of *Deinococcus* in the production of EPS material, but the colors of the colonies are pink. They are known to be extremely

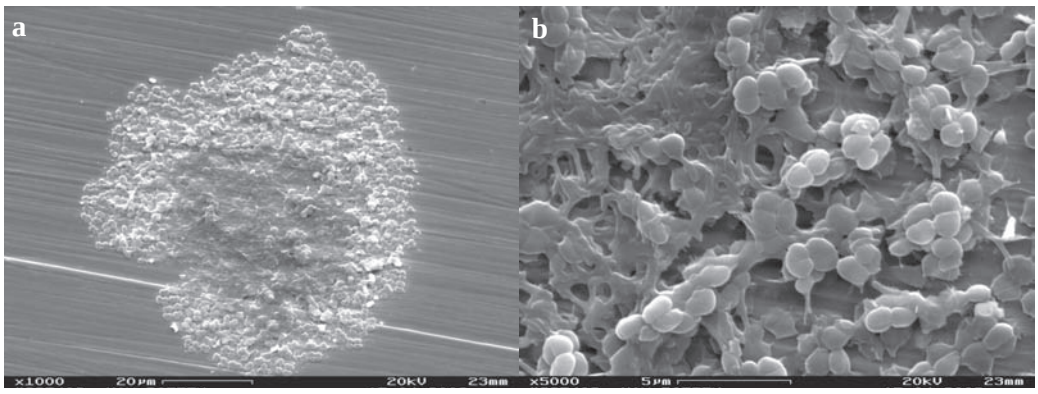


Figure 13 a and b: Bacterial deposit, 8 days old on a coupon immersed in the white water channel in a paper mill using recycled fibers (two different magnifications x1000, and x5000) (SEM-micrograph).

tolerant against stress [Makarova *et al.* 2007] and their protection mechanism seems to be protection of proteins against oxidation due to high levels of manganese [Potera, 2007]. This may explain the tolerance against the oxidative halogen releasing treatment program running in this mill where this bacterial deposit was found, and why it seems to be a monoculture. The steel surface looks very clean after 8 days, except for the bacterial cluster. In paper IV the occurrence of this type of bacteria in the slime deposits of different paper machines was analyzed to be about 15 %. They might have been underestimated due to the sampling of slime deposits when due to the firm attachment of *Deinococcus* as explained by Kolari *et al* (2002).

Another example of a *Deinococcus*-like bacteria deposition can be found in Paper IV, Figure 3 A-B.

Examples of bulk deposit which are not caused by microorganisms can be found in Paper II, Figure 10, and 11 a-c where thick deposits of resin compounds are shown. The speed map 11b clearly shows the presence of calcium carbonate, and the shape of the particle reveals that it is PCC. The organic content of the deposit was analyzed by Py-GCMS and was showed to be extractable material (resin acids and fatty acids) from mechanical pulp, see Figure 14. In addition, fiber analysis was made with light microscopy. The results showed that the majority of the fiber and fiber parts in the deposit came from TMP (spruce) (data not shown).

This shows that the deposit consisted of a resin, where PCC served as a collector added to the mechanical pulp. It is known that PCC has a high affinity to sizing agents [Petander *et al.* 1998], but it is also introduced to the industry as a pitch (wood resin) collector in the paper industry (information received from a paper mill). In this case it could be proven that the reason for the deposit problems at the machine was organic material (wood resin) coagulated by PCC.

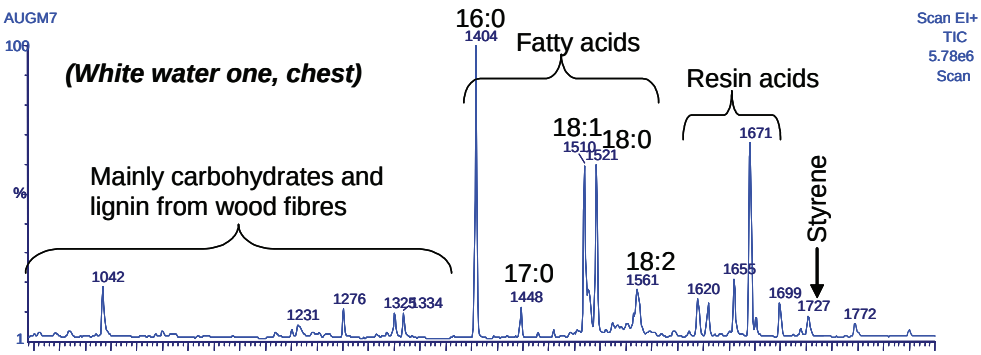


Figure 14: Py-GCMS analyze to determine the organic content of the deposits found in the deposits on a paper machine producing LWC paper (wood containing paper, coated). Deposit can be seen in Paper II, Figure 10 and 11 a-c.

Figure 15 shows a sizing (ASA/AKD) deposit after 6 days falsely interpreted to be a biofilm deposit, and treated with different types of biocides. The presence of sizing agents was determined by Py-GCMS (data not shown).

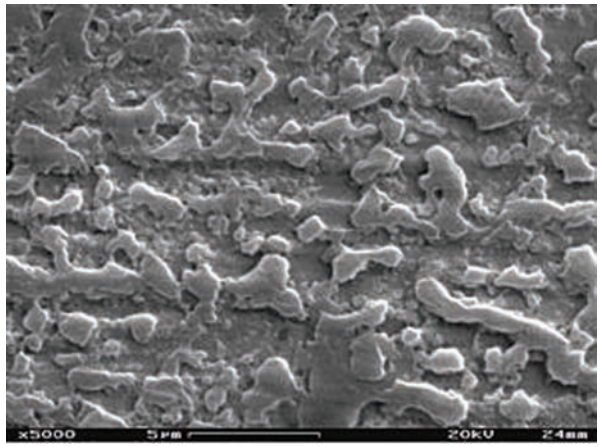


Figure 15: ASA/AKD deposit from a coupon immersed 6 days in the outlet of a dilution headbox in a finepaper mill (bleached softwood/hardwood chemical pulp; (SEM micrograph, magnification x5000).

5.2.4. Influence of raw water quality

Raw water as a contamination source has been mentioned in several studies [Paper I; Väisänen *et al.* 1998; Kanto *et al.* 2001; Blanco *et al.* 2004]; however, it has not been considered to be a major source of contamination. The general view is that the small amount of microbes in the raw water, compared to the amount in the circulation waters, cannot have an impact on the machine runnability [Simons *et al.* 2003; Flemming *et al.* 2004], because the total aerobic plate counts in the raw water are much lower than in the process waters. Process waters contain between 10^6 and 10^8 CFU g^{-1} or mL^{-1} [Suihko *et al.* 2004; Väisänen *et al.* 1998]. Figure 16 shows measurements done over two years fo the CFU in the raw water in a board mill in north of Europe. It can clearly be seen that the counts vary considerably through out the years. Raw water and process water of this board mill was also studied by coupon analysis (Figure 4 a-c, Paper II).

Coupon analysis as a complement to cultivation increased the understanding of the influence of the water quality. The warm water (Paper II, Figure 4 b-c) was used in the high-pressure showers for cleaning of the press felts. Paper II, Figure 5 a-b shows how the raw water can contaminate additives used at the machines. This case shows how a dilution tank with retention polymer can be contaminated by a high load of fungi. The fungus was very difficult to analyze by normal cultivations, but with the help of microscopy and coupon methods, the problem could be indicated and solved. The source of the contamination was the water used as dilution water for the retention polymer. Alternative methods for the analysis of the water systems are necessary, which is also suggested by Lahtinen *et al.* (2006). Several severe problems are coming from insufficient treatment, which shows the importance of the topic.

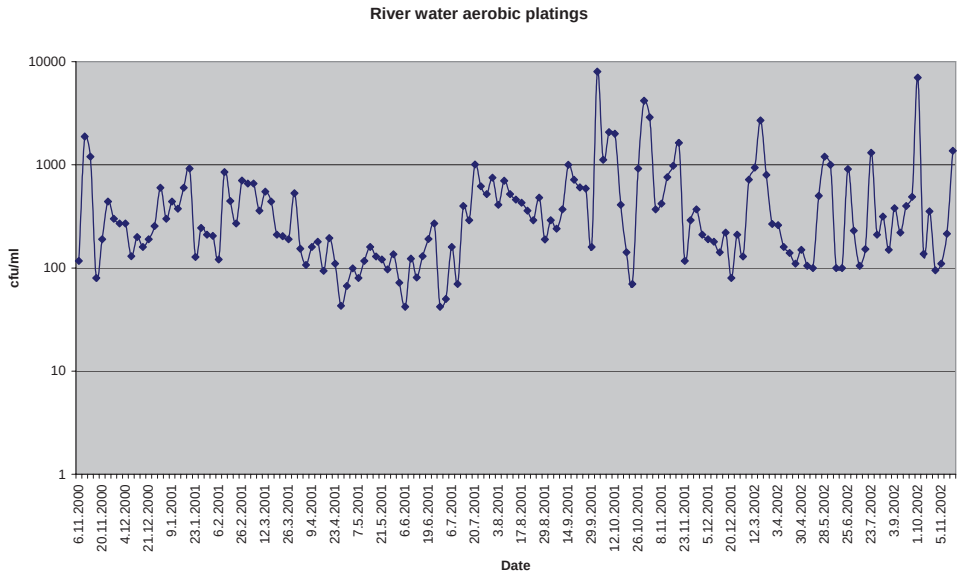


Figure 16: Total aerobic plate count of the raw water (surface water) of a board mill over 2 years. The cultivation was done on Plate count agar (Merck KGaA, Darmstadt, Germany) and the plates were incubated at 37 ºC for three days.

When insufficiently treated water from the tertiary circuit is taken back into the process to save water, it can create severe contamination and thereby production problems. Figure 17 shows an example of water returning from the microbiological treatment step (tertiary circuit). Cultivation of the water showed < 1000 CFU mL^{-1}. The coupons placed in the waters for 9 days contained about 30 filamentous bacteria and 300 rod shaped bacteria per area unit (50 µm x 50 µm = 0,0025 mm^2). That equals 12 000 filamentous bacteria and about 120 000 rod shaped bacteria per mm^2. Most likely only parts of the microbial content could form colonies by cultivation.

Because the water from the tertiary circus was taken back to the process, the filamentous bacteria were found forming complex deposit with fibers in the machine circuit together with fines and fillers (Figure 18). This shows the importance of correct treatment of tertiary circuit water taken back into the process and sufficient methods for the analysis to prevent problems.

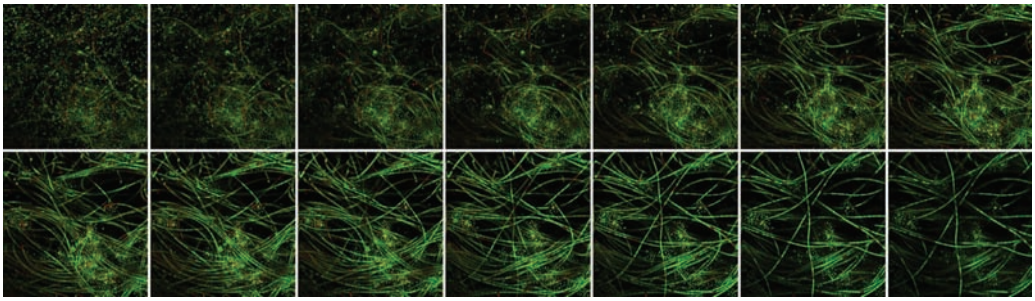


Figure 17: Analysis of the deposit formation in the tertiary circuit in a board mill. Coupon placed in the bio-water reused in the mill for 9 days, analyzed by CLSM 400 time magnification (live/dead stain). Green fluorescent = living cells, red fluorescent = dead cells. Step size in z-direction was 0.54 µm, and thickness of the deposit was ca 76 µm. All pictures are not displayed.

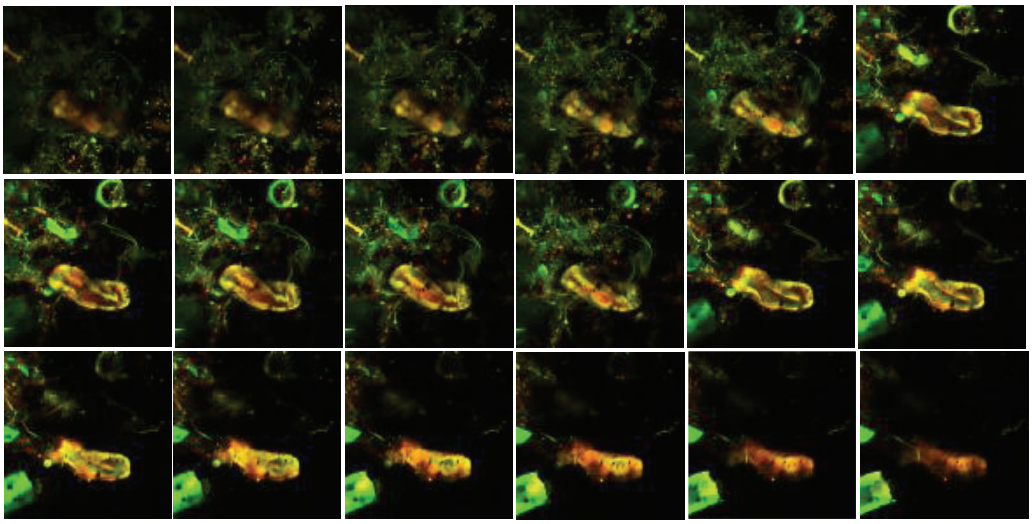


Figure 18: Analysis of the deposit formation in the white water circuit in a board mill with tertiary circuit. Coupon placed in the white water channel for 7 days, analyzed by CLSM 400 time magnification (live/dead stain). Green fluorescent = living cells, red fluorescent = dead cells. Step size in z-direction was 1.08 µm, and the thickness of the deposit was ca 20 µm.

Flemming *et al* (2004) wrote that several of the filamentous bacteria isolated from a paper mill survived 30 minutes at high levels of free halogen (0.8-1.6 ppm), which has to be considered when treating these types of bacteria. *Sphingomonas sp*., *Cytophagales, Bacillus macroides*, and *Methylobacterium mesophilicum* were found to be contaminating species in fresh water by the 16S rRNA gene analysis [Flemming *et al*. 2004]. Similar results were obtained by Oppong *et al* (2003), by 16S rRNA gene analysis of filamentous bacteria from mill slimes, although some additional species have been indicated like *Paenibacillus sp, Flavobacterium columnare, Norcardiopsis alba* and *Streptomyces albidoflavus*, however, sometimes with a similarity of less than 96%. The result of the analysis could be misleading because the colonies were isolated from agar plates. Ramothokang and Drysdale (2003) wrote about the difficulties in growing filamentous bacteria on nutrient media.

5.3. Effect of closing water circuits on the microbiology of paper/board mills

5.3.1. Characterization of the recycling waters of closed cycle paper mills

A paper/board mill with a closed water circuit (MHS), and a paper/board mill (MSS) discharging about 4 m^3 of water per ton of produced product were chemically characterized and the analyses were done on the clarified water and the white water of the two paper/board mills, which can be found in Table 15. Compared to the MSS mill the water of the MHS mill was more acidic 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 was composed of volatile acids (lactic, acetic, propionic and butyric acids). The content of TOC, the contents of the anion sulfate, the cations sodium, potassium, magnesium, iron, calcium, and organic acids (lactic, formic, butyric) were

Table 15: Characterization of the primary (white water) and the secondary (clarified water) circuit waters of two closed cycle paper/board mills.

Mill	**MHS**		**MSS**	
	Crude samples			
Analyzed item	**Clarified water**	**White water**	**Clarified water**	**White water**
pH	5.9	5.6	7.2	7
T° C	54	52	42	46
Conductivity (mS cm^{-1})	40,4		8.5	
TOC (mg L^{-1})	10370	10025	1040	790
AOX (mg L^{-1})	7.85	6.9	8.5	5.6
Samples filtered through 1.6 µm pore size filter				
TOC (mg L^{-1})	10080	10325	920	540
AOX (mg L^{-1})	5.3	3.9	6.6	5.1
Chloride (mg L^{-1})	1075	1106	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})	1420	1504	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})	1205	1220	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}	2510	2627	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 P L^{-1})	<0.5	<0.5	2.2	1.5
Lactic acid (mg L^{-1})	6760	6583	220	190
Formic acid (mg L^{-1})	330	258	<10	25
Acetic acid (mg L^{-1})	2775	3190	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

tenfold or higher in the waters of the MHS mill than in those of the MSS mill. 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). The MHS mill waters thus represent a more extreme environment than the water from the MSS mill. Geller (1984) published differences in VFA´s in recycled paper processes discharging 0 m^3 and 20 m^3 per ton paper/board produced (Table 1). A discharge of 20 m^3 can be considered to be an open mill. The values of Geller (1984) for the 0 m^3 discharge mill showed approximately the same amount of acids responsible for the rancid smell of the paper products (butyric and propionic acid) as well as the amount of acetic acid compared to the MHS mill. There is, however, a big difference in the amount of lactic acid, where Geller (1984) claimed that the open mill contained double the amount of the closed one. None of the lactic acid values reached the values analyzed in the circulation waters of the MHS mill. Explanations could be differences in process temperature, very big storage towers for the pulp in the open paper mill, or other differences in the process parameters. There is unfortunately no information about the mills included in the paper written by Gellar (1984).

In Table 16 additional data are given from measurements in a paper/board mill discharging <0.5 m^3 per ton product produced that had installed what Hamm and Schabel (2007) called a kidney, a tertiary circuit. This mill is from now on called MRO.

The amount of acids in the white water of the MRO mill is clearly lower than in the MHS mill, which can not be explained by the small amount of water discharged from the former. The result shows the benefits of reusing bio-water from the tertiary circuit. The values are in the same range as seen in Table 2 of a mill with the tertiary circuit installed, except for the acetic acid, which is much higher in the measurements described by Hamm and Schabel (2007). However, the measurement method was not described in the article, which makes it impossible to explain the differences tot he MRO mill. Lactic acid was also not determined by Hamm and Schabel (2007).

5.3.2. Microbiological comparison of waters and deposits in closed paper mills

The clarified and white waters of the mills MHS and MSS were analyzed for cultivatable bacteria and fungi (Paper III, Figures 1 and 2). The densities of bacteria capable of anaerobic growth were high (10^7-10^8 CFU mL^{-1}) in the circulation waters, which were also found by Jung and Kutzner (1978) in their study of a closed paper circuit. Studies done on open paper mills reveals contamination levels of bacteria capable of anaerobic growth seldom exceeding 10^6 CFU mL^{-1} (data not shown). The MSS mill waters contained a high density of H_2S producing anaerobic bacteria. The ratio of anaerobic colony counts compared to aerobic was high in the waters of the MHS mill. The absence of H_2S producing bacteria has been explained in another closed circuit by Geller (1984), where he concluded that, sulfate reducing bacteria did not dominate in the circulation waters. He also wrote that the paper mills with the highest H_2S problems are those bleaching with dithionite (reductive bleach). The prevalence of anaerobic growth and the high numbers of H_2S producers may explain the odor problems in the MSS. The MSS mill waters contained about tenfold more coliform bacteria and H_2S producers than those

of the MHS mill. This could be due to a more efficient survival of coliform bacteria originating from the raw material (= recycled paper) or that coliforms propagated under the anaerobic conditions and the lower temperature of mill MSS (Table 15). Anaerobic conditions exist in the mills due to the big water volumes that do not get aerated more than at the paper/board machine, and the low amount of fresh water used. The high amount of the VFA in the circulations also indicates growth of anaerobic bacteria.

DNA was obtained from samples taken from mills MSS and MHS and analyzed using16S rRNA targeted primers by q-PCR. The numerical results for eubacteria matched closely with those of the cultivatable bacteria in the white water. This must mean that a major portion of the detected ribosomal gene copies originated from cultivable bacteria. In the present study we found that the numbers of 16S rRNA gene copies in the recycling waters of both closed cycle mills were close to the numbers obtained by cultivation, when the presence of 2 to 10 gene copies assumed per cultivatable bacterial cell. A significant part of the16S rRNA genes thus represented viable bacteria.

Table 16: Characterization of the primary (white water) and the tertiary (Bio-water) circuit waters of a closed cycle paper/board mill.

Mill	**MRO**	
Crude samples		
Analyzed item	**White water**	**Bio- water**
pH	6.4	7.5
T° C	45	
COD (mg L^{-1})	9890	318
Samples filtered through 1.6 µm pore size filter		
COD (mg L^{-1})	7832	287
Chloride (mg L^{-1})	139	209
Nitrate (mg L^{-1})	<1	<1
Sulfate (mg L^{-1})	304	151
Phosphate (mg L^{-1})	<1	<1
Sodium (mg L^{-1})	378	424
Ammonium (mg L^{-1})	<1	24.4
Potassium (mg L^{-1})	26.5	23.9
Magnesium (mg L^{-1})	32.2	38.5
Calcium (mg L^{-1})	781	340
Iron (mg L^{-1})	1.8	0.3
Lactic acid (mg L^{-1})	2700	<1
Formic acid (mg L^{-1})	8	<1
Acetic acid (mg L^{-1})	670	<1
Propionic acid (mg L^{-1})	240	<1
Iso-butyric acid (mg L^{-1})	19	<10
Butyric acid (mg L^{-1})	85	<1

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 to 10^9 gene copies g^{-1}. The clarified waters from these mills showed only traces ($< 1.0 \times 10^4$ mL^{-1}) of archaean DNA. It thus seems that an archaeal population had accumulated at sites where there was little moving water, like in the deposits analyzed for these two mills.

The diversity of prokaryotes in the mill was analyzed samples by cloning and sequencing of 16S rRNA genes DNA. Waters from the clarifier, not influenced by 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 MHS. Twelve were unique and interpretable and some were closely related. A total of 114 eubacterial clones of the16S rRNA genes were retrieved from the MSS mill 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 of the archaeal clones 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, despite of several attempts with products obtained using different PCR conditions.

The bacterial sequences obtained from the mill MHS DNA grouped into four branches (Paper III, Figure 3,). The branch C contained sequences resembling those reported from anaerobic reactors for brewery sewage treatments. Branch D contained sequences with a distant similarity ($\leq$ 96%) to *Enterococcus cecorum*, with narrow diversity. Branch B was closest related but yet distant ($\leq$ 93% similar) to *Clostridia* known to produce short organic acids. The fourth branch (A) had closest resemblance to a bacterial clone B9 (AY426453) found in a full-scale anaerobic bioreactor treating paper mill wastewater [Roest *et al.* 2005]. Sequencing of the mill MHS bacterial clones revealed that most of the sequences belonged to either uncultured or unknown species. This might reflect the extremophilic conditions prevailing in this machine environment.

After multiple cloning efforts 43 of the obtained clones were retrieved from the MSS mill clarified water. According to the tree analysis (Paper III, Figure 4) the sequences were assigned to four branches, where one group of 29 clones was found to be $\geq$ 99 % similar to *B. coagulans* ATCC 7050 and was displayed as one sequence. Five sequences were <97% similar to *B. coagulans* but yet closest to this species. The branch G represented another *Bacillus* group, similar to *Bacillus thermoamylovorans*. This species is known to utilize starch at high temperatures [Combet-Blanc *et al*. 1995]. Interesting is also that the mill MSS contained unknown bacteria closest but distantly related to *Enterococcus cecorum*. The mill MSS encountered no odor problems in the product but sometimes had problems with “rotten egg” odor that could have been explained by the H_2S producers in the water cycles. Absence of the odor problems in the product may be due to the high process pH and the lower amount of organic acid in the MSS circulation waters compared to the MHS circulation waters.

Traditional paper mills discharging 10-20 m^3 of effluent per ton paper produced are known to have a high microbiological diversity when studied with culture independent

methods [Lahtinen *et al.* 2006], or with cultivation methods [Väisänen *et al.* 1998]. Lactic acid and lactates (pKa 3.79), propionic acid and propionates (pKa 4.87) are synergistically active in inhibiting growth of bacteria (including gram-positive cocci, *Clostridia, Enterobacteriace, Pseudomonas*) moulds and yeasts at concentrations of 0.1% to 5%, being most effective at acid pH (< 6) [Davidson and Taylor, 2007]. The high content of these organic acids, low inorganic P and N content may explain the low diversity of bacteria. This might explain why, in spite of the high contents of anaerobic and aerobic bacteria, a low diversity was indicated by sequencing of the samples. The only taxon that were obtained as multiple clones from the MHS mill were closest but still distantly related to *Enterococcus cecorum*, about 95-96% similar [Suihko *et al.* 2004]. *Enterococci* are known as lactic acid resistant, good growing in CO_2 rich environment, including fermented foods, animal gut [Fortina *et al.* 2004; Butaye *et al.* 1998;; Benz *et al.* 1998], and reported common in sewage plants of paper mills in Canada [Gauthier and Archibald, 2001]. *Enterococci* can degrade humic acid under formation of lactates [Benz *et al.* 1998]. Only one sequence of *Clostridium spp*. was found, although it was assumed to have been connected to the organoleptic problems in the end product [Maukonen *et al.* 2004]. The < 95% sequence match and the moderate phylogenetic distance to the described species of *Clostridium* indicate a potential new genus [Suihko *et al.* 2004]. One earlier study [Jung and Kutzner, 1978] investigated the different groups in a closed paper mill and determined the dominating group belong to *Bifidobacterium*, by phenotypic properties. Could the *Enterococcus cecorum* like taxon identified by cultivation independent methods be identical with the described *Bifidobacterium* type?

The 4 m^3 effluent mill, MSS, yielded not only *Enterococcus cecorum* like sequences but also *Bacillus coagulans* and *Bacillus thermoamylovorans* upon cloning. These species indicate the presence of thermophilic starch degrading populations capable of creating lactate [Combet-Blanc *et al.* 1995 and 1999]; like has been reported for mills discharging more water per ton produced paper/board that the MSS mill [Desjardins and Beaulieu, 2003; Väisänen *et al.* 1998]. Another finding previously described from paper mill recycling water was the presence of a sequence similar to *Sphingomonas sp*. [Desjardins and Beaulieu, 2003; Lahtinen *et al.* 2006; Väisänen *et al.* 1998].

In contrast to the mills included in this study, Lahtinen *et al.* (2006) found a high diversity of bacteria in open cycle mills. The bacteria in this study were predominantly gram-positive, whereas predominantly gram-negative in the study of Lahtinen *et al*. (2006). No *Actinobacteria* were found in contrast to results from recycle paper and board mills presented by Suihko *et al.* (2005).

Lahtinen *et al* (2006) also described the difference of results obtained by cultivation and by modern technologies (like DNA extraction with LH-PCR analysis, cloning and sequencing), and showed that it is difficult or even not possible to grow many cultures of the bacterial populations occurring in the biofilms. New types of bacteria like *Rubellimicrobium thermophilum* that are continuously found in the paper mills indicate that we have a long way to go before we understand all the Microbially caused variations in the paper process waters and deposits in the paper industry [Denner *et al.* 2006].

6. CONCLUSIONS

1. Steel coupons placed in situ analyzed with different microscopic methods was best suited for the evaluation of interactions between all components in the paper/board machine circulation leading to the formation of deposits, including the function of different treatment programs.

2. An on-line device that can be built directly into the process enables us to gain reliable data and make decisions about countermeasures before disturbances occur. The monitoring system was based on the measurement of backscattered radiation caused by the biofilm/deposit attaching to the stainless steel surface of the sensor.

3. The first layer of deposits formed in paper machine systems varies from machine to machine, and should be determined before any treatment decisions is made. This requirement is not industry specific and is also important for several different research areas as shown for the attachment of *Staphylococcus epidermidis* [MacKintosh *et al.* 2006] where the "conditioning film" is of great importance.

4. Using steel coupons *Deinococcus* like bacteria are confirmed *in situ* to be the primary attacher in paper machines in many cases where biological or mixed deposits are formed..

5. *Deinococcus* like bacteria are also found in the presence of oxidizing treatment systems, possibly explained by the protein protection mechanism described by Potera *et al.* (2007).

6. Bacterial EPS material in combination with substances in the circulation waters creates the matrix of the deposit.

7. Organic material is often found to be the conditioning layer of deposits in the paper/board machine circuits. Among these we find the sizing agent, pitch deposit, and other hydrophobic agents i.e. originating from recycled paper. Due to the pH level and amount of calcium circulating in the system, they deposit often in form of calcium soaps.

8. Sizing agents can build thick chemical deposits, which could be mistaken as microbiological deposits.

9. Different inorganic compounds can act as a conditioning layer creating a good base for bacterial attachment. One example is the use of Alum as fixative, which was used at almost all paper machines in the past when they were running at acidic pH. It is sometimes still used, even at neutral pH, but the risk of deposition of aluminum hydroxide is high.

10. PCC with its affinity for sizing agents often takes an actively part in coagulating hydrophobic particles, which in turn increases the affinity to the surfaces in the machine systems.

11. The treatment of the raw water, including circulated bio-water, has to be properly done to prevent runnability and quality problems. It has to be focus on the analytical methods to indicate bacteria capable of biofilm formation.

12. Closure of the water circuits can lead to increasing growth of extremophilic bacteria. The closure from 4 m^3 to 0 m^3 water discharge increases the levels of salts, organic carbons and organic acids up to tenfold or more, which can be decreased when a tertiary circuit is installed.

13. In a closed machine system, the number of gram-positive bacteria dominates over the number of gram-negative bacteria.

14. Archaea have been detected for the first time in paper machine environments. They occur in deposits where water flow is low. The extreme environment in the paper machine circulations most likely supports archaeal growth.

15. In contrast to cultivation methods, cultivation independent methods are a useful tool to determine the bacteria in paper machine circuits, and to understand the mechanism of deposit formation.

7. OUTLOOK

Future studies should concentrate further on the mechanism of the deposit formation at paper machines in combination with the developments of deposit control agents, to meet the industrial demands of efficiency, as well as environmental sustainability. This might also include new treatment (e.g. diamond-like carbon, fluoropolymers) methods of the steel to prevent or influence the attachment mechanism of the microbes and other substances as described by Raulio *et al* (2008); Kallio and Kekkonen (2005). The understanding and implementation of such studies would lead to less toxic antifouling products, as well as individually designed more cost efficient programs for each paper machine in the future.

It is important to carry out direct measurement in the system (Paper I). Developments of an online monitor that can determine the different components in a deposit will continue.

Further studies with 16S rRNA gene analysis to confirm the genus of the *Deinococcus* like bacteria found on the coupon surfaces should be done. For even further understanding of its role in the deposit and biofilm formation in the paper industry (Paper I, II and Paper IV).

It would be of great interest to use other modern methods to analyze further samples from closed mills to determine if the diversity is as low in these processes as it seems. Chaudhary *et al.* (1997) wrote, “Every paper mill is unique as far as the presence of different microorganisms is concerned.” and “Thus it becomes imperative to understand the problem of a particular mill and recommend individual control strategies accordingly.”, i.e. by detecting the microorganisms and investigating their specific action and behavior.

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