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MICROBIOLOGICAL CONTROL OF PIGMENTS AND FILLERS IN PAPER INDUSTRY

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ABSTRACT

Microbial contaminants will decrease the quality of pigments and fillers in paper and board industry. Microbe-caused spoilage of these raw materials as well as indirect deteriorative effects of these spoiled additives on the machine housekeeping, production and hygienic properties of paper products are well-known problems at the mills.

The traditional analysis of colony forming units helps both the manufacturer of raw material and his client to understand, what are the major contaminants and their densities in the raw material studied. These methods, unfortunately, are unsatisfying when rapid microbiological analyses are needed in urgent situations (trouble shooting, prevention of process contamination). Their shortage as indicators of the effects of biocides on the activity of microbes is also evident. Three "rapid methods" were regarded as promising

alternatives of colony count analyses in the control of paper and board machine's processes: detection of microbial growth by impedance method or turbidity test and measurement of biomass by luminometric ATP assay. When tested for mineral additive control, difficulties were arisen in every case: inhibitive effect of sedimentation of the sample in direct impedance measurement, poor interpretation of slow growth responses in indirect impedance method and the adsorptive effects of turbidity on the light emission (ATP assay) or on the background absorbance (turbidometry). For these reasons, strong dilutions of samples were needed and only high microbial densities (over 1 000 000 CFU/ml) were practically measurable by these methods. Integration of total impedance change may help to estimate low microbial densities by impedance methods, and more effective extraction and fractioning of microbial ATP may increase the sensitivity of ATP assay. Turbidometry seems to be a rather finished method, but its weak point is the long incubation period needed. Some efforts to develop all methods for the control of mineral additives seems thus to be necessary.

INTRODUCTION

The main mineral additives used as pigments and fillers in paper industry are clays and carbonates. Kaolines are clay products of mining industry and thus carry varying densities of bacteria and fungi from the earth. Carbonates may be mined, but the calcium carbonate produced chemically by precipitation will be relatively microbe-free.

The rapid growth of the alkaline paper and board making has been widely reported in the middle of last decade. Benefits of the alkaline sizing papermaking process compared to the traditional alum-rosin process have been claimed to be reduction of the costs of the products, lowering of the energy requirements, improvement of the brightness, stabilisation of the chemical and physical phenomena in the headbox, decrease of the paper degradation (due to the greater resistance of

fibers to oxidation), lowering of the water consumption and effluent treatment cost as well as improvement of the elastic properties of cellulose fibers giving the possibility to improve the paper strength (1, 2).

Disadvantages of alkaline paper production have also been noticed very soon: dirt and stickiness at various points along the paper machine, wet press picking, decreased paper opacity in some cases, abrasion of the plastic wire and dusting of the product during printing (2). A compromising alternative of the alkaline and acidic processes, the neutral or "pseudo-alkaline" process, has been applied to diminish the negative effects of pure alkaline process (2, 3). Still, criticism against the over-optimistic expectations of alkaline paper makers have been published: retention of pigments has not been very effective, synthetic sizes have reacted in a wrong way and in a wrong place, mixing of white waters having different pH values as well as mixing of alkaline waters with alum-containing ones have produced deposits etc. (4).

The progress towards higher alkalinity in paper and board manufacture processes has favoured the growth of bacteria in the processes because of decreased temperature, increased load of mineral fractions and more optimal pH range of the environment (1, 4, 5). The microbiological quality of mineral fillers like ground calcium carbonate, titanium dioxide and clays may be severely affected for various reasons and needs a control system of its own (5).

Rather few studies have been done to evaluate the effects of microbial growth and metabolism of unclean filler minerals on the process concerned, though the microbial densities and their effects on the value of these products is quite well-known (6).

Only slight increase in viscosity and no effects on the brightness of kaolin due to the high densities of bacteria (up to 10^7 CFU/ml) were seen (6). Nevertheless, it is evident that this kind of high bacterial load

affects the process itself by contaminating all areas of the water circulation in the machine. Not only chemical deposits of mineral additives but also biofilm and slime formation were seen (7, 8). The last, unfavorable stage of events will be a dirty machine producing paper or board having poor visual and hygienic quality.

The high microbial contents of mineral particles is due to the affinity of microbes to clay particles (Figure 1). Clay colloids in the earth are coated with metal hydroxides and sesquioxides, carrying net electronegative charges which are similar with those of the bacteria. Nevertheless, cohesion of these two partners occurs because of the "bridging" effect of di- and polyvalent cations (9). A model has also been suggested to account for the persistence of at least some of the enzymes accumulated in soil by being trapped by the humus fraction on the surface of the mineral particles: this layer will serve as a welcome substrate for the microbes of the earth (10).

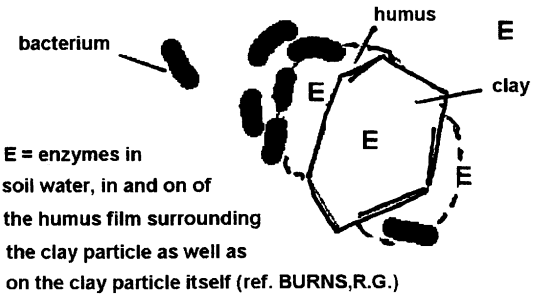

Figure 1. Distribution of enzymes in the water phase of soil, on and in the humus colloids surrounding mineral particles and on the surfaces of minerals (10).

During production of the mineral pigments, no specific treatment to decrease the contents of microbes is applied. The delivering companies will add biocidal compounds to their products before the delivery to the mills, with the aim of avoiding the situation that the densities of microbes would exceed the limits specified by the client. Microbiological guidelines for mineral additives do not exist for the most part of paper industries. The areas of sensitive industries are those with hygienic quality requirements for the end product, the paperboard or board used to package food and drink.

In the dry end of the board machine surface temperature of the paperboard will reach 100°C and higher, but the residence time in the hot area is short in modern machines and the hygienization will therefore remain incomplete. As inorganic materials, eg. clay, are known to protect microbes against heat-killing (9), the hygienic quality of the inorganic pigments and fillers can be expected to be of great importance to end product hygiene. Because threshold values of colony counts have been laid for food packaging board (11), the production of this kind of material especially needs a continuous microbiological control beginning from the raw materials, ending to the final packages to be filled with edible liquids.

Traditional colony counting (12,13) will give rather accurate estimates of densities of different microbial groups but gives no indication on the metabolic activity of the population. Automated systems for colony count estimation and collection of data will hardly be applied to analyse mineral pigment samples because false positive items may appear on the colony count plates. The most negative feature of colony count technique is the the long incubation period needed.

"Rapid Methods for Microbiology" are more and more popular in various areas of clinical, industrial and environmental microbiology. The lack of their applications for paper and board industry has prevented their use in mineral quality control until now. Some promising methods are still to be tested for this purpose, eg. Direct

Epifluorescence Technique (DEFT), Adenosine Triphosphate (ATP) Assay, Rabit[R] system (trademark of an automatized analytical equipment measuring the microbial effect on impedance of the substrate) and Bioscreen[R] (trademark of an automatized turbidometry).

In some cases, especially concerning food packaging board production, the commercial negotiations force the manufacturers of pigments and fillers to keep their product very clean (11,14,15). Rather low densities of microbes should thus be detected in these turbid substances which dictates the features of analytical system needed: it must detect some thousand cells per gram of mineral or pigment product without false positives, it may not be disturbed by biostatic agents included in these raw materials, is shall be fast, it shall measure not only densities but also (when testing biocides) activity of microbial flora etc. Different methods have different benefits and shortages when applied to the control of mineral products, and a combination of several methods may be needed for the fast and accurate evaluation of mineral's microbiological quality.

For the reasons stated above, methods applicable for the microbiological analysis of large number of samples and preferably giving results in hours rather than days, are needed. Several such methods have been developed for the needs for clinical research and food industry, but experiences on their applicability on paper industry raw materials have not been reported. Though an instant ON LINE system for the microbiological control of incoming raw materials for the paper mills may not be within reach, a shorter than an standardised 3-d incubation period will undoubtely be needed. This paper will describe and evaluate applications of those methods for paper industry and compare their performance to that of the traditional viable counting method.

MODIFIED STANDARD COLONY COUNTING METHODS FOR MINERAL PIGMENTS

A modified method for the microbiologial quality control of mineral products for the manufacture of paper and board was developed at the Research Centre of the paper and pulp company, ENSO Oy. This procedure is based on the aerobic total tount of colonies (12,13) and is presently applied by two other microbiological laboratories of the company in Imatra. The steps of this assay are as shown in Table1.

1	Suspend the mineral pigment in aqua dest., 1:10[1] (w/w)
2	Mix the suspension for 15 s. using a stirrer
3	Dilute the suspension (v/v) with phosphate buffer (equal volumes of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4) by the factors 1:10, 1:100 and 1:1000
4	Mix 1 ml of the pre-stirred (15 s) diluted suspension with 20 ml of agarized nutrient at 44.5 °C in a Petri dish
5	Allow the mixture to solidify in room temperature and incubate at +30 °C for 3 d

[1] for dry samples only

Table 1. Preparation of mineral samples for colony count analyses. The agarized tryptone yeast glucose broth had the following composition: tryptone, 5 g; yeast extract, 2.5 g; glucose, 1.0 g; agar 15 g; aqua dest, 1000ml.

The mean contents of solids in slurries was 50 %. This causes a slight inaccuracy between dry and slurry-type samples. No correcting factor has been used for slurries because dry weight analyses was not done.

QUALITY CONTROL OF COLONY COUNT METHODS

Microbiological laboratory procedures in Research Center and mills of Enso Oy are controlled every third month using statistical methods (16). In these quality control measures, original colony counts of fixed sample dilutions are studied by: 1) χ^2 test (comparison of observed and expected colony counts to detect "outliers"), 2) examining cumulative colony counts derived by laboratories (confirmation of indepedence of the results of the laboratory) 3) comparing variances of the normal and the Poisson variations of colony counts for each sample, V_n/V_P, indicating deviations between laboratories compared with the Poisson-type standard deviation of cumulated colony counts of the same sample. A rate of no more than 5 is allowed for the variable V_n/V_P.

Special attention is being paid to the accurate estimation of real colonies on nutrient medium. Aggregates of mineral particles may look very similar to bacterial colonies especially when both are detected inside the medium. A magnifying glass as well as frequent training with sterilised samples or colony count cultures before incubation (both showing aggregates of minerals etc.) help to keep the false positives in control.

DENSITIES OF BACTERIA IN CALCIUM CARBONATE ADDITIVES

Total Counts of Colonies in clay and carbonate additives on the screening period 1986 - 1992 were analysed by the methods described above (Figures 2 and 3).

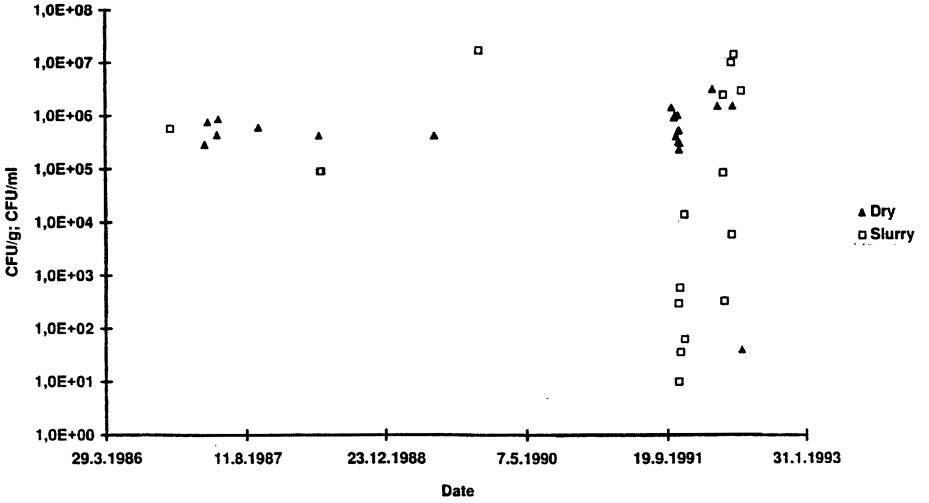

Figure 2. Total counts of colonies in dry and slurried calcium carbonate products during the period from 1986 to 1992. n_{dry} = 22, n_{slurry} = 27.

High colony counts with mean values $7.5*10^5$ CFU/g (dry products) and $1.8*10^6$ CFU/ml(slurried products) were detected when random samples during first period 1986 - 1992 were analysed (Figure 2). The colony counts of slurried products were slightly higher but more variable than those of dry products. The wider range of colony counts in slurries than in dry products may be caused by the varying usage of biocidic compounds for storage of slurries from time to time. Depending on the effectivity of a certain biocide against prevailing microbial population as well as the distribution of the biocide in the slurry, the prevention of microbial growth may be more or less successful.

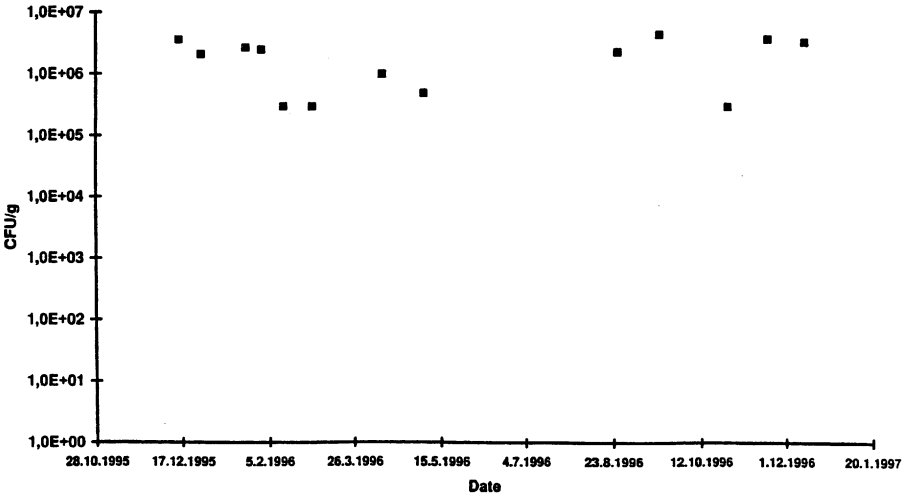

Figure 3. Total counts of colonies in solid calcium carbonate products during the period from 12/95 to 12/96. n = 13.

Another, more recent one-year screening period for the quality confirmation of dry calcium carbonate products gave rather the same results as the previous one: mean viable count value was $2.1*10^6$ CFU/g (Figure 3). The time series of this winter-to-winter quality control data gave no indication of seasonal variation among results. The quality of incoming, dry calcium carbonate lots thus indicated no improvement during the total time span 1986 - 1996.

DENSITIES OF BACTERIA IN CLAY ADDITIVES

Figure 4 shows an overview on the hygienic quality of clays. The trend observed indicated improvement in the quality of both dry and slurried clay products.

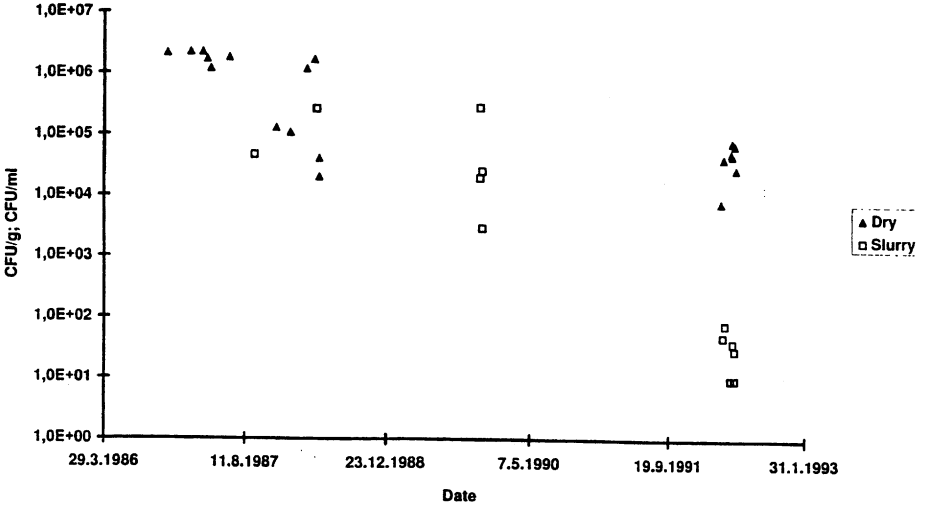

Figure 4. Total counts of colonies in dry and slurried clay products during the period from 1986 to 1992. n_{dry} = 20, n_{slurry} = 14.

The mean values of total colony counts ($7.4*10^5$ CFU/g for dry, $4.6*10^4$ CFU/ml for slurried products) shows slightly better condition of slurries compared with the dry products. A second period for the screening of dry clays was carried out during the period 1/96 - 12/96 and is shown in Figure 5.

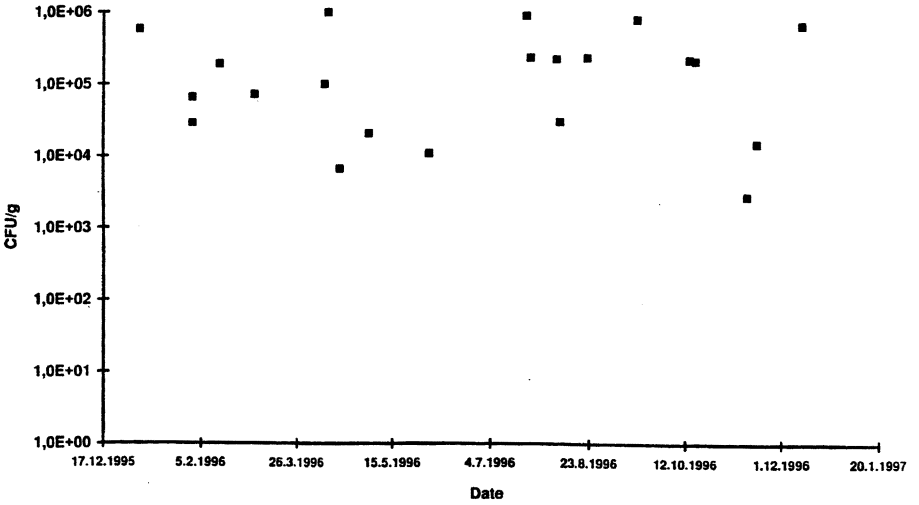

Figure 5. Total colony counts of solid clay products during the period from 1/96 to 12/96. n = 21.

The second period shows that there was no real improvement in the hygienic quality of dry clays - as was the case also with the dry calcium carbonate lots. The mean colony count of $2.7*10^5$ CFU/g is only slightly lower than during 1986-1992. Also in this case, there was no dependence between the season and the quality.

Regarding total colony counts of mineral pigments, wide variation of the results is evident. Products with very low microbial densities (below < 10 CFU/ml which is the practical threshold level for this analysis) were also detected. This range of bacterial densities from < 10 to hundreds of thousands per gram can be covered by colony count techniques when series of sample dilutions are used, but for the alternative, direct and (usually) less sensitive rapid methods, this may be a hard task to fulfill.

MEASUREMENTS OF MICROBIAL GROWTH IN MINERAL SAMPLES BY THE IMPEDANCE METHOD

Rapid methods of microbiology have some common special features compared with the traditional colony count methods, independent on the specific system achieved: they will measure biochemical reactions instead of just counting the colonies, they are much faster than cultivation methods with several days' incubation periods and they usually can measure the microbiological variables directly from the sample instead of needing any preparation of the sample before inoculation on or into the nutrient medium.

A common challenge of any rapid method is to have some correlation with the colony count results - no matter, there hardly will be such a dependence in every case between the microbes' biochemical activities and their cultivatibility. Inspite of the universal acceptibility of the colony count methods in many areas of quality control, rapid methods very often are still asked to give fast estimates of colony counts.

Impedance microbiology bases on the experiments made as early as 1889 when the British researcher G.N.Stewart published an article called "The Change Produced by the Growth of Bacteria in the Molecular Concentration and Electrical Conductivity of Culture Media" (17). This title includes the idea of impedance microbiology: microbial activity affects the conductivity of growth medium by breaking down large, electroneutral macromolecules into small, charged metabolites (H^+ included). During years, experiences have cumulated and led to the final idea of the semi-continuous measurement and automatic registration of impedance - a function of resistance and capacitance, both affecting the AC - in a series of cuvettes (18):

$$Z = (R^2 + (1/(2\pi fC))^2)^{0.5} \qquad (1)$$

where Z is the impedance in ohms, R is the resistance in ohms, f is the frequency of the AC in cycles/second and C is the capacitance in farads.

In practical applications, the inverse variable 1/Z expressed in μS units is preferred because it indicates the basic phenomenon, the increase of AC caused by conductive metabolites more clearly than Z.

Impedance microbiology is a respected method in rapid detection of pathogenic organisms today (19, 20). Hygienic impedance analyses in clinical and food microbiology usually will be preceded by enrichment procedures to select the organisms to be detected and increase their densities.

Unfortunately similar enrichment cannot be done in microbiological control of paper industry minerals because the need for very fast reporting of the results. The wide variety of microbes with different growth rates and different wishes for their enrichment environment also prevent the practical use of enrichment techniques: variation in growth responses from sample to sample would cause a confusion in the attempts to estimate original densities of microbes in the incoming samples.

A four-month test period for the evaluation of Rabit^{R} (Don Whitley Scientific Ltd.) impedance measurement as a replacement of viable colony count analyses for mineral samples has been carried on in ENSO Oy Research Centre. This method measures either the increase of impedance in the cultivation broth caused by the degradative action of microorganisms on the macromolecules of the medium or alternatively the decrease of electrical conductivity in KOH agar bridge caused by the neutralising action of major metabolic end-product of microbial respiration, CO_2 (19). These methods will be called direct and indirect conductimetry, respectively.

In Rabit^R system, automatic detection of growth is expressed as soon as the preselected change of impedance has been detected three times, one after another. This period from the start to the moment of detection will be called time of detection (TTD) and expressed in hours and minutes. The higher the density and activity of micro-organisms in the sample, the shorter will be the time needed for the detection of growth.

The main steps of sample and incredient preparation before measurement by impedance methods as well as set-up of critical values for TTD measurement are described in Table 2.

Preparation of growth medium (direct or indirect)
direct measurement: preparation of the growth medium (Don Whitley Impedance Broth), sterilisation of the cuvettes and the medium by autoclaving (121 °C for 15 min) and aseptic dosing of the medium into the cuvettes (a 9 ml)
indirect measurement: preparation of growth medium (Tryptone Yeast Glucose Broth, see Table 1), dosing of the medium into the inner test tubes (a 4 ml), preparation of KOH agar (see appendix A) and sterilisation of inner test tubes and cuvettes by autoclaving (121 °C for 15 min)
temperation of test tubes filled with medium (min. 1 h in room temperature) before starting the analysis

Table 2. Preparation of the measuring unit, ingredients and the samples for the impedance measurement.

Input of parameters for incubation and growth indication (control unit)

type of measurement (direct/indirect)

incubation period (24 hours will be optimal)

incubation temperature (37 °C for mesophilic, 50 °C for thermophilic population)

sensitivity of growth indication (an impedance increase or decrease of at least 20 µS during three progressive periods of 6 minutes)

Preparation of the sample for the analyse

homogenisation and dilution of the sample by common buffered diluents

inoculation of growth medium with the sample or sample diluent

Table 2. Preparation of the measuring unit, ingredients and the samples for the impedance measurement...continues.

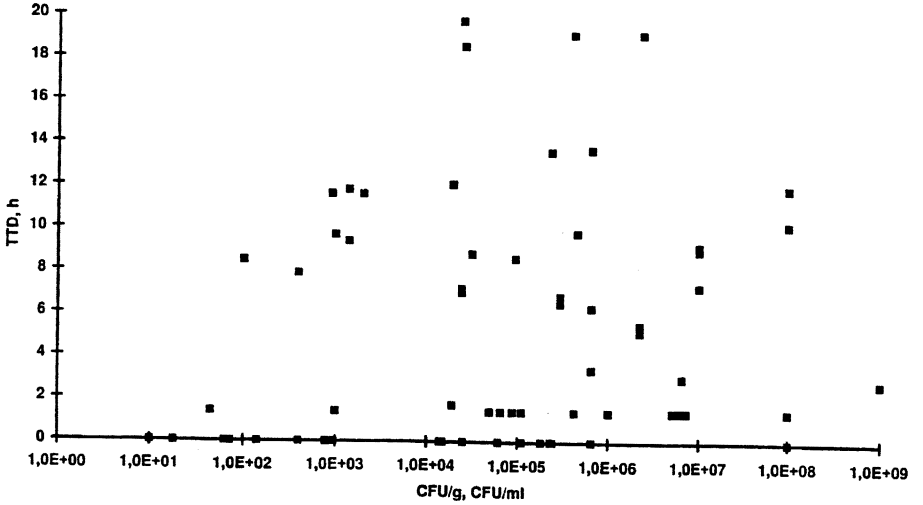

Figure 6. Total count of colonies vs. TTD values of indirect impedance measurements of paper industry samples. n = 48.

Total data of the indirect impedance analyses is collected in Figure 6. The origin of the samples (mineral fillers, starches, resins, white waters of paper and board machines as well as broth cultures of pure and mixed populations) was very variable. It is obvious that both the microbiological and the chemical heterogenity of samples resulted in wide variation and mutual independence of viable colony counts and detection times when this combined data was examined.

The direct measurement of impedance happens on the bottom of the cuvette, where also the heaviest fraction of raw materials sediments and disturbs the measuring event. For this reason, indirect measurement has been suggested by manufacturer for this kind of samples. however, a smaller set of samples with different origins and properties has been studied by using the direct method, too (Figure 7).

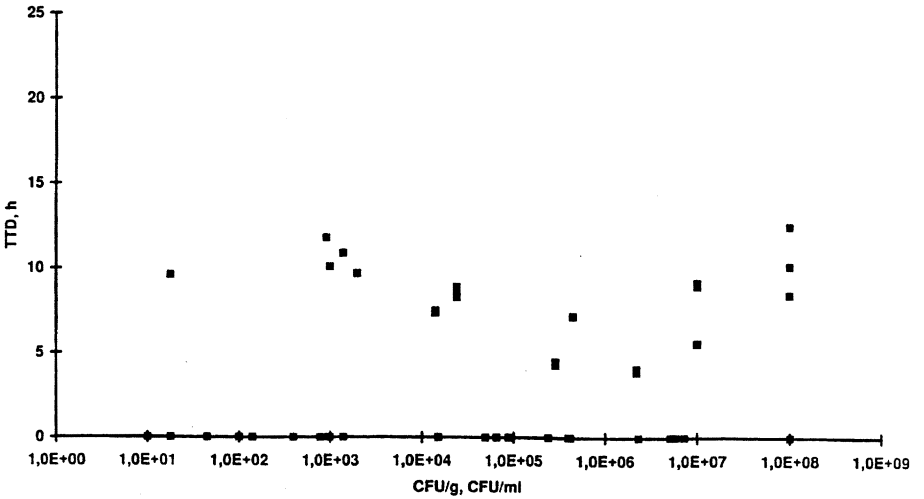

Figure 7. Total count of colonies vs. TTD values of direct impedance measurements of paper industry samples. n = 21.

Independence of the TTD values on the CFU values is clearly visible in figure 7. The relatively big portion of measurements with no detection of growth in this material may be explained by the very slow and/or narrow change of impedance during test period.

When only mineral fillers were the object of a comparison, the majority of the viable count analyses gave a zero result and no conclusions of the dependence of impedance alteration on the density of microbes could be done. Comparison of CFU and TTD values is shown in Figure 8.

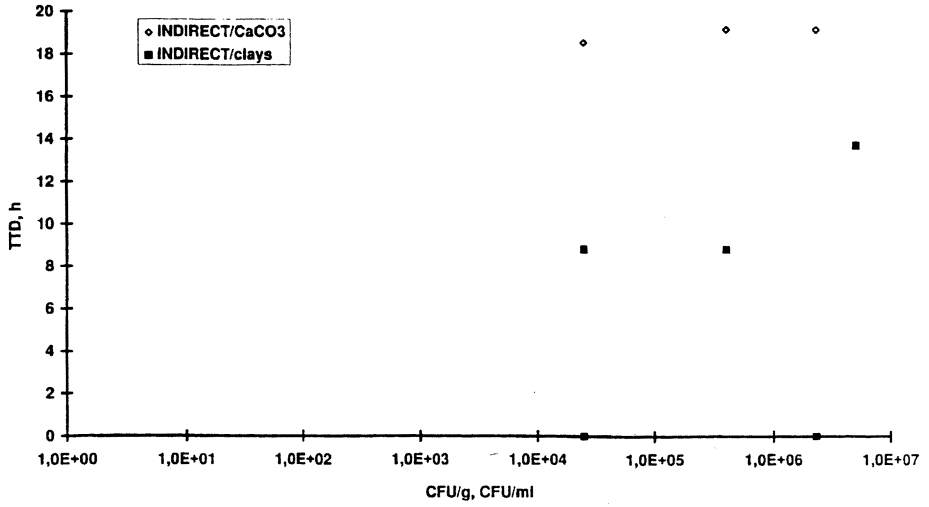

Figure 8. Total count of colonies vs. TTD values of direct and indirect impedance measurements of calcium carbonate and clay samples. n = 6.

In this rather limited material, a range of viable colony counts from $> 10^4$ to $> 10^6$ CFU/ml (or CFU/g) was detected by the TTD. This level of contamination was reflected to the CO_2 production of $CaCO_3$ and clay samples in different ways: with the same CFU value, detection of growth in calcium carbonates was seen at later stage than with kaolins. This delay in calcium carbonate samples may be due to the high background of CO_2 released by the carbonate itself, which masks the microbial carbon dioxide production - though this hypothesis has not been confirmed.

The results from experiments with three different strains of machine contaminating bacteria in Tryptone Yeast Glucose Broth exhibited a close dependence of TTD on the diluting factor of the broth culture

when dilutions were made by phosphate buffer and measuremen. was done by indirect method. Also the decrease of impedance during 6 hours from the start are in connection with the dilution of the sample (Figure 9).

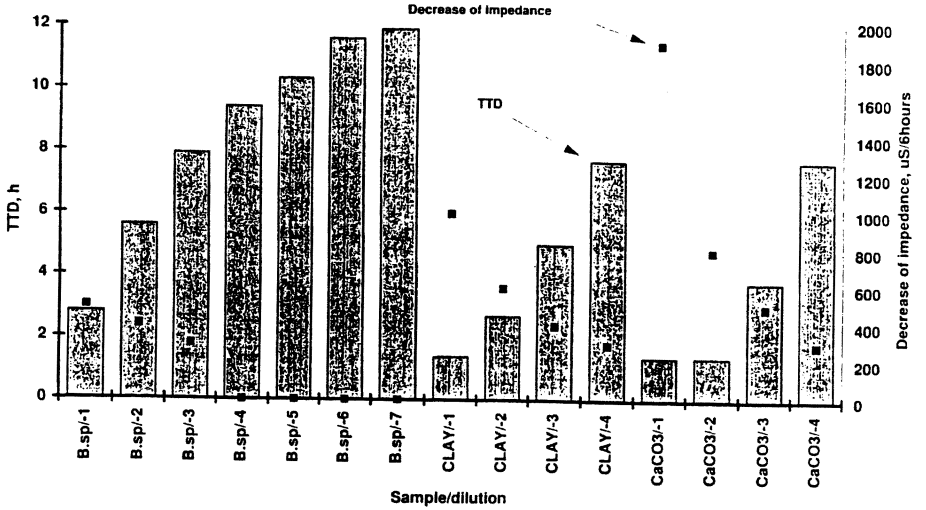

Figure 9. TTD values and the decrease of impedance (indirect method) of calcium carbonate contaminating *Bacillus sp* strain and unidentified pure cultures from clay and calcium carbonate samples. Dilutions of the bacterial cultures was made by phosphate buffer at logarithmic steps from 10^{-1} to 10^{-7}.

It is obvious that dependence of impedance changes and microbial densities really exists when samples with low turbidity and rather high microbial activity are studied. When impedance measurement is applied to mineral control, several confusing factors connected to the variation of biological and chemical features of samples will soon appear. It seems that the preparation of the samples and the adjustion of the critical parameters of the measurement (threshold value in impedance decrease, length of period between measurements,

temperature etc.) shall be studied more deeply. Replacement of the TTD value by absolute impedance decrease measured during the early hours of analyses looks like a tempting alternative.

MEASUREMENTS OF BIOMASS BY ADENOSINE TRIPHOSPHATE (ATP) ASSAY

Adenosine triphosphate is an universal biochemical compound for storage and release of energy in all living cells. It has been widely adopted in environmental, medical and industrial areas of research, and instructions and applications for different practical tasks have been published by the manufacturers of analytical devices and reagents (22, 23).

Release of luminescence light by firefly luciferin-luciferase under the control of microbial adenosine triphosphate (ATP) follows the equation below (24):

$$\text{MgATP} + \text{luciferin} \rightarrow \text{PP}_i + \text{AMP luciferin} \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light} \qquad (2)$$

where MgATP is magnesium salt of adenosine triphosphate, PP_i is pyrophosphate (released from MgATP), luciferin is the substrate of firefly luminescence reaction, oxyluciferin is luciferin oxidised in the luminescence reaction, luciferase is the enzyme of firefly luminescence reaction and AMP is adenosine monophosphate.

The reaction above includes oxidation of luciferin by oxygen. ATP serves as a converter of luciferin into a form capable of being catalytically oxidised by the luciferase in this system.

Luminescence of this ATP-mediated reaction will be measured utilising an instrument for the amplification and quantification of released light, a luminometer. There are several types of luminometers beginning from simple, trasportable equipments for measurement of single samples to large ones having automatised measurements and data collection for sample series. In this research, Wallac^R 1250 and Bio-Orbit^R 1253 were used for emission measurements. These luminometers indicate the light released during a measuring period of 10 seconds either in millivolts (1250) or R(elative)L(ight)U(nits) (1253). The previous instrument can be connected to Bio-Orbit^R light integration program where measuring period is adjustable (Figure 10).

Like the conductimetry, ATP assay has a relatively long history beginning from 1888 when the physicist Eilert Weidemann distinguished "cold" light from the light arising from incandescence (25). During the years, the different types of luminescence were detected, among them chemiluminescence. In this type of reactions, the electronically excited state of an atom is first generated by a chemical reaction, after which the energy is released as light without production of heat. The use of firefly luciferase in the quantitative assay of ATP was then the final result of several researchers' efforts.

References indicating the benefits of ATP assay in the screening of urinary tract infections have led to discussions concerning the value of this method in early diagnoses of positive cases among big number of samples as well as ideas for the development of immobilized assay systems and reagents with better purity and efficiency for extraction of ATP and luminescence reaction (26).

Another example of the utilisation of ATP assay in the control of large number of samples is the dairy industry. Whilst the detection of ATP by luciferase reaction is very sensitive (regarding the general rule, 10 000 cells/ml * 1 femtogram ATP/cell = 0.01 ng ATP/ml could be detected), the non-microbial ATP from other sources like somatic cells of milk may cover the microbial ATP severily (27).

When experiments are carefully planned and possible inhibitory effects of the samples are excluded, a close connection between total count of colonies (when > 20 000 CFU/ml) and the concentration of ATP can be detected (25). Very similar threshold level for the detection of yeast ATP beginning from 10 000 CFU/ml was noticed for contaminated orange juice (28).

ATP assay has been applied to paper industry already in the beginning of last decade and regarded as one of the rapid methods suitable for the estimation of microbial activity in paper machines (29). This method has since then been used for the antimicrobial sensitivity testing of process samples. It also has found an application in the control of waste water treatment by activated sludge process in paper industry (28).

Considering the promising references, ATP assay was tested as a control method for calcium carbonate and clay hygiene in ENSO Oy Research Centre. The four-step assay is shown below (28):

$$[ATP]_t = (c/(d-c))*[ATP]_s*df \quad (3)$$

$$[ATP]_c = ((c-b)/(d-c))*[ATP]_s*df \quad (4)$$

where $[ATP]_t$ is the total ATP concentration, $[ATP]_s$ is the concentration of cell-bound (extracted) ATP, $[ATP]_s$ is the concentration of standard ATP, df is the inverse of the dilution factor and a,b,c and d are the emissions on successive analyse steps, which are the luminometric measurements of the untreated sample (a) and the same sample containing luciferine-luciferase (b), luciferine-luciferase and ATP extractant (c) and luciferine-luciferase, ATP extractant and standard ATP (d).

All ingredients (sample, luciferin-luciferase, ATP extractant and standard ATP) are dosed in the same cuvette in 100 µl amounts. Luminescence during 10 seconds period is measured in every step after a delay of 15 seconds, with the exeption of the 30 seconds delay period before the third step. All reagents are manufactured by Bio-Orbit Oy, Turku, Finland.

The effect of microbial ATP on the luminescence in a process water sample could be seen in Figure 10, where periods of 30 seconds were used to collect the luminescence responses at each step described in Table 3. The poor yield of light in step c (first repeat) caused by too short an extraction time (< 30 sec.) is clearly seen when comparing it with the good response in step c (second repeat) when extraction period of > 30 seconds was used.

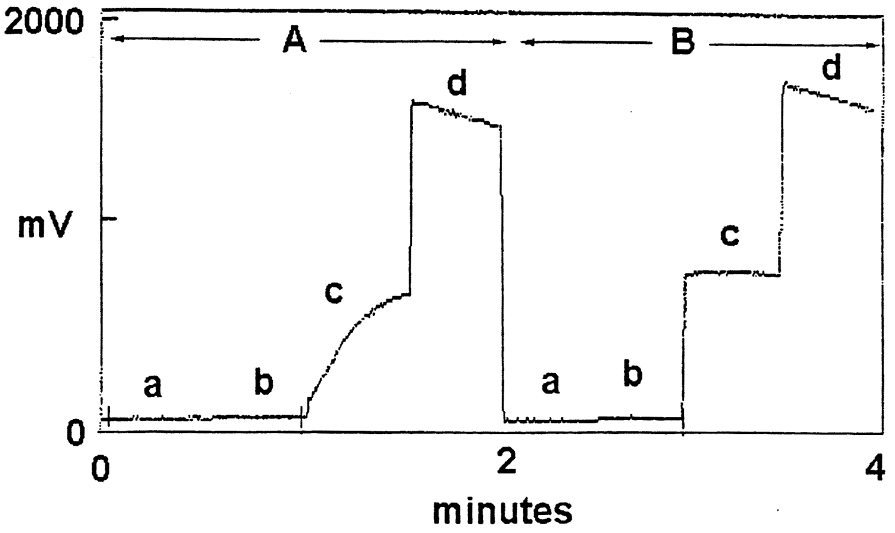

Figure 10. Integration of luminescence during measuring periods a-d (see Table 3) of 30 seconds. Integration has been interrupted for reagent addition, mixing and ATP extraction periods.

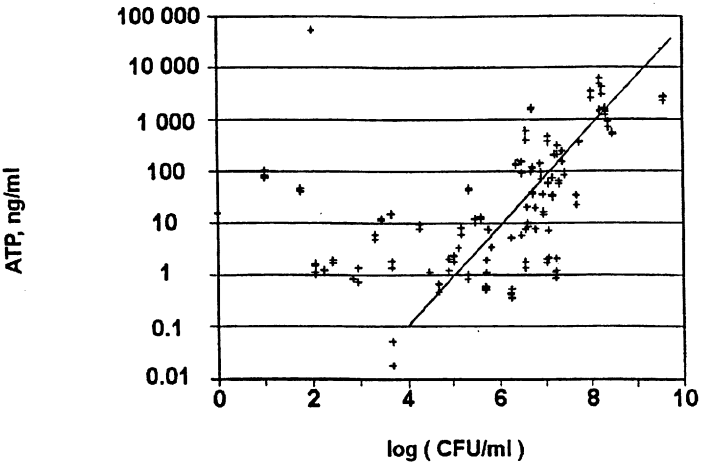

Figure 11. Log values of total count of colonies vs. ATP concentrations in paper industry samples during one year test period (1989). n = 121.

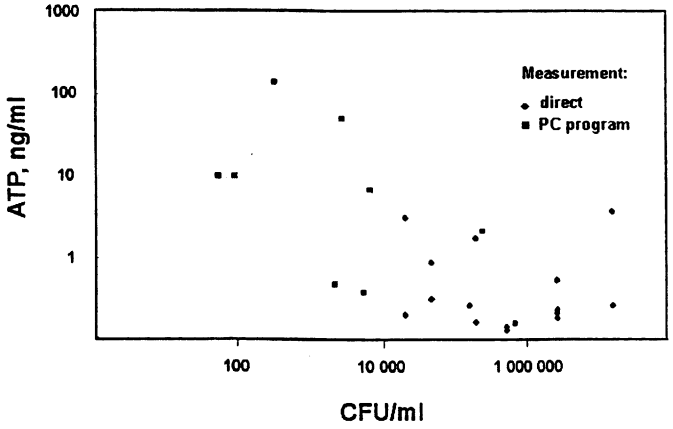

Figure 12. Log values of total count of colonies vs. ATP concentrations in calcium carbonate and clay samples during three month test period (1995). n = 25.

The previous experiments with several types of paper industry samples have shown that ATP densities give an estimation of total count of colonies when density of viable bacteria exceedes 10 000 CFU/ml (Figure 11). In opposite, poor dependence of ATP on CFU in raw material tests was evident (Figure 12). This phenomenon was caused by the distribution of colony counts on and below the threshold level of ATP measurement. The weakening of response was partly due to the dilution factor of at least 1:100, which was needed because the strong turbidity of mineral samples studied. If 10 000 CFU/ml is regarded as the lowest decimal colony count level for microbial ATP assay (27,28), then dilution factor of 1:100 strongly decreases the sensitivity of the test and lowest value of total viable count to be detected accurately by luminescence method will be as high as 1 000 000 CFU/ml, respectively.

Another factor affecting the yield of ATP is it's strong tendence to attach on the mineral particles (31). When released from the cell, very small amount of ATP will be left in the supernatant during sentrifugation or filtrate passing 10 µm pores (32).

MEASUREMENT OF GROWTH BY AUTOMATED TURBIDOMETRIC METHOD

Automated turbidometric methods are usually easier to perform and they require less materials and shorter incubation time than the plate count techniques. Their disadvantage is that the turbidity of the sample sets limitations to their usage. We studied the application of automated turbidometry for microbiological quality control of calcium carbonates. The analytical system consisted of Bioscreen C^R (Labsystems Oy, Helsinki, Finland), which can run 200 samples simultaneously.

To test the effect of the precence of calcium carbonate on the detection of growth of *B.cereus*, three growth media were used: (1) Tryptose-glucose-yeast extract (TGY), (2) 0.5 % soluble starch and 0.05 % yeast extract and (3) CTMP filtrate and white water (1:1, pH 7.2). Test organisms were *Bacillus cereus* MC23 of paper machine origin, and *B. cereus* ATCC 9139 (freeze dried calibration culture in milk powder). Fifty µl of technical calcium carbonate containing 0, 0.1 or 1 % $CaCO_3$ and 1500 CFU *Bacillus cereus* MC23 or 2 CFU *B. cereus* ATCC 9139 were added in each microtitre plate well holding 300 µl of the medium. The plates were incubated in Bioscreen C^R at 30 °C for 24 h with continuous shaking and the optical density (wide band filter) was continuously recorded.

Growth was detected in TGY and starch - yeast extract -medium (Fig. 13a-d) but not in CTMP filtrate - white water medium (increase in optical density less than 0.1, data not shown). Initial (mean) values of the optical density were 0.15, 0.2 and 0.8 in the wells holding 50 µl 0, 0.1 and 1 % $CaCO_3$, respectively. In rich medium (TGY) the wells holding *B. cereus* MC23 (inoculum level 1500 cfu per well, Fig. 13a) or *B. cereus* ATCC 9139 (inoculum level 2 cfu per well, Fig. 13b) increased turbidity irrespective of the concentration of $CaCO_3$. During the incubation, the optical density in the wells with 50 µl 1 % $CaCO_3$

reached a value of 1.6. In practice, this is close to the useful range accurately measurable by the instrument. Therefore the samples to be tested should not contain significantly more than 1 % of $CaCO_3$.

To see how sensitive the automated turbidity assay was to the growth medium, starch - yeast extract -medium was used. Growth of both tester strains was measurable with 0.1 % $CaCO_3$ (50 µl added in 300 µl) but with 1 % $CaCO_3$, growth of *B. cereus* ATCC 9139 (inoculum level 2 cfu per well) was not measurable. (Figure 13 c,d). The freeze dried calibration culture of *B. cereus* ATCC 9139 contained milk powder which increased slightly nutrients to the medium. To maximize the sensitivity of the assay, we therefore recommend rich medium for the quality control of calcium carbonates.

Our experience shows that for reliable results, the level of turbidity increase indicating growth should exceed 0.1 optical density units during the assay. The results shown in Figure 13 b and d demonstrate that 15 (83 %) out of the 18 wells holding (on average) 2 cells of *B. cereus* ATCC 9139 per well were easily recorded positive in 24 h incubation. This can be considered as detection limit of the method. In the assay, maximum 0.5 mg dry calcium carbonate can be used per well (equal to 50 µl of 1 %) in order to avoid too high initial turbidity, which means that the method should detect during 24 h incubation contamination in 80 % of samples containing at least 4000 CFU per gram dry calcium carbonate. If the contaminants in the calcium carbonate are other than *B.cereus*, or if there are biocides present, the sensitivity of the method may be different. The initial number of cells in the wells does not affect the shape of the turbidity curve, but the smaller the inoculum, the longer it takes before the instrument detects growth. Our results showed that in rich medium (TGY) measurable growth was detected in 8 h when the initial cell number was 1500 per well (Figure 13 a) and in 14 - 18 h when the initial cell number was 2 per well.

In conclusion, the use of automated turbidometric method for quality control of calcium carbonates requires dilution to concentration 1 % $CaCO_3$. Using rich growth medium, the test detects the contamination of 4000 CFU or more per gram dry calcium carbonate during one day.

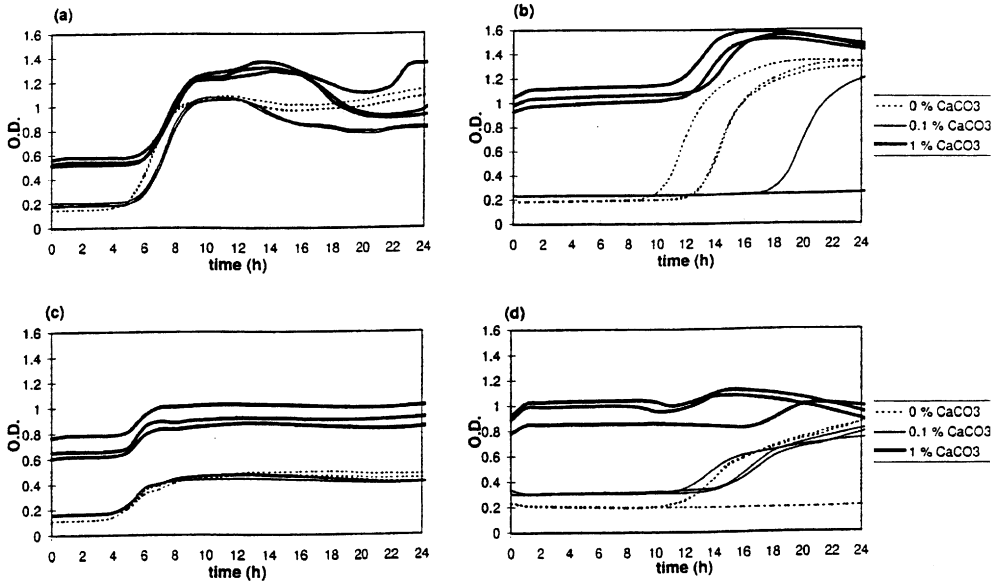

Figure 13. Turbidity curves at 30 °C measured by automated turbidometer. The 50 µl samples added to 300 µl of medium in each well contained the inoculum and 0, 0.1, or 1 % $CaCO_3$. (a) TGY medium, *B. cereus* MC23, inoculum 1500 CFU per well, (b) TGY medium, *B. cereus* ATCC 9139, inoculum 2 CFU per well, (c) starch -yeast extract -medium, *B. cereus* MC23, inoculum 1500 CFU per well, (d) starch - yeast extract -medium, *B. cereus* ATCC 9139, inoculum 2 CFU per well.

CONCLUSIONS

There are some questions to be answered when rapid methods are regarded as alternatives for traditional colony count techniques:

- What parameter should be measured? Is it the density of microbes or their activity in the samples, which will affect the quality of raw material and be related to the raw material's effects on the manufacturing process and products, which should be measured?

- How fast should the microbiological analyses of incoming minerals be carried out, when all aspects (especially the chances to start the preventive activities against contamination during only some hours after the delivery of the mineral to the paper mill) is taken into account?

- How high are the costs of alternative vs. traditional methods?

- Is there some other microbiological tasks (testing of biocides, identification of organisms) than the exclusive quality control to fulfill, needeng the assistance of colony count methods?

The good features of rapid methods - speed, capacity, automation - will usually be encountered by weaknesses: sensitivity to disturbing factors (turbidity, tendence of the samples to sedimentate), poor responses when low microbial densities are present, high prices of equipments etc.

The experiences at ENSO Oy Research Centre indicate that there very often is a poor correlation between viable colony counts and activity of the population in raw material samples to be checked. Some hypothesis for the independence of these two microbiological features exists:

- A varying portion of microbes are in dormant state as spores: they may be detected by colony count methods but they need time to germinate before they will carry on the physiological events measured by rapid methods

- All microbes will not be detected by total count of colonies but are still active in the samples - examples of these groups are filamentous fungi and majority of actinomycetes

These two hypotheses are in agreement with soil microbiology, where two survival types of micro-organisms in soil could be detected (33):

- zymogenous (allochthonous) organisms which are present in low numbers but are capable to grow (and die!) very fast when favourable nutrients are available

- autochthonous organisms which grow much slower than allochthonous ones but whose densities are more stable in the soil

The previous organisms may have an active outgrowth on nutrient medium and may also be metabolically active when testing by rapid methods. Latter, in opposite, have slow growth rates and may be hidden in analyses. Responses of these groups (and members of them) may vary regarding their cultivatibility on solid media (traditional colony count analyses) and broth media (a common choose in rapid methods).

In addition, also biocidic treatments may prevent the microbial activities when original samples without any dilutions are carried out by rapid methods. Dilutions of samples and favorable conditions in the nutrient medium may diminish the effects of biocides, no matter colony count methods or alternative techniques are applied.

It seems that all the rapid methods so far tested - conductimetry, luminometry and turbidometry - give good estimates of colony count when "easy" samples like broth cultures or higly diluted, microbial-rich samples are studied. In opposite, the real, "difficult" samples are those which should be controlled in industry - and as fast and accurately as possible. In mineral pigment samples, rather good sensitivity for growth have been detected by Bioscreen^{R} test where just a few cells per microtitre well were indicated in an overnight incubation. ATP method gives the results immediately but needs at least 10 000 CFU/ml to show any real response. Rabit^{R} system also worked out, but in this case, the correlation between CFU and TTD values was poor in both direct and indirect analyses.

In every alternative method, there seems to be chances to improve the response of microbial contents on the variable measured. In conductimetry, the prevention of sample sedimentation and the measurement of the in- or decrease of impedance during test period instead of trying to detect the start of the growth on the growth curve may give positive results. In luminometry, the prevention of rather irreversible ATP attachment on the mineral particles as well as improvement of ATP extraction from the cells (34) may help. Turbidity test could also be improved by carefully screening the best choices for nutrient medium to have as good increase of turbidity caused by microbial population as possible, compared with the background noise caused by the solids of the samples. Rich mediums are thus preferred in this method.

The primary problem in applicativity of these methods should be solved by the cooperation of device manufacturer and applying customer. This, no doubt, is very important especially in paper industry where the overall knowledge of microbiology usually is rather rare and variety of processes to be controlled very wide. When the solution is the right one, the higher material costs of the new method will soon be encountered by more effective hygiene control with lower costs of personnel, easier updating and applicativity of data etc. The

manufacturer of rapid methods inevidently should observe the needs of the enormous field of universal paper and board industry to be ready for the new challences: increasing quality criteria for raw materials because of more delicate products, decreasing water consumption in the production which is likely to cause new kinds of microbiological problems and increasing use of tricky raw materials like hygienic labile recycled fiber brands.

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Transcription of Discussion

Microbiological Control of Pigments and Fillers in the Paper Industry

Juha Mentu, Microbiologist/Laboratory Manager, Enso Group Oy, Finland

Professor Jean-Claude Roux, EFPG, France

According to your experience have you studied the impact of the presence of these microbes on the sheet paper properties and what can you recommend to a papermaker?

Juha Mentu

Considering the effect of microbial growth on paper and board products, the main factor here will be of course the agglomeration of fibres and fillers and fines which will cause the holes in paper and board. But, if you think of other technical paper properties, maybe the flows of raw materials and the non-beneficial effects of microbial growth on viscosity and on the break down of some components like starches which are very sensitive to microbial growth, are also affecting. They will have non-beneficial effects on the whole of the paper production. A third thing will be the accumulation of bacterial spores which will spoil the hygienic quality of the board.