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EVALUATION OF THE SUITABILITY OF THE DEVELOPED METHODOLOGY FOR NANOPARTICLE HEALTH AND SAFETY STUDIES

Virpi Väänänen[1], Elina Rydman[1], Marit Ilves[1], Kati Hannukainen[1], Hannu Norppa[1], Atte von Wright[2], Ulla Honkalampi[2], Irina Tsitko[3] and Juulia Rouhiainen[4]

[1] Finnish Institute of Occupational Health
[2] Biosafe –Special Laboratory Services Oy
[3] VTT Technical Research Centre of Finland
[4] Pöyry Management Consulting Oy

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SUMMARY

The goal of the SUNPAP project is to proceed from research to pilot scale and finally to the end products. One of the main targets of the project is the risk assessment to guarantee the safe introduction of nanotechnologies in to the forest industry value chain. Because there are no explicit international guidelines for risk assessment of nanoparticles, it was necessary to outline a risk assessment methodology for this project.

A qualitative risk assessment was employed in SUNPAP, because the toxicological data about nanofibrillated cellulose (NFC) was scarce, and no information about the release of NFC, nor there measured airborne concentrations of NFC in the workplaces exist. Qualitative risk assessment was based on hazard testing done and by using Control banding approach. Control banding was originally developed to assist industry in ensuring the safety of workers in situation when little hazard information was available. Currently, control banding approach has been developed to be used with nanoparticles. In SUNPAP two control banding tools were tested: CB Nanotool and Stoffenmanager Nano 1.0.

The health hazard testing protocol used in SUNPAP was seen as suitable for a first step hazard assessment of NFC. In *in vitro* tests NFC was marginally toxic or not toxic, and the results were clearly weaker than the used positive controls (carbon nanotubes). The nematode model indicated that NFC does not have systemic effects. However, the pharyngeal aspiration studies indicated some inflammatory responses in the lungs of the mice. Studied NFC contained biocide, which did not have effects on the tests. However, the studied NFC contained some bacteria and may have contained also chemical residues from the TEMPO treatment; hence the slight inflammatory effect was associated with a combined exposure to NFC, bacteria and possible chemical residue rather than NFC alone.

In exposure assessment, first step was to define the exposure scenarios, which included two different types of NFC manufacturing in laboratory/pilot scale and various steps in manufacturing two different NFC applications. The exposure scenarios included information from the laboratory and pilot scale plants, expert judgements and modeled information for production plants for applications. The conclusion based on Stoffenmanager Nano was that the possible exposures were assessed to be low in all the scenarios defined. Reasons for the low exposure level were the facts that the amount of workers in each scenario was low, NFC was used in the processes in wet stage, production systems in some scenarios were partly closed, fume hoods and mechanical ventilation were used, and regular maintenance and cleaning were assumed.

The hazard assessment done in SUNPAP project was like the first tier in the health hazard testing of the product development. More toxicological tests are needed to study NFC, because some positive effects were seen in the hazard tests. The defined exposure scenarios were based on qualitative information delivered by experts. More accurate exposure scenarios are needed for quantitative risk assessment. The used risk evaluation followed the new guidelines of the ISO/TR 13121 made for product development (ISO/TR 13121 Nanotechnologies-Nanomaterial risk evaluation).

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1 INTRODUCTION

Nanotechnology represents a promising industry that can have enormous positive impact to several economic and social sectors. Due to the small size, nanoparticles can exhibit novel properties that are remarkably different from the corresponding bulk material. Nanoparticles are more reactive than their corresponding conventional form, mainly due to large surface area. For the industry, new cellulose-based nanomaterials offer great potential for creation of new products with unique properties. However, besides expected benefits, there are some concerns about possible risks associated with production, use and recycling of such new materials. Thus, the development of nanomaterials should closely go together with risk assessment, to ensure the safety of nanomaterial production and products.

Work on hazard and risk evaluation is clearly lagging behind the rapid development of new nanomaterials. Currently, risk assessment is facing many uncertainties which make evaluation of the potential hazard of nanoparticles or risk of exposure very difficult (SCENIHR 2009). Data available in open literature are mostly focused on the toxicological properties of certain groups of nanoparticles such as metal oxides, silver nanoparticles, and carbon nanomaterials (Pronk 2009; Stone 2010; Christensen, Johnston et al. 2011; Mikkelsen 2011), and most of the studies published have utilized *in vitro* cell culture systems. *In vivo* experiments in animals are fewer, and there is lack of data on exposure to nanoparticles/nanomaterials in workplaces.

There is some evidence suggesting that nanomaterial properties (e.g. size, surface properties, tendency to aggregate/agglomerate etc.) influence their hazard. However, until these relationships are properly understood, one should be very cautious about generalizing the results and extending them to all nanomaterials. Due to the unique properties of nanomaterials, risk assessments should be conducted on a case-by-case basis (Stone 2010). At any rate, detailed physico-chemical characterization of each individual nanomaterial is absolutely important for risk assessment (Bouwmeester, Lynch et al. 2011). There are still technological difficulties in this area. Until recently, the absence of unequivocal definition of nanomaterials made the decision on parameters to be measured difficult. On October 18th, 2011, the EU Commission adopted a recommendation on the definition of a nanomaterial. A nanomaterial was defined as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm" (Anonymous 2011). The Commission also mentioned that in some cases with high environmental, health, or safety concerns the number size distribution threshold may be between 1 and 50 %.

Cellulose as such has been considered a safe natural material. Some results have, however, indicated that some cellulose fibres may cause tumours (sarcomas) by inhalation exposure (Cullen 2002; Cullen, Miller et al. 2002). The safety assessment of nanofibrillar cellulose (NFC) depends on the specific characteristics possibly introduced by the small particle size and shape, and the accompanying bioactivities. Inorganic nanoparticles or nano-/microfibers are not particularly suitable as risk assessment

models for NFC, because of different chemical composition, size range, shape and physico-chemical properties. Nevertheless, there is some concern that NFC might act similarly to other fibrous nanoscale structures such as carbon nanotubes and asbestos causing fibrosis and cancer. Carbon nanotubes are known to be cytotoxic, and there is evidence that they may cause oxidative stress and DNA damage (Donaldson, Aitken et al. 2006; Yang, Liu et al. 2009). No studies on the toxicity or toxicokinetics of NFC have been available. In the only published study (Vartiainen 2011), microfibrillated cellulose (MFC; also referred to as nanocellullose in the article) did not have cytotoxic or inflammatory effects in cultured cells *in vitro*.

There is no information on measurements of exposure to NFC in industrial scale processes, because the material is still under development. In exposure scenarios, it may be assumed that the most probable route for exposure of workers at workplaces is via lungs and skin. However, exact levels of occupational exposure to NFC and possible emission to the environment during manufacturing cannot be estimated, before the manufacturing process is well defined. In the current report, the first effort has been made to estimate occupational risk of NFC. The risk assessment was performed based on all the information thus far available - mainly generated during the SUNPAP project. Due to the many gaps mentioned above, it is currently not possible to conduct a quantitative risk assessment. Therefore, a qualitative approach was employed for this report, to evaluate the possible risks occurring during laboratory scale and pilot scale production of NFC and some applications.

2 METHODS

2.1 Hazard assessment

The crucial safety aspects of NFC depend on its ability to interact with cells either directly or indirectly (for example, via formation of reactive oxygen species). The *in vitro* cytotoxicity and immunotoxicity tests, together with the physical characterization of the materials, will give indication whether the NFC fibres will be able to cause cellular damage and whether their systemic effects will be likely. Also a nematode model based on a well known test organism (*Caenorhabditis elegans*) that can be used to investigate both potential systemic effects and neurotoxicity will be employed for the safety assessment. While the exposure of the workers is likely to happen via the lungs, the inhalatory exposure is of special importance considering the occupational safety. Therefore, an inhalatory toxicity study with experimental animals is included in the test program. The hazard testing scheme is outlined in Figure 1. Briefly, the chosen approach relied on *in vitro* tests for cytotoxicity, immunotoxicity, and genotoxicity, followed by an assay for systemic effect in a nematode model, and assessment of pulmonary toxicity in mice.

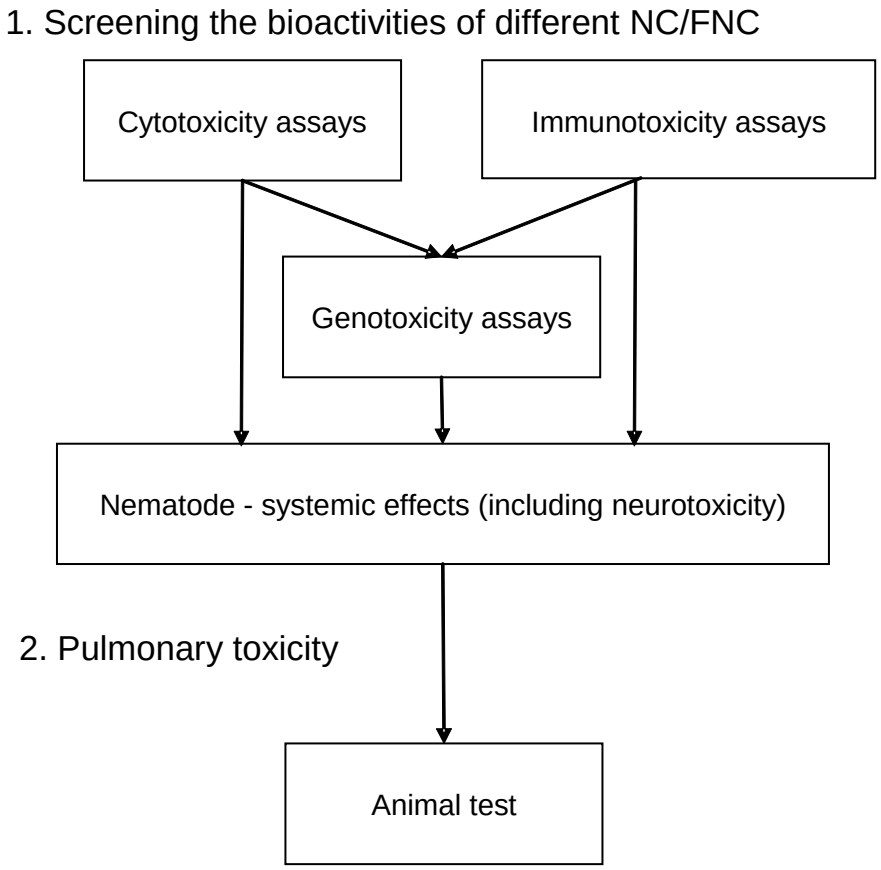

Figure 1. Hazard assessment testing scheme.

2.1.1 *In vitro* studies

In vitro methods are essential in toxicological research, as they allow one to investigate the mechanisms of action of hazardous substances in less complex systems than a whole organism. In an organism (*in vivo*), thousands of genes, RNA molecules, proteins, ions, and other molecules are interacting with each other and with the surrounding environment, to regulate processes throughout the system. This complexity makes it challenging to study basic biological functions *in vivo*, and hence *in vitro* settings are used to simplify the system under study.

In SUNPAP, several kinds of NFC materials have been studied in order to find out their technical properties and suitability for different applications. Studied materials were characterized according particle size profiles, viscosity, zeta potential and conductometric titration. Materials produced by different processes were chosen for studies within Module 4:

- NFC-VTT - produced using Masuko grinder (5 passages) without pre-treatment. No biocide was added to the suspension.
- NFC-CTP/VTT – produced using Masuko grinder (5 passages) with enzymatic pre-treatment. Biocide was added to the pulp after pre-treatment.

And three batches of TEMPO-treated NFC:

- NFC-TE/VTT – produced using Masuko grinder (3 passages) with TEMPO-mediated oxidation as a pre-treatment. No biocide was added to the suspension.
- NFC-TE/CTP B1 - produced using a lab scale high-pressure homogenizer with TEMPO-mediated oxidation as the pre-treatment. The sample was dialysed in order to remove traces of remaining reactant. No biocide was added to the suspension.
- NFC-TE/CTP B2 - produced in pilot scale with a high-pressure homogenizer with TEMPO-mediated oxidation as the pre-treatment. The pulp sample was washed four times before homogenization but can contain small residues of the reaction chemicals used in pre-treatment (TEMPO, sodium bromide and sodium hypochlorite). Moreover, samples with and without a biocide addition.

The cytotoxicity, genotoxicity and immunotoxicity of the selected NFCs were first studied using *in vitro* -models.

2.1.1.1 Cytotoxicity

The cytotoxicity of the NFC samples was assayed using a human cervical cell line (HeLa-cells) for the detection of acute toxic effects. The specific assays were the Highest Tolerated Dose (HTD) –test and the Total Protein Content (TPC) -test. The HTD test gives a qualitative estimate on toxicity, based on the observed changes in the cellular morphology of the exposed cells, while the TPC-assay gives a quantitative estimate of the toxicity using the protein content of the cells as the end point. The Boar Sperm Motility Inhibition (BSM) test was additionally used to detect sub-lethal effects seen as the impairment of sperm movement. All these tests have previously been validated in the project by using industrial microfbrillated cellulose sample (MFC-ARBOCEL) as a model nanocellulose and known cytotoxic nanomaterials (carbon nanotubes) in addition to standard chemical positive controls. The test materials were suspended either in the cell culture medium (HTD- and TPC-assays) or in sterile water (BSM-test) at appropriate concentrations. After mixing the suspensions were vortexed at highest setting and ultrasonicated for 5 min to ensure an even distribution of particles.

2.1.1.2 Genotoxicity

The purpose of *in vitro* genotoxicity studies was to reveal whether NFCs have genotoxic potential, a characteristic linked with genotoxic carcinogens. The genotoxicity of the NFCs studied was assessed at the level of DNA and chromosome, taking into account the possibility for indirect action mediated by the generation of reactive oxygen species (ROS). The studies were performed on human bronchial epithelial BEAS 2B cells which represent a model for epithelial target cells of the bronchioles. The techniques used were the alkaline single cell gel electrophoresis (comet) assay for DNA damage, a modification of the comet assay for oxidative DNA damage, and the micronucleus assay for chromosome damage. The comet assay and the micronucleus assay in BEAS 2 B cells had earlier been used in studies with other types of nanomaterials, such as metal oxide nanoparticles and carbon nanomaterials, and the techniques had been modified for

testing of small particles (Falck, Lindberg et al. 2009; Lindberg, Falck et al. 2009). To further assess the suitability of the assays, preliminary studies were performed using microcrystalline (Avicel) and nanocrystalline cellulose (whiskers) as model materials. For tests with NFCs, further washes were required in the preparation of microscopical specimens for the micronucleus assay, to reduce the opaque NFC layer that was formed on top of the cells when traditional slide preparation techniques were used.

For the *in vitro* treatments, a stock dispersion was prepared on each NFC sample. The dispersion of the materials was enhanced by using bovine serum albumin and sonication. A dilution series was prepared for the various doses of NFCs tested in BEAS 2B cells. The dispersions were used immediately after preparation. Before the actual genotoxicity tests, the cytotoxicity to BEAS 2B cells of each NFC material was examined, to find a suitable dose range for the genotoxicity assays. Semiconfluent BEAS 2B cell cultures were exposed to various doses of the different NFC materials for 24 and 72 h on 24-well plates (20 000 cells/well). Cytotoxicity was assessed by counting the total number of cells and living cells using propidium iodide staining and fluorescence microscopy with phase-contrast. Propidium iodide stains only dead or dying cells. The cell count obtained after NFC treatment was compared with the cell count of untreated control cultures. As none of the NFCs was able to reduce cell number by ≥ 50%, the highest tested dose was set at 250 μg/cm^2. In addition, a number of lower doses were studied.

The comet assay was used to study DNA damage and oxidative DNA damage after a 24-h treatment of BEAS 2B cells with the NFCs in 24-well plates. The assay reveals strand breaks (SBs) in nuclear DNA of the cells. Untreated controls were included in all series. 20 mM hydrogen peroxide was used as a positive control treatment. After the 24-h treatment, the cells were mounted onto a gel on a microscopic slide and subjected to alkaline conditions to remove other cellular components than nuclear DNA (nucleoid). In electrophoresis, DNA containing strand breaks migrated out of the nucleoid, forming a "comet tail". The DNA was stained with a fluorescent dye (propidium iodide), and the proportion of DNA in the tail (in comparison with total DNA of a nucleoid) was measured from coded samples in a fluorescence microscope using a semiautomated analysis system. Oxidative DNA damage related to the possible formation of reactive oxygen radicals was assessed similarly except that formamidopyrimidine DNA glycosylase (Fpg), a DNA repair endonuclease, was used that cuts DNA at the site of oxidative adducts, creating DNA single-strand breaks. The proportion of oxidative DNA damage was obtained by comparison with results obtained without Fpg.

Micronuclei (MN) in BEAS 2B cells *in vitro* were studied using the cytokinesis-block micronucleus assay. This approach is based on the OECD guideline for *in vitro* micronucleus test. MN are formed from chromosome or chromatid fragments and whole chromosomes that lag behind in cell division. Therefore, the MN assay reveals the ability of the NFC materials to induce structural chromosome damage (clastogenic effect) or numerical chromosome alterations (aneugenic effect). BEAS 2B cells were treated with the test materials for 48 h in T25 flasks. Cytochalasin-B (Cyt-B) was added for the last 24 h of the treatment, to induce binucleation of dividing cells. MN were exclusively analyzed in such cytokinesis-blocked binucleate cells, to ascertain that all cells analyzed have divided once after the treatment. MN can be formed only in dividing cells.

Untreated controls were included in all series. Mitomycin C was used as a positive control treatment in the MN assay. MN were analyzed from coded slides (1000 binucleate cells per culture) stained with acridine orange using a fluorescence microscope. Acridine orange stained DNA green and RNA (cytoplasm) red, which facilitated reliable identification of binucleate cells and MN.

2.1.1.3 Immunotoxicity

Sample preparation
NFC dispersions were prepared shortly before cell exposures. The material samples were dispersed in cell culture media which was supplemented with growth factor and antibiotics that support the maintenance of exposed cells. In addition bovine serum albumin was added to achieve well-dispersed suspensions. At first, a stock dispersion was prepared and sonicated. Thereafter, a dilution series (30, 100 and 300 µg/ml) was prepared and treated in a similar manner as the stock suspension. The dispersions were used immediately.

Cell stimulations
In the current project, macrophages were used to test immunological responses caused by NFC. These immune cells act as the first line of defence in the organism and play an important role between innate and adaptive immunity. Macrophages are phagocytic cells that are responsible for engulfing and digesting pathogens and foreign particles. When these cells detect external hazards, they start to release signalling-molecules (cytokines and chemokines) that recruit other immune cells to the affected site.

Human macrophages were obtained from monocytes isolated from healthy donor blood by allowing the monocytes to differentiate to macrophages. Thereafter, half of the macrophages were preactivated with lipopolysaccaride (LPS), a compound occurring in bacterial wall. This step was used to simulate real-life situation; the approach allowed one to study the release of more stringently controlled cytokines, e.g. interleukin (IL)-1β. Exposure to NFC lasted for six hours, and three different dispersion concentrations were used to assess dose-response relationship. The duration of the exposure was chosen based on previous studies which had shown that this time point was most suitable for investigating immunotoxic effects caused by fibrous nanomaterials.

Sample analysis
After the NFC exposure, the cell culture medium was collected to analyze proteins that had been secreted from the cells in response to the nanomaterial. Thereafter the cells were lyzed and the lysates were collected for messenger RNA (mRNA) analysis. The expression and secretion of tumor necrosis factor (TNF)-α and IL-1 β, two most relevant pro-inflammatory cytokines secreted by macrophages, were then studied. A detailed description of methods has been published earlier (Palomaki, Valimaki et al. 2011).

2.1.2 In vivo studies

Relevant *in vivo* models are essential in the examination of disease mechanisms and in developing strategies to prevent or treat diseases, as *in vitro* systems cannot provide the complex and tightly regulated environment of organs and cells present in the living organism. *In vivo* models are especially important in the case of nanomaterials, since knowledge of their behaviour in the pulmonary systems and elsewhere in the body is extremely limited. Furthermore, comparative *in vivo* data are not available to evaluate the performance of *in vitro* assays to predict toxic effects detectable *in vivo*. It should also be noted that the *in vitro* assays and the nematode tests are readily suitable for the testing of several materials, while the *in vivo* exposure studies are technically demanding and can be utilized only for selected materials. The NFC material used in both in the *in vivo* studies of SUNPAP was NFC-TE/CTP which contained 50 ppm Busan 1009 biocide.

2.1.2.1 Nematode model

The nematode (*Caenorhabditis elegans*) is a simple organism, possessing, however, digestive tract, xenobiotic metabolism, and primitive nervous system. That is why it can be used to assess the systemic effects of chemicals and materials. The particular nematode stock used in the studies expresses the Green Fluorescent protein in their nervous system, fluorescent ganglia indicating functional and intact neural cells.

The exposure to NFC-TE/CTP (with but also without biocide) was carried out by suspending the test material into a buffer (3.0g KH_2PO_4, 6.0 g Na_2HPO_4, 5 g NaCl, in 1 L of 1 mM $MgSO_4$ solution). The suspension was vortexed and ultrasonicated for 5 minutes. Subsequently the suspensions were pipette into microplate wells into which adult nematodes (10 per microplate well) were transferred. The viability, behaviour and reproduction of the nematodes were followed for 48 hours. In the end of the experiment the fluorescence of the ganglia was checked microscopically.

2.1.2.2 Pharyngeal aspiration exposure of mice

Pharyngeal aspiration has certain advantages over inhalation exposure. With aspiration, the actual dose delivered to the lungs can essentially be ascertained, and the technique permits the introduction of a range of doses of aqueous materials into the lungs. In these experiments female C57Bl/6 mice were treated by single pharyngeal aspiration with four different doses of (10, 40, 80 and 200 µg per mouse) of NFC-TE/CTP with biocide (50 ppm). The two lowest doses were relevant for immunotoxic endpoints and the two highest were added to achieve reliable genotoxic endpoints. This single exposure technique with relevantly early sacrifice of the animals makes it possible to assess acute inflammatory responses.

As materials need to be diluted and dispersed into something inert, phosphate-buffered saline (PBS) was used as a control treatment. As NFC-TE/CTP contained Busan 1009, mice exposed to this biocide were included as an additional group, to control for the

possible effects of the biocide. The amount of Busan 1009 given was equal to the amount that the highest NFC dose contained. To get an idea of the scale of toxicity, mice were also exposed to a known toxic fibrous multiwalled carbon nanotube (MWCNTs), as a fibre control.

Samples were collected from the mice 16 h after the exposure. This timepoint was chosen as a compromise to get reliable results for both the immunotoxicological and genotoxicological endpoints. At this timepoint, a good picture of the infiltrating cells could be obtained, while the levels of cell-signalling molecules (cytokines) could sometimes have reached their highest levels at an earlier timepoint.

2.1.2.2.1 Genotoxicity

The purpose of the *in vivo* genotoxicity studies was to examine, whether NFC is able to produce genotoxic effects in bronchoalveolar lavage (BAL) cells of mice after exposure by pharyngeal aspiration. BAL cells were chosen for the analysis, because they consist mostly of macrophages which are the first line of defence against pulmonary exposure to particles. Macrophages will receive most of the NFC dose of the animal and represent, therefore, a worst-case scenario as concerns *in vivo* exposure of pulmonary cells. Genotoxicity was assessed by the analysis of DNA damage and oxidative DNA damage in BAL cells, using the alkaline comet assay.

2.1.2.2.2 Immunotoxicity

For immunological endpoints, infiltrating cells in BAL fluid, mRNA levels of major inflammation related cytokines in the lung tissue and selected cytokines also on the protein level were analysed. In addition, lung histology samples were studied. From these endpoints, a good overall picture of the inflammatory status of the mice at this time point could be seen.

2.1.3 Conclusions of the hazard assessment

The outcome of the toxicological test is shown in Tables 1 and 2.

The NFCs did not have cytotoxic effects *in vitro*, indicating that they do not directly interfere with vital cellular functions (Table 1). Only marginal signs of cytotoxicity could be seen at maximum concentrations tested (1-2 mg/ml), and these effects were more likely to be artefactual than specific, resulting from purely physical interactions of fibres and exposed cells. In conclusion, the cytotoxic hazard of the NFC samples studied appeared to be remote, suggesting their direct interactions with cellular processes is negligible.

Results on the genotoxicity of NFCs suggested a slight ability to induce DNA damage or oxidative DNA damage in cultures of human bronchial epithelial cells *in vitro*, although the results were somewhat variable (Table 1). In this respect, the NFCs seemed to

resemble most nanomaterials which have been positive in the comet assay. However, it should be remembered that the comet assay measures DNA damage that is repairable and may not necessarily lead to chromosome damage or gene mutations. No positive results were obtained in the micronucleus assay with the BEAS 2B cells, suggesting that the NFCs did not have the capacity to induce chromosome damage.

Only one of the samples (NCF-TE/CTP) was able to slightly increase the level of TNF-α mRNA and protein and IL-1β mRNA *in vitro*. This material and most of the other NFCs tested showed the presence of bacteria. Hence, the slight inflammatory effect was associated with a combined exposure to NCF-TE/CTP and bacteria rather than the NFC alone, which should be kept in mind when the inflammatory effects of NFCs are considered.

According to the nematode test *in vivo* the test material NFC-TE/CTP (with and without biocide) did not cause mortality, affect the movement, interfere with the production of eggs and larvae, or quench the fluorescence of ganglia during the 24-h exposure at a concentration of 0.5 mg/ml. Exposure of nematodes to carbon nanotubes at nearly cytotoxic concentrations did not induce any effects either. While no measurement of the amounts ingested NFC was feasible, the nematodes are known to indiscriminately swallow particles of the size of a bacterium or smaller. Therefore, the exposure model can be considered representative.

NFC-TE/CTP containing biocide was also studied in mice *in vivo* after exposure by pharyngeal aspiration. The results showed a clear induction of pulmonary inflammation, with a dose-dependent increase in neutrophils, lymphocytes, and macrophages in BAL fluid and in the mRNA level of the pro-inflammatory cytokine IL-6 in lung tissue (Table 2). However, no increase in the level of DNA damage in BAL cells was seen in the same animals. These results clearly showed that NFC-TE/CTP containing biocide had inflammatory potential when given to mice by pharyngeal aspiration. The biocide alone did not have an effect. Despite the biocide, the material contained some bacteria, and it could contain small residues of the reaction chemicals from the TEMPO treatment. It could not be determined, if the slight inflammatory effect was due to NFC, bacteria or residues of the reaction chemicals.

Table 1. Summary of the *in vitro* tests performed with NFC.

Test material	**Cytotoxicity** (maximum dose 1-2 mg/ml)			**Genotoxicity and cytotoxicity in human bronchial epithelial BEAS 2B cells** (maximum dose 250 µg/cm^2)				**Immunotoxicity in human monocyte-derived macrophages[1]**			
	HTD	TPC	BSM	Cyto-toxicity	DNA damage: comet assay (lowest positive dose, µg/cm^2)	Oxidative DNA damage: Fpg-modified comet assay (lowest positive dose, µg/cm^2)	Chromosome damage: micronucleus assay	mRNA expression		Protein secretion	
								IL-1β unprimed/primed with LPS	TNF-α	IL-1β unprimed/primed with LPS	TNF-α
NFC-VTT	-	-	-	-	-	+ (5)	-	-/-	-	-/-	-
NFC-CTP/VTT	-	-	-	-	+ (2.5)	-	-	-/-	-	-/-	-
NFC-TE/CTP B1[1]	-	-	-	-	+ (5)	(+) (5)	-	(+)/(+)[4]	(+)[4]	-/-	(+)[4]
NFC-TE/VTT	-	-	-	-	+ (5)	-	-	-/-	-	-/-	-
NFC-TE/CTP B2[2]				-	+ (15)	-	-	-/-	-	-/-	-
NFC-TE/CTP B2[3] (with biocide)				-	+ (5)	-	-	-/-	-	-/-	-

-, negative; (+) equivocal; +, positive; ++, highly positive.
[1] Batch one; NFC was dialyzed in order to remove traces of remaining reactant. No bioside.
[2] Batch two; NFC was produced in pilot scale, washed four times but can contain small residues of the reaction chemicals (TEMPO, sodium bromide and sodium hypochlorite). No biocide.
[3] Batch two; NFC was produced in pilot scale, washed four times but can contain small residues of the reaction chemicals (TEMPO, sodium bromide and sodium hypochlorite). A biocide was added to the suspension.

[4] The test system is sensitive to bacteria and their residues; the materials tested contained some bacteria, and therefore the positive reactions are related to a combined exposure to bacteria, their residues and NFC rather than NFC alone. The NFC-TE/CTP which showed an inflammatory effect was from a different source than the NFC-TE/CTP (with and without biocide) on the last two lines of this table - the latter NFC-TE/CTP was also tested *in vivo* (Table 2).

Table 2. Summary of the *in vivo* tests performed with NFC-TE/CTP.

Test material	**Nematode test**	**Genotoxicity in broncho-alveolar lavage (BAL) cells**		**Immunotoxicity***											
		DNA damage (comet assay)	Oxidative DNA damage (Fpg-modified comet assay)	mRNA expression in lung tissue			Protein secretion in BAL fluid		BAL cells				Histology of lung tissue	
				TNF-α	IL-1β	IL-6	TNF-α	IL-1β	ne	ma	eo	ly	HE	PAS
NFC-TE/CTP B2 [1]	-	-	-	-	-	++	-	-	++	+	-	+	(+)	-

-, negative; (+) equivocal; +, positive; ++, highly positive.

*ne, neutrophils; ma, macrophages; eo, eosinophils, ly; lymphocytes; HE, hematoxylin-eosin staining; PAS, periodic acid Schiff staining.

[1] Batch two; NFC was produced in pilot scale, washed four times but can contain small residues of the reaction chemicals (TEMPO, sodium bromide and sodium hypochlorite). A biocide was added to the suspension. In spite of biocide, the NFC contained some bacteria and therefore the positive reactions were related to a combined exposure to bacteria, their residues and NFC rather than NFC alone.

2.2 Exposure scenarios and exposure assessment

During the SUNPAP project, NFC was manufactured in laboratory and pilot scale and used in up-scaling trials for two different type of applications. To study potential exposure during NFC manufacturing and NFC applications, seven different exposure scenarios were formed. They covered two different types of NFC manufacturing methods in laboratory and pilot scale. In addition, scenarios were formed for two separate application cases covering different steps in manufacturing (Table 3).

Table 3. Description of the scenarios used in exposure assessment

Scenario 1.	NFC-TE/CTP production
Scenario 2.	NFC-VTT production
Scenario 3.	Preparing NFC for coated board
Scenario 4.	Coated board Production
Scenario 5.	Maintenance in production of Coated board
Scenario 6.	Preparing NFC for Non-woven
Scenario 7.	Non-woven Production

At this stage, only occupational exposure was estimated in each scenario separately. As there were no measurements quantifying possible exposure in the workplaces, exposure of workers was assessed based on qualitative information and expert judgements. Exposure estimation was based on possible exposure routes (inhalation and dermal), consideration of the work processes (etc. grinding, pouring, spraying paper machine felts with clean shower waters or cutting), amounts of material used, dustiness or mistiness of the used material, time of exposure, and number of workers. The engineering controls (local ventilation, enclosure system, fume-hood, and personal protection equipment) used were taken into account. Within the present project we did not measure the airborne NFC levels in different procedures. The available data are limited to only one publication by Vartiainen et al. (Vartiainen 2011) where the level of nanoparticles in air during production of MFC using Masuko grinding was reported. However, this data was far from sufficient.

In the exposure assessment, Control Banding approaches were used in estimating the level of exposure. The estimated levels of exposure were low in all defined scenarios, reflecting the facts that NFC is used in wet stage and the number of workers is low in all scenarios.

2.3 Risk assessment

2.3.1 The use of Control banding methods

For qualitative risk assessment of nanomaterial the control banding (CB) strategies has been proposed. This concept was originally developed for safety control of bulk materials in small enterprises to assist the industry in ensuring the safety of workers in situation when little hazard information was available. It is well suited for industry working on development of new chemicals/materials. Lately this approach has been modified to make it applicable for nanomaterials (Maynard 2007). CB is an approach which combines risk assessment and risk management. CB method offers simplified solutions for controlling risk in the workplace in the absence of adequate toxicological and exposure data. CB tool can be used both to conduct a preliminary risk assessment and to point out the information gaps that need further studying. Currently, this approach is particularly useful in nanotechnology applications, considering the uncertainty over risk associated with nanomaterials and how these risks can be assessed and managed. The main goal of the qualitative risk assessment is to assess the likelihood of the danger under particular exposure scenario and give some recommendation on possible control measures. The principle of such approach is that the higher the hazard, the stricter the controls need to be.

There are several CB tool used internationally and nationally. In present project we used two different CB tools, CB Nanotool and Stoffenmanager Nano Module 1.0. CB Nanotool was developed by Paik et al. (Paik, Zalk et al. 2008) and applied to different nanomaterials and evaluated by Zalk et al. (Zalk, Paik et al. 2009). Stoffenmanager Nano Module 1.0 has been designed by Dutch authorities together with TNO and Arbo Uni to assess exposure to manufactured nanomaterials via inhalation. The principles of the tool are described by Van Duuren-Stuurman et al (Van Duuren-Stuurman, Vink et al. 2012).

2.3.1.1 CB Nanotool

The CB Nanotool can be applied to all nanomaterials and covers different scenario. The tool is user friendly and freely available. CB Nanotool requires introduction of information on the toxicity of the materials, some information on the material characterization, and the process information, but also allows some assumptions. This is rather convenient, when some information is missing. However, at the same time it can introduce some misjudgements and, therefore, the CB Nanotool should be used cautiously.

CB Nanotool is based on determination of severity and estimation of probability. For each factor scores are given. The combination of the severity and probability scores determines the Risk Level (RL) which corresponds to certain control approach. There are four RLs in CB Nanotool (Table 4).

Table 4. Risk level matrix employed in CB Nanotool. Adopted from Paik et al., 2008.

Probability / Severity	Extremely unlikely (0-25)	Less likely (26-50)	Likely (51-75)	Probable (76-100)
Very high (76-100)	RL3*	RL3	RL4	RL4
High (51-75)	RL2	RL2	RL3	RL4
Medium (26-50)	RL1	RL1	RL2	RL3
Low (0-25)	RL1	RL1	RL1	RL2

*the overall Risk Level (RL) without controls and recommended engineering control based on RLs are as follows:
RL1 (total scores < 125): General ventilation
RL2 (total scores 125-150): Fume hood or local exhaust ventilation
RL3 (total scores 150-175): Containment/Enclose the process

CB Nanotool assesses the hazard level of nanomaterial (severity) by evaluating number of severity factors: toxicity (carcinogenicity, mutagenicity, reproductive and dermal toxicities), physico-chemical properties, such as nanomaterial shape, size and surface reactivity, of nanomaterial as well as some toxicity data and the occupational exposure limit (OEL) on the bulk material. The developers of this tool gave rather high weight to physico-chemical properties of the nanomaterials, based on available literature on the effect of particle's size, shape and surface chemistry on their biological activity.

The exposure probability is evaluated based on information on the amount of handled material, number of employees, frequency of operation and its duration, and level of dustiness/mistiness. The later factor is most important in exposure evaluation and has the highest weight.

During hazard level estimation we have found that CB Nanotool was rather sensitive to the fibre size of the NFC. Change in fibre's diameter from 11-40 nm to > 40 nm reduced Severity Band from "high" to "medium". This consequently decreased overall risk level from RL3 to RL2. It is important to be aware of such sensitivity when analysing NFC samples which might be not very homogeneous in size. In case of two NFC samples (NFC-VTT and NFC-TE/VTT), it was difficult to estimate more or less precise fibre diameter. In this case, it had to be assumed that the size of the fibres was >40 nm. For two other NFC samples, the size was more precisely estimated to be 10-40 nm in diameter. In case, when the sample/material is not homogeneous and contains different kind of fibres and particles, the decision of the size might be challenging. To make such decision the expert should probably look at the amount of material handled. CB Nanotool gives the maximum points if >100 mg of nanomaterial is handled. This may help the expert to estimate the amount of NFC with certain size needed to be taken into account. For instance, for the heterogeneous NFC sample in which the fibrils of 11-40 nm in diameter are only 0.1% of total sample and the amount of NFC used/produced during the operation is 10 kg (dry NFC), the size in severity factors should be reported as 11-40 nm.

Another challenge was the description of surface reactivity, a parameter that had a high impact on the outcome. Surface reactivity should be described as “low”, “medium” or “high”. Surface activity can be defined by markers of inflammation if such data are available (Paik et al 2008). However, while there are no quantitative means to define surface reactivity, it is depending on the expert's judgement.

Hazard data available from the toxicological studies of SUNPAP were limited and not sufficient for CB Nanotool. However, CB Nanotool gives weight to “unknown” (75% of point of high rating). We found that the same severaty band "high" was given for NFC (fibrous nanomaterial, fibre diameter 11-40 nm) when all toxicological properties were marked as “unknown” or “yes” (positive).

As mentioned above, in exposure probability estimation the dustiness/mistiness factor is rated relatively high. Generally, liquid-suspended materials (the case of NFC production) are rated in this category lower than dry non-agglomerated ones. However, the level of mistiness should be estimated by evaluating the surfaces within working area. This shows that the risk assessment using CB Nanotool should be conducted by a person with a good knowledge on the process. The possibility to visit the production/application sites during the operation is a crucial. Within SUNPAP project we have visited pilots with Masuko and fluidizer and were able to check along of other parameters the levels of surface contamination.

2.3.1.2 Stoffenmanager Nano Module 1.0

The Stoffenmanager Nano Module 1.0 tool can be used for all types of synthesized nanoparticles and –fibres whether or not they are agglomerated or aggregated. It focuses in to the inhalatory exposure assessment. The tool is web based and freely available but requires registration. Stoffenmanager Nano asks for information of the material characterization, the process information and the measures used for the reduction of potential exposure. In some cases it also requires some information about the toxicity of the materials. In all cases the tool also allows assumptions or “not known” as an answer. Stoffenmanager Nano is aimed to be a tier 1 tool for prioritising risk. Later it could be developed to a quantitative risk assessment tool (Van Duuren-Stuurman, Vink et al. 2012).

Stoffenmanager Nano is based on the determination of hazard and exposure through various attributes. For each answer a score is given. These scores ultimately result to a certain hazard band (A-E) and to certain exposure band (1-4) described in table 5. The combination of these bands determines overall risk priority (1-3) (Table 5).

Table 5. Bands used in the Stoffenmanager Nano Module 1.0. Adopted from Van Duuren-Stuurman et al., 2011.

Hazard band / Exposure band	A	B	C	D	E
1	3	3	3	2	1
2	3	3	2	2	1
3	3	2	2	1	1
4	2	1	1	1	1

Hazard band: A= lowest hazard, E= highest hazard. Exposure band: 1=lowest exposure, 4=highest exposure. Overall result: 1= highest priority, 3= lowest priority

Stoffenmanager Nano assesses the hazard level of the nanomaterial by evaluating factors including: physico-chemical properties such as solubility and fibre shape and size.

The exposure probability is evaluated based on the process information such as description of the source process, size of the working space, maintenance schedule, description of the handling process, duration of the handling, frequency of the handling, distance of workers to the emission source, the product type, concentration, dustiness or moisture content, viscosity, control measures like personal protective equipment and ventilation.

Seven separate scenarios were assessed with Stoffenmanager Nano during the SUNPAP project. It was noted that the tool offers limited possibilities to take into account the toxicological results obtained from the project. If the assessed material is seen to be fibre or fibre like, no information about the hazard is asked for. On the other hand, if the material is not fibre, only little information of the hazard is asked for. The developers of Stoffenmanager Nano have given a rather high weight to the particle shape and size in determining the potential hazard. In addition fibres are always placed in to the highest hazard band based on the paradigm that "all insoluble fibres thinner than 3 nm and longer than 20 nm are biopersistent in the lungs and therefore highly hazardous" (Van Duuren-Stuurman, Vink et al. 2012). Concerns are related to the potential asbestos-like carcinogenic effects after inhalation of insoluble manufactured nano-objects (MNOs). It is also stated by Van Duuren-Stuurman et al (Van Duuren-Stuurman, Vink et al. 2012) that it's possible that nano-fibres which are not associated with persistence and carcinogenic effects are misclassified in the hazard band E. This possibility of misclassification should be assessed separately in the case of NFC. The approach used in Stoffenmanager Nano might change when there is more information about the toxicity of different nanosized fibres.

According to these results, it seems that the Stoffenmanager Nano tool gives quite conservative estimations to nanosized fibres about the risk priorities and thus executes the precautionary principle. It has to be noted that it may not be the most suitable tool for assessing the risk related to non-toxic biobased materials such as NFCs as there are

no possibilities to utilize the information available from the hazard assessment studies conducted within the project.

However, Stoffenmanager Nano assesses exposure in occupational environment in detail. It takes into account the source of emission, transmission and immission (Van Duuren-Stuurman, Vink et al. 2012). All the scenarios assessed with the Stoffenmanager Nano resulted in to the low exposure band. This result was affected by the facts that NFC was handled as dilution with low concentrations and high viscosity. The process didn´t include e.g. high pressure and it was assumed that regular maintenance practices were in place in addition to good ventilation. It seems that the most important information related to exposure was collected in the tool. In a general level almost all the information required by the tool could be easily obtained and used to assess the exposure with the tool. The only issue noted in the assessment of exposure was the limited possibilities to select appropriate localised control measures and ventilation used which probably have effect to the exposure.

2.3.2 Combined protocol for risk assessment

In the final risk assessment protocol, the results from the health hazard assessment of the SUNPAP and the exposure assessment of defined scenarios by using CB-methods (Stoffenmanager Nano) were combined. The reasons for this combination were that the CB-methods used did not include all the toxicological information that was obtained in the SUNPAP project, and the toxicological information that is needed for CB-Nanotool is not available yet. The hazard banding in CB-Nanotool is based on physico-chemical properties, carcinogenicity, reproductive toxicity, mutagenicity and dermal toxicity of the nanomaterial and also parent material. These toxicological endpoints of nanomaterials are seldom known. The hazard assessment protocol in SUNPAP consisted of screening the bioactivities of NFC with cytotoxicity, genotoxicity and immunotoxicity *in vitro* and *in vivo* assays and screening of systemic effects with nematode model (described in Chapter 2.1).

In the SUNPAP project all NFCs were produced in short trials in pilot scale and it was not possible to assess exposure to NFC by measuring airborne concentrations at workplaces. Therefore, the potential exposure was assessed with the help of Stoffenmanager Nano, which is like first tier in the occupational exposure assessment. In the first tier it is essential to gather all the information from the work environment, the processes, possible exposure routes, the materials used, the number of workers, the duration of the work, and the engineered controls used. Stoffenmanager Nano gives for this information comparable estimation of the potential exposure (exposure band) in the studied working conditions.

2.3.3 Nanotechnologies - Nanomaterial risk evaluation by ISO/TR 13121

The International Organization for Standardization (ISO) has published a Technical Report ISO/TR 13121 Nanotechnologies-Nanomaterial risk evaluation (First edition 2011-05-15) . This report is based on the Nano-Risk Framework, an approach created by the Environmental Defense Fund and DuPont. This approach can be used in evaluating and managing potential risks of manufactured nanomaterials, especially in the product development.

ISO/TR 13121 gives a tiered approach to the health hazard testing aligned with product development. An organization may apply Tier 1 testing (general assessment of cytotoxic potential using in vitro and in silico methods) during the research and development stage, move to Tier 2 testing (e.g. in vitro pulmonary, cancer, neurological, reproductive, cardiovascular, and developmental toxicities) when the nanomaterial/product moves to a prototype stage, apply Tier 3 testing as the nanomaterial/product is at test marketing, and complete Tier 4 testing when the nanomaterial/product is moved to commercial launch. At each step the hazard testing gets more demanding, more specific and more expensive. For exposure assessment, the process of developing an exposure profile is described. It is important to assess the potential for release of manufactured nano-materials, define the routes of exposure, engineering measures used, and personal protection equipment. Also processes and the effectiveness of the engineering controls should be defined. The standard also includes guidance on how to obtain exposure information in every stage of the lifecycle of the nanomaterial: manufacture (e.g. production volumes, industrial functions, stage of development, physical form of nanomaterials, maximum concentration, number of employees), processing, use, distribution/storage, waste processing, and post use management. Also workplace monitoring is described in the standard.

3 DISCUSSION ABOUT THE SUITABILITY OF THE USED METHODS AND METHODOLOGIES

3.1 Hazard assessment

Cytotoxicity assays *in vitro* did not indicate an effect of NFC on cell viability, whereas a cytotoxic effect was seen with carbon nanotubes, indicating that the test system is capable of detecting the cytotoxic effects of fibrous nanomaterials.

Immunotoxicological assays *in vitro* showed a slightly positive response with one NFC especially when LPS priming (data not shown) was used, suggesting that this NFC can induce a marginal inflammatory response. However, as the NFC sample contained bacteria and bacterial components, the possible effects of NFC could not be separated from those of bacteria. The fact that carbon nanotubes, known to be strong inducers of inflammation *in vivo*, induced a strong response also *in vitro*, suggested that the immunological *in vitro* tests are capable of detecting immunotoxic fibers. The *in vitro* immunotoxicity assays were technically applicable to NFCs. There are presently no international guidelines for *in vitro* immunotoxicological testing, and the assays have not been validated for assessing nanomaterials. However, previous studies have suggested that the assay based on LPS priming, mimicking co-exposure to microbial components (the usual situation *in vivo*), is a promising new approach for *in vitro* identification of the inflammatory effects of nanomaterials.

In vitro genotoxicity assays suggested a slight induction of DNA damage or oxidative DNA damage by all NFCs tested, similar to a number of other inert nanomaterials. The results of the micronucleus assay were consistently negative. The slightly positive comet assay observed with the NFCs *in vitro* reflects the potential of these materials to damage DNA. However, as the micronucleus assay was negative, NFCs were not able to induce misrepaired DNA damage resulting in chromosome breakage or damage to the mitotic apparatus resulting in whole chromosomes micronucleation. The mechanisms of DNA damage induction by small "inert" particles *in vitro* are presently unclear. Both the comet assay and the micronucleus assay were technically applicable to NFCs. The *in vitro* micronucleus assay is an OECD test that has been validated for genotoxicity testing of chemicals. No genotoxicity assays have presently been validated for nanomaterials but the methods seem to be applicable.

The nematode assay was not positive with either NFCs or carbon nanotubes. A possible reason is that high concentrations of this type of test materials are not feasible due to sedimentation of the material. Positive results have recently been reported with inorganic nanoparticles (REF), indicating that there may be qualitative differences between organic and inorganic materials in this assay.

It is concluded that *in vitro* assays can technically be used to show cytotoxicity, immuno-toxicity and genotoxicity of NFCs. NFCs showed slightly positive genotoxic and (for one NFC) immunotoxic responses *in vitro*, suggesting that the materials are not completely inert. It is presently unclear if such weak positive findings are predictive of an *in vivo*

response and how well the *in vitro* assays are, in general, able to identify hazardous nanomaterials.

In the present project, NFC-TE/CTP was studied both *in vitro* and *in vivo*. Exposure of mice to this material by pharyngeal aspiration resulted in a clear induction of pulmonary inflammation. However, no DNA damage was seen in BAL cells. With the experimental conditions used, the *in vitro* immunotoxicity assays (negative) and the *in vitro* DNA damage assay (positive) were not predictive of the *in vivo* outcome with NFC-TE/CTP, while the negative *in vitro* micronucleus assay agreed with the negative *in vivo* DNA damage assay. However, too few data are presently available, to generally judge the performance of each *in vitro* assay in correctly detecting nanomaterials that will or will not induce inflammation or genotoxic effects *in vivo*. Data on the correlation between *in vitro* and *in vivo* assays with nanomaterials is urgently needed.

A confounding factor in *in vitro* (and to an extent also *in vivo* trial) is the frequent microbial contamination of NFC-samples (filamentous fungi, bacteria and yeasts). Since this contamination is in practice impossible to eliminate, it often necessitates the inclusion of a biocide in the test material. This has to be taken into account when designing the experiments and the proper controls. Although the growth of micro-organisms can be controlled in the experimental setting, microbial cells (dead or alive) and their metabolites can influence the results (particularly the immunological effects). Consequently, it is not, at the moment, possible to totally discriminate between the effects of NFC and the combined effects of NFC and the microbial contaminants. Of course, the microbially contaminated material is the one to which the exposure in occupational settings occur, and in that sense the results are relevant regarding the practical implications. Anyway, culturing the samples on appropriate media to evaluate the microbial load is recommended as a part of the sample characterization. The NFC-sample that was used in *in vivo* studies may have contained also the residues of the reaction chemicals used (TEMPO, sodium bromide and sodium hypochlorite), even if this NFC –sample was washed in four stages.
In vivo, analysis of BAL fluid for inflammatory cells and pro-inflammatory cytokines and chemokines, as performed in this project for NFC-TE/CTP, if often part of standard *in vivo* toxicology studies complementing histopathology examinations when studying pulmonary irritant aerosols and agents that may be deposited and retained in the lower respiratory tract (poorly soluble particles). These techniques are well established, and a number of previous studies have shown pulmonary inflammation after exposure of rodents to various nanomaterials and other small particles.

However, *in vivo* genotoxicity studies using BAL or lung cells in assessing local genotoxic effects of pulmonary exposure to nanomaterials are still few. Nevertheless, such techniques are very promising, and should be more widely applied and developed. The mouse model used in SUNPAP, combining BAL analyses of inflammatory parameters and DNA damage in the same animals may provide a suitable approach for studying the immunotoxic and genotoxic effects of nanomaterials *in vivo*. Such studies could also be used to assess the tentative association between inflammation and genotoxicity in particle carcinogenesis.

3.2 Risk assessment

A qualitative risk assessment was employed in SUNPAP, because the toxicological data were scarce and there were no measured airborne concentrations of NFC in the workplaces. Qualitative risk assessment was performed by using Control Banding (CB) approach for nanomaterials and combining CB-method and the hazard assessment done in this project. CB-approach was originally developed to assist industry in ensuring the safety of workers in situation when little hazard information was available. Currently, CB-approach has been modified to be used with nanoparticles. In SUNPAP we tested two CB-tools: CB Nanotool developed by Paik et al. (2008) and Stoffenmanager Nano Module 1 developed by Dutch authorities.

The hazard assessment methods used in SUNPAP were seen as suitable for as a first step in hazard assessment of NFC. In *in vitro* tests NFC was not toxic or marginally toxic, and the results were clearly weaker than the used positive controls (carbon nanotubes). The nematode model indicated that NFC does not have systemic effects. However, the pharyngeal aspiration studies with mice indicated acute inflammatory effects of inhalation of NFC, which is the most likely route of exposure at the workplaces. Workers may also be exposed to NFC after dermal contamination and splashes of NFC. However, according the present knowledge, the healthy skin is a good barrier to the nanomaterials (Christensen, Johnston et al. 2011).

In the exposure assessment, the first step was to define the exposure scenarios, which included two different types of NFC manufacturing in laboratory and pilot scale and also various steps in manufacturing two different NFC applications. The information used in the scenarios included information from the laboratory and pilot scale plants, expert judgments and modeled information for production plants for applications. Four different exposure scenarios were tested with CB Nanotool and seven different exposure scenarios with Stoffenmanager Nano. The conclusion was that the possible exposures were placed into the low exposure band in all the scenarios defined. Reasons for the low exposure bands were the facts that the amount of workers in each scenario was low, NFC was used in the processes in wet stage, production systems in some scenarios were partly closed, fume hoods and mechanical ventilation were used, and regular maintenance and cleaning were assumed.

General comment on CB Nanotool is that it focuses more to the hazard assessment taken in account some toxicological data and physico-chemical properties of nanoparticles. CB Nanotool is sensitive to fibre’s size. This presented some difficulties with NFC, which is not very homogenous, and the size of fibres varies a lot in NFC-materials. CB Nanotool is also sensitive to description of surface reactivity which is dependent on the expert's judgement. The hazard data from toxicological studies obtained in SUNPAP are not sufficient for CB Nanotool and the use “unknown” as an input for toxicity in hazard evaluation resulted in severity estimated as “high”.

Stoffenmanager Nano focuses on the occupational inhalatory exposure. Exposure banding includes the source of emission, transmission and immission. It also takes into account more hazard and exposure bands than CB Nanotool. The tool offers very limited

possibilities for taking into consideration the toxicological results of the SUNPAP project, especially in the case when a fibre or fibre-like particles are assessed. Stoffenmanager Nano tool assigns all insoluble nanofibres, also NFC, to the highest hazard band because of the concern for asbestos-like carcinogenic effects after inhalation (Van Duuren-Stuurman et al., 2012). The hazards estimated within SUNPAP project suggests that NFC is not very hazardous material, even that it had slight toxic effects *in vitro* and inflammatory response from the *in vivo* tests. However, the exposure banding part is quite detailed and the most important information related to the exposure was collected with the tool. Stoffenmanager Nano tool placed all the defined exposure scenarios of NFC in the lowest exposure band. In general, this tool may not be the most suitable for assessing hazard risks related to biobased materials such as NFCs as there are no possibilities to utilize the information available from the hazard assessment studies conducted within the project.

The overall risk related to the assessed exposure scenarios and used materials are, thus, assessed to be low/moderate. The hazard assessment done in SUNPAP project was like the first tier in the product development. More toxicological tests are needed to study NFC, because some inflammatory responses were seen in the lungs of the mice. The first step in occupational exposure assessment is to define exposure scenarios. In the project, exposure scenarios were defined according to Stoffenmanager Nano tool, which placed all scenarios into the lowest exposure band. At the moment, the quantitative exposure assessment is not possible. The used risk evaluation followed the guidelines of the ISO/TR 13121 made for product development (ISO/TR 13121 Nanotechnologies-Nanomaterial risk evaluation).

4 CONCLUSIONS

The risk assessment is a combination of exposure and hazard assessment. In SUNPAP project, the hazard testing methodology of NFC included *in vitro* cytotoxicity, genotoxicity and immunotoxicity test to give indication whether NFC will cause cellular damage and whether their systemic effects will be likely. In addition a nematode model based test organism was used to investigate potential systemic effects and neurotoxicity. Because the exposure to NFC is likely to happen through inhalation, inhalatory toxicity study with animals was included to assess acute responses.

The exposure assessment was based on the gathered information and expert's judgement of possible exposure scenarios. When pre-commercial and commercial production is started real exposure measurements can be carried out and the measured exposure levels should be used in the risk assessment.

The two common control banding models (CB-Nanotool and Stoffenmanager Nano Module 1) tested in risk assessment of NFC indicated higher risks than in the full production will probably be the outcome, because the engineered control measures are usually planned better in the full production than in the lab or pilot scale production. The risks would be lower if the fact that the biobased NFC materials are not hazardous contrary to the assumptions made solely based on their physico-chemical characterization and the assumed toxicity of fibrils. Therefore, more advanced tools for organic nanomaterials are needed, in which the analysed results of the hazard assessment are also taken into account.

Risk assessment is a demanding process in a product development, where a hazard assessment should follow steps in a development and exposure profiles cannot be preciously defined. The used methodologies were suitable as a first tier hazard and risk assessment and they followed the latest ISO/TR 13121 made for nanomaterial risk evaluation.

5 ABBREVIATIONS

BAL *Bronchoalveolar lavage*
BEAS 2B *Human bronchial epithelial cell line*
BSM *Boar Sperm Motility*
CB *Control Binding*
DNA *Deoxyribonucleic acid*
Fbg *Formamido pyrimidine*
HTD *Highest Tolerated Dose*
IL-1β *Interleukin-1 beta*
ISO *International Organization for Standardization*
LPS *Lipopolysaccharide*
NFC *Nano-fibrillar cellulose*
MFC *Microfibrillated cellulose*
MN *Micronuclei*
MNO *Manufactured Nano Object*
mRNA *Messenger ribonucleic acid*
MWCNT *Multiwall carbon nanotube*
OECD *The Organisation for Economic Co-operation and Development*
OEL *Occupational Exposure Limit*
PBS *Phosphate-buffered salina*
RL *Risk Level*
RNA *Ribonucleic acid*
ROS *Reactive oxygen species*
SCENIHR *Scientific Committee on Emerging and Newly Identified Health Risks*
TEMPO *2,2,6,6-Tetramethyl-piperidin-1-oxyl*
TNF-α *Tumor necrosis factor-alpha*
TPC *Total Protein Content*
TR *Technical Report*

REFERENCES

1. "ISO/TR 13121 Nanotechnologies - Nanomaterial risk evaluation. First edition 2011-05-15."

2. Anonymous (2011). "Commission recommendation of 18 October 2011 on the definition of nanomaterial." Official Journal of the European Union **54**: 38-40.

3. Bouwmeester, H., I. Lynch, et al. (2011). "Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices." Nanotoxicology **5**(1): 1-11.

4. Christensen, F. M., H. J. Johnston, et al. (2011). "Nano-TiO--feasibility and challenges for human health risk assessment based on open literature." Nanotoxicology **5**(2): 110-24.

5. Cullen, R. T., B. G. Miller, et al. (2002). "Tumorigenicity of cellulose fibers injected into the rat peritoneal cavity." Inhal Toxicol **14**(7): 685-703.

6. Cullen, R. T., Miller, B.G., Jones, A.D. and Davis J.M.G. (2002). "Toxicity of Cellulose Fibres." Ann. occup. Hyg. **46**(1): 81-84.

7. Donaldson, K., R. Aitken, et al. (2006). "Carbon nanotubes: a review of their properties in relation to pulmonary toxicology and workplace safety." Toxicol Sci **92**(1): 5-22.

8. Falck, G. C., H. K. Lindberg, et al. (2009). "Genotoxic effects of nanosized and fine TiO2." Hum Exp Toxicol **28**(6-7): 339-52.

9. Lindberg, H. K., G. C. Falck, et al. (2009). "Genotoxicity of nanomaterials: DNA damage and micronuclei induced by carbon nanotubes and graphite nanofibres in human bronchial epithelial cells in vitro." Toxicol Lett **186**(3): 166-73.

10. Maynard, A. D. (2007). "Nanotechnology: the next big thing, or much ado about nothing?" Ann Occup Hyg **51**(1): 1-12.

11. Mikkelsen, S., Hansen, E., Christensen, TB., Baun, A., Hansen, SF and Binderup M-L. (2011). Survey on basic knowledge about exposure and potential environmental and health risks for selected nanomaterials. Environmental Project No. 1370 2011, Danish Ministry of the Environment, Environmental Protection Agency.

12. Paik, S. Y., D. M. Zalk, et al. (2008). "Application of a pilot control banding tool for risk level assessment and control of nanoparticle exposures." Ann Occup Hyg **52**(6): 419-28.

13. Palomaki, J., E. Valimaki, et al. (2011). "Long, needle-like carbon nanotubes and asbestos activate the NLRP3 inflammasome through a similar mechanism." ACS Nano **5**(9): 6861-70.

14. Pronk, M. E. J., Wijnhoven, S.W.P., Bleeker, E.A.J., Heugens, E.H.W., Peijnenburg, W.J.G.M., Luttik, R. and Hakkert, B.C. (2009). Nanomaterials under REACH. Nanosilver as a case study. . RIVM report.

15. SCENIHR (2009). Risk Assessment of Products of Nanotechnologies. Scientific Committee on Emerging and Newly Identified Health Risks, European Commission Health & Consumers DG.

16. Stone, V., Hankin, S., Aitken, R., Aschberger, K., Baun, A., Christensen, F., Fernandes, T., Hansen, SF., Hartmann, NB., Hutchinson, G., Johnston, H., Michetti, G., Peters, S., Ross, B., Sokull-Kluettgen, B., Stark, D. and Tran L. (2010). Engineered Nanoparticles: Review of Health and Environmental Safety (ENRHES).

17. Van Duuren-Stuurman, B., S. R. Vink, et al. (2012). "Stoffenmanager Nano Version 1.0: A Web-Based Tool for Risk Prioritization of Airborne Manufactured Nano Objects." Ann Occup Hyg 56(5): 525-541.

18. Vartiainen, J., Pöhler, T., Sirola, K., Pylkkänen, L., Alenius, H., Hokkinen, J., Tapper, U., Lahtinen, P., Kapanen, A., Putkisto, K., Hiekkataipale, P., Eronen, P., Ruokolainen, J. and Laukkanen, A. (2011). "Health and environmental safety aspects of friction grinding and spray drying of microfibrillated cellulose." Cellulose **18**(3): 775-786.

19. Yang, H., C. Liu, et al. (2009). "Comparative study of cytotoxicity, oxidative stress and genotoxicity induced by four typical nanomaterials: the role of particle size, shape and composition." J Appl Toxicol **29**(1): 69-78.

20. Zalk, D. M., S. M. Paik, et al. (2009). "Evaluating the Control Banding Nanotool: a qualitative risk assessment method for controlling nanoparticle exposures." J. Nanoparti. Res. **11**(7): 1685-1704.