Nanofibrillated cellulose: results of *in vitro* and *in vivo* toxicological assays

Final Conference 20.6.2012

Hannu Norppa
Nanosafety Research Center

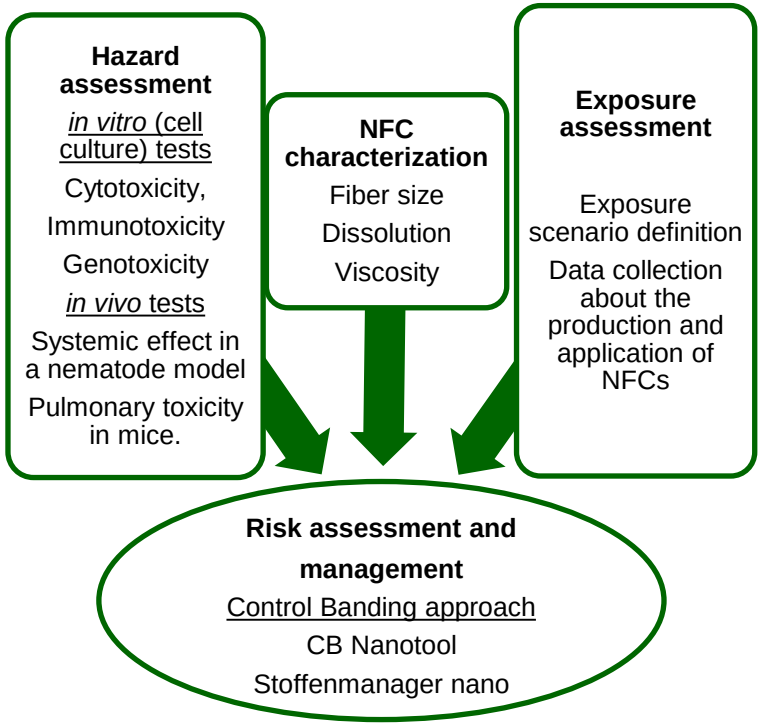
Finnish Institute of Occupational Health

BioSafe
special laboratory services

Hazard assessment approach for NFC

Methodology

- ***In vitro* assays for cytotoxicity, genotoxicity, and immunotoxicity**
- **A nematode model for systemic effects and neurotoxicity**
- **Pharyngeal aspiration study with mice for pulmonary immunotoxicity and genotoxicity**



NFC samples studied

NFC-VTT

- Masuko grinder (5 passages)
- No pre-treatment, no bioside
- Bacteria

NFC-CTP/VTT

- Masuko grinder (5 passages)
- Enzymatic pre-treatment, bioside added to the pulp
- Bacteria

NFC-TE/VTT

- Masuko grinder (3 passages)
- TEMPO-mediated oxidation as a pre-treatment, no bioside
- Bacteria and yeast

NFC-TE/CTP samples studied

NFC-TE/CTP B1

- Lab scale high-pressure homogenizer
- TEMPO-mediated oxidation as pre-treatment
- Dialysed to remove traces of reactant
- No biocide added
- Bacteria

NFC-TE/CTP B2 ± biocide Busan 1009

- Pilot scale high-pressure homogenizer
- TEMPO-mediated oxidation as pre-treatment
- Washed four times but might contained residues of reagents (TEMPO, sodium bromide and sodium hypochlorite)
- Without biocide: bacteria, yeast and mold
- With biocide: some bacteria

Cytotoxicity

- **Highest Tolerated Dose (HTD) assay**
  - Qualitative morphological changes in human cervix carcinoma HeLa cells under an inverted phase contrast microscope
  - Comparison with untreated cells and evaluation on a scale ranging from 0 to 4
- **Only marginal effects seen at top doses (→ 2 mg/ml) of NFCs, probably as a result of mechanical influence**
- **Total Protein Content (TPC) assay**
  - A quantitative test measuring growth inhibition of HeLa cells, total protein content as the indicator of the cells' viability
  - Based on reaction of fluorescamine with compounds containing primary amino groups to produce fluorescent products.
- **None of the NFCs indicated a cytotoxic effect**
- **Boar Sperm Motility Assay (BSM)**
  - A qualitative test particularly suitable for testing of suspensions
  - Boar spermatozoa's movement inhibition indicates mitochondrial or membrane damage
- **No toxic effects seen**

Immunotoxicity – pulmonary inflammation

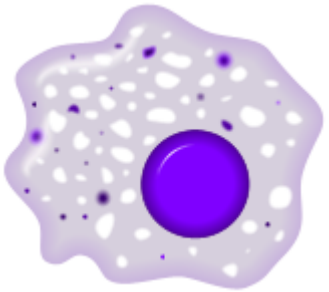
In vitro studies

- Macrophages exposed to several doses of nanocelluloses in cell culture
- **Cytotoxicity** assessed by photometric and luminometric methods
- Macrophage activation assessed by measuring the **expression of essential cytokines**
  - at the mRNA level by real-time quantitative PCR
  - at the protein level by ELISA

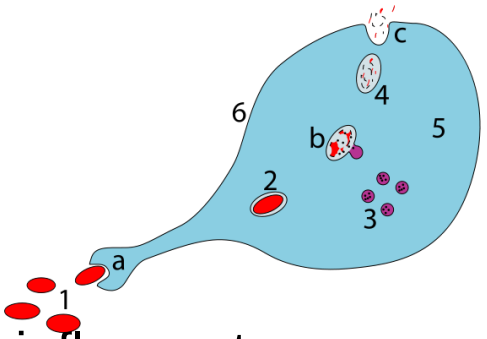
In vivo studies on NFC-TE/CTP B2 + biocide

- Material for *in vivo* studies chosen by the Consortium
- Mice exposed by pharyngeal aspiration
- Inflammatory parameters analysed

Macrophages



- act as the first line of defense against pathogens and particles in the lungs
- ingest pathogens and particles and break them down



- release proinflammatory cytokines which promote inflammatory processes
  - TNF-alpha, IL-1beta, and IL-6 determined in the present study

In the immunotoxicological part of the present study, human macrophages derived from blood monocytes of were used

Figures: Wikimedia Commons

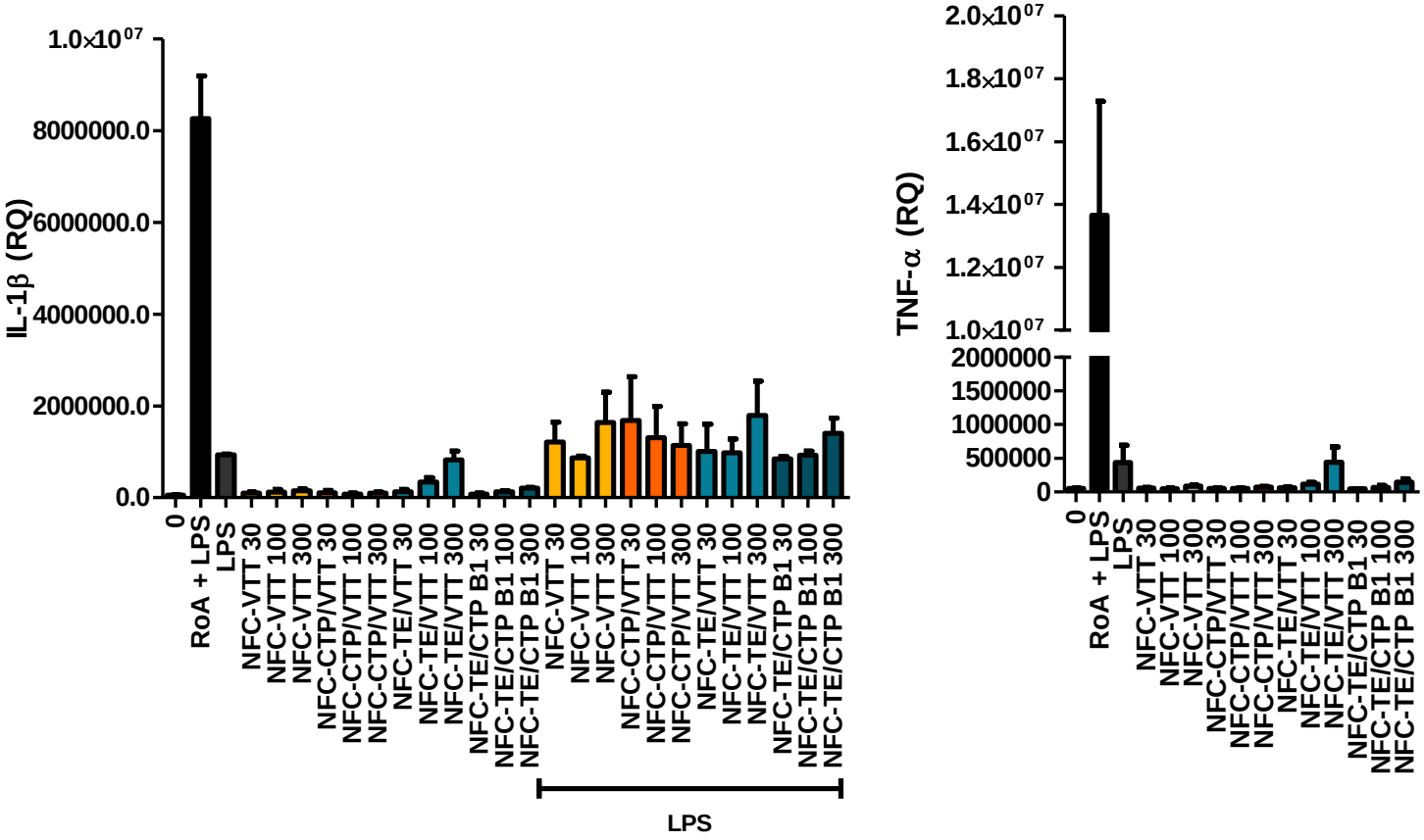
Cytotoxicity in human macrophages

- LDH release from cells expressed as percentage of total LDH, measured photometrically
- ATP in cells expressed as relative luminescence (percent of unexposed control), measured luminometrically
- Human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected
- Cytotoxicity measured by lactate dehydrogenase (LDH) leakage and decrease of ATP (luminescent cell viability assay)
- Roridin A (RoA) and silica used as positive controls

NFCs were not cytotoxic to macrophages at doses up to 300 µg/ml

Ilves et al., in preparation

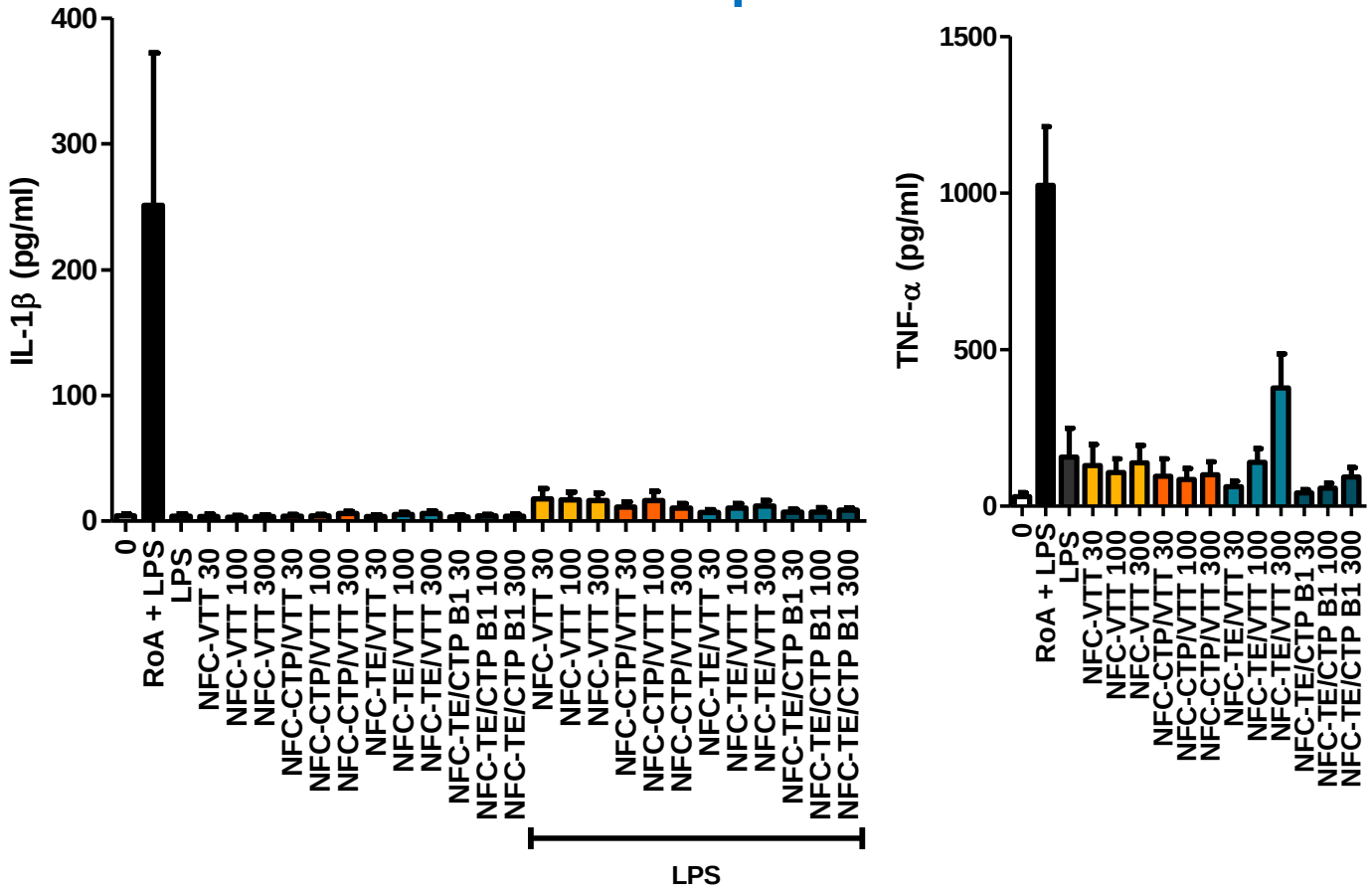
mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFCs



- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, mRNA measured by PCR
- **NFC-TE/VTT slightly increased IL-1-β and TNF-α mRNA (due to bacteria & yeast?)**

Ilves *et al.*, in preparation

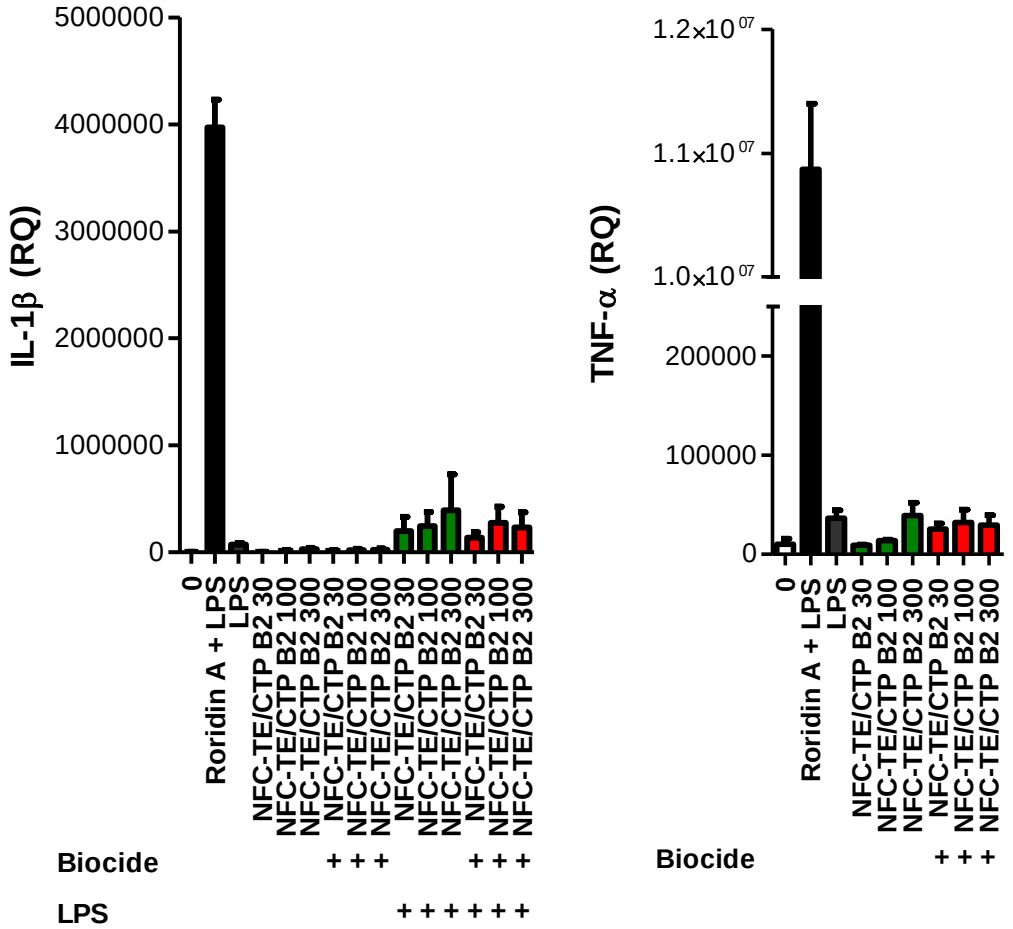
Secretion of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFCs



- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, pro-inflammatory cytokines measured by ELISA
- **All NFCs slightly induced TNF-α and, in LPS-primed samples, IL-1-β**

Ilves *et al*., in preparation

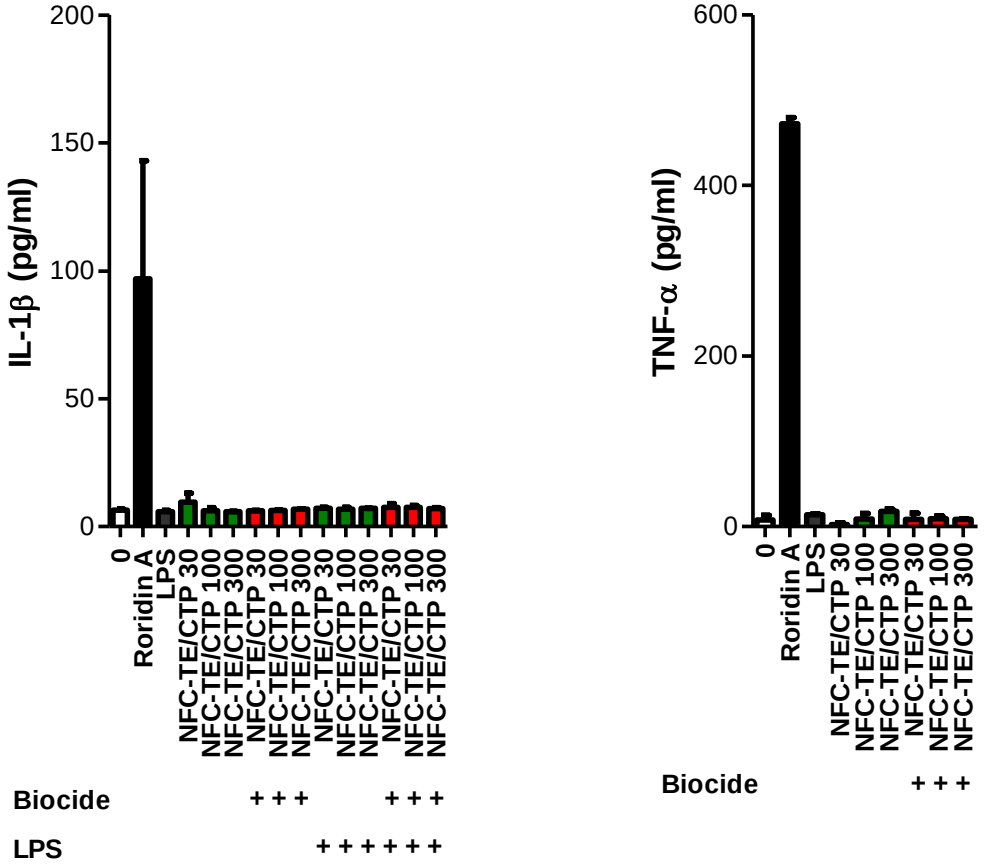
mRNA expression of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFC-TE/CTP B2 biocide



- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, mRNA measured by PCR
- **No significant effects observed**

Ilves *et al.*, in preparation

Secretion of pro-inflammatory cytokines IL-1β and TNF-α in exposure to NFC-TE/CTP B2 ± biocide



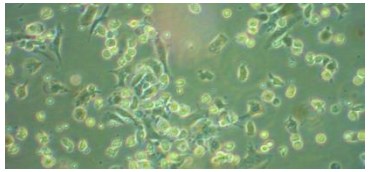
- Primed and unprimed human monocyte-derived macrophages exposed for 6 h
- Cell culture supernatants collected, pro-inflammatory cytokines measured by ELISA
- **No effects observed**

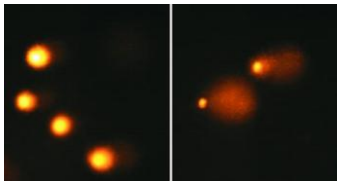
Ilves *et al*., in preparation

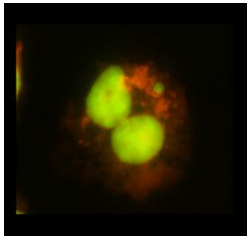
Genotoxicity – identifying possible carcinogens

In vitro studies

- Human bronchial epithelial BEAS 2B cells exposed to several doses of nanocelluloses in cell culture
- **Cytotoxicity** (cell count) utilized for dose finding
- **DNA damage** examined by the Comet assay
- **Oxidative DNA damage** studied by the enzyme-modified Comet assay
- **Chromosome damage** assessed by the micronucleus assay
- **Cell cycle delay** studied by the cytokinesis-block proliferation index







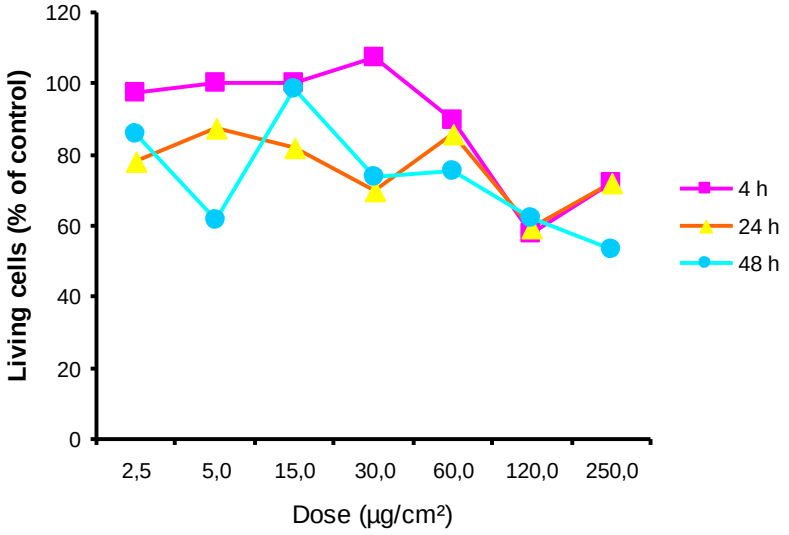
In vivo studies on NFC-TE/CTP B2 + biocide

- One NFC material chosen for *in vivo* studies by the Consortium
- Mice exposed by pharyngeal aspiration
- DNA damage and oxidative DNA damage studied in bronchoalveolar lavage (BAL) cells

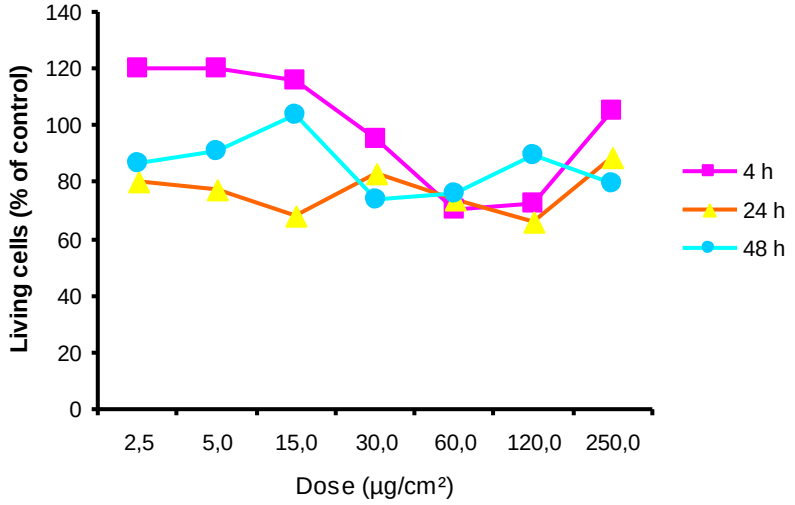
Cytotoxicity of NFC-TE/CTP B2 ± biocide in human bronchial epithelial BEAS 2B cells

- **Double staining:** Propidium iodide stains dead and dying cells, Hoechst stains cell nuclei
- Cell count in fluorescence microscope

NFC-TE/CTP B2 with biocide



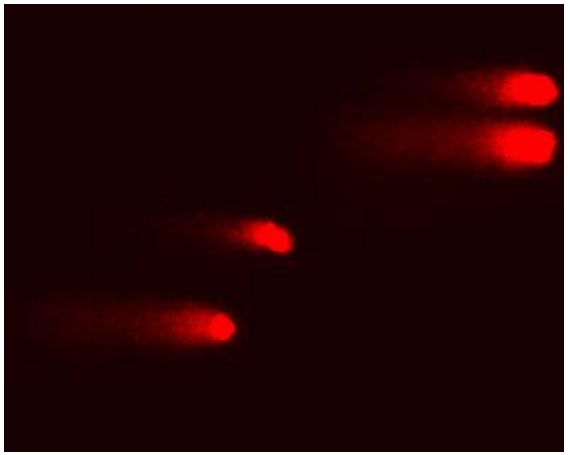
NFC-TE/CTP B2 without biocide



Cytotoxicity of the NFCs in BEAS 2B cells was generally low

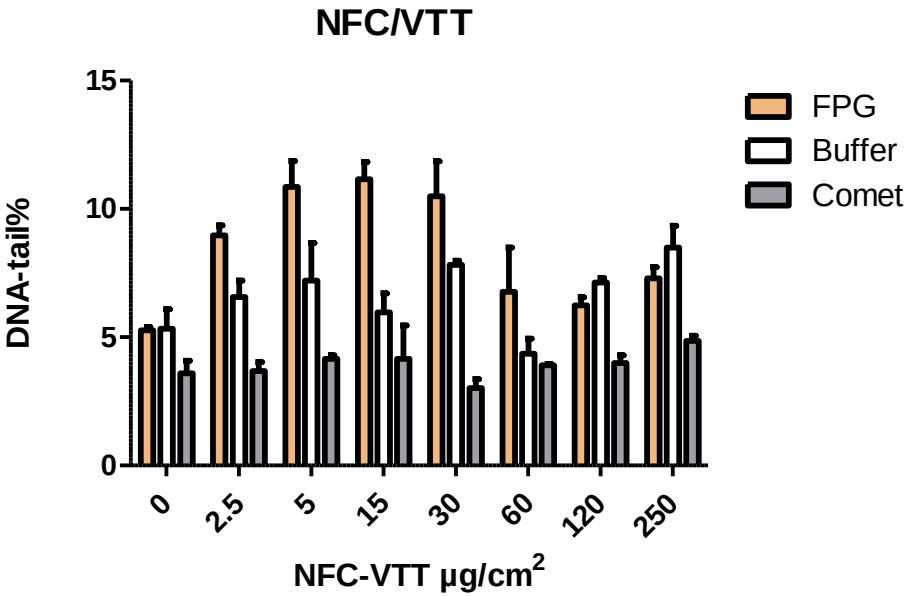
Hannukainen *et al*. in preparation

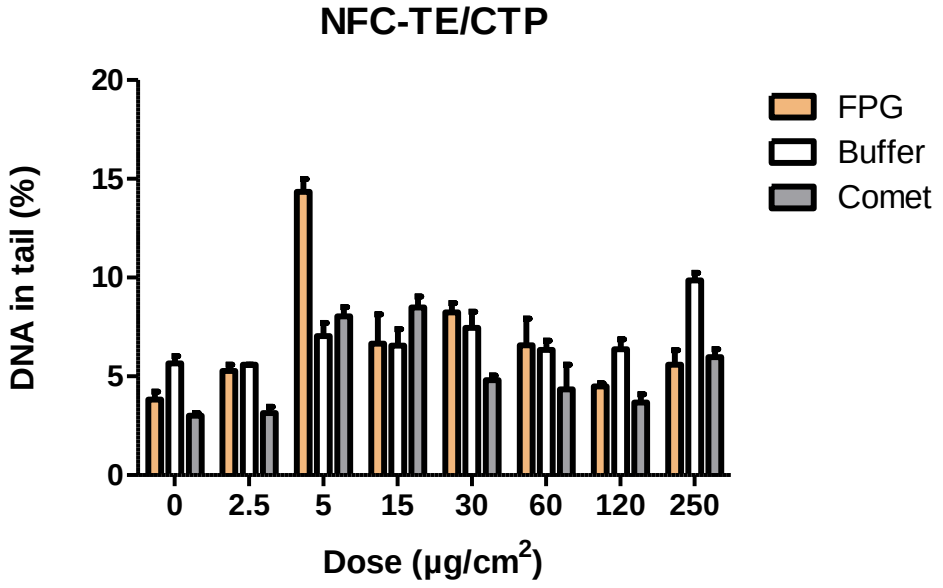
DNA damage detection by the comet assay



- DNA with strand breaks wanders out of the nucleus in electrophoresis
- The proportion of DNA in "comet tail" reflects the amount of DNA damage
- Oxidative DNA damage visualized by turning oxidative DNA adducts to strand breaks by a specific enzyme (FPG)
- Analysis in fluorescence microscope using a semiautomatic interactive software

DNA damage induced *in vitro* by NFCs

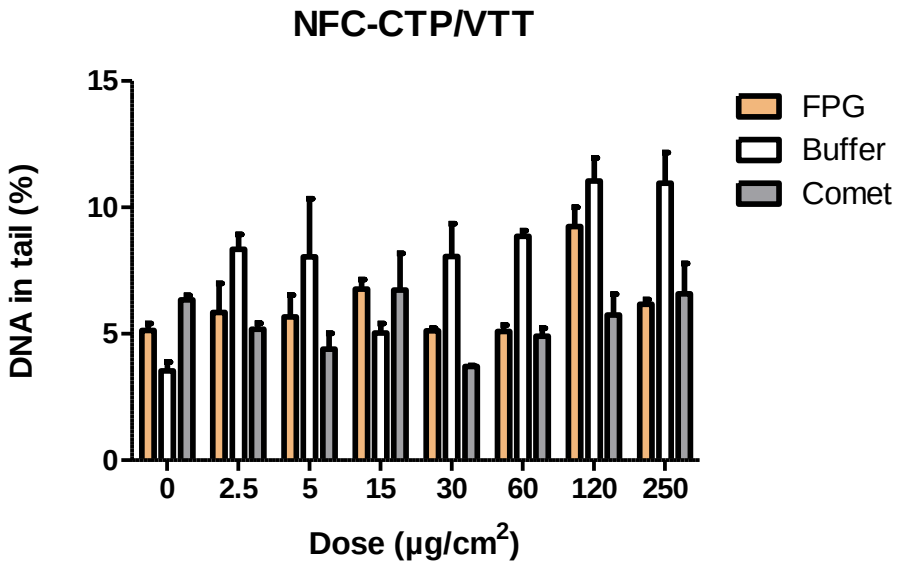


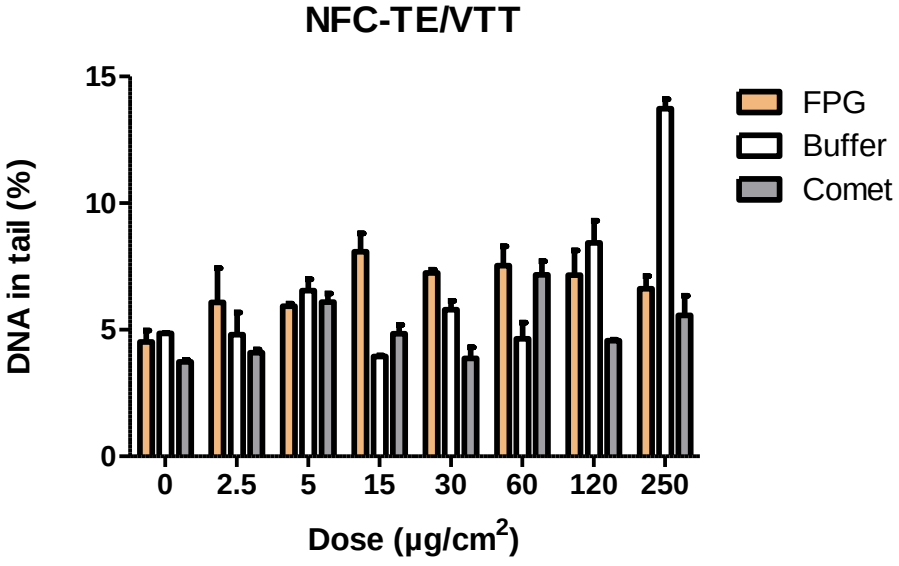


- **No increase in DNA damage**
- **Slight increase in oxidative DNA damage (FPG vs Buffer)**

- **Slight increase in DNA damage in the comet assay**
- **Slight Increase in oxidative DNA damage at one dose**

DNA damage induced *in vitro* by NFCs

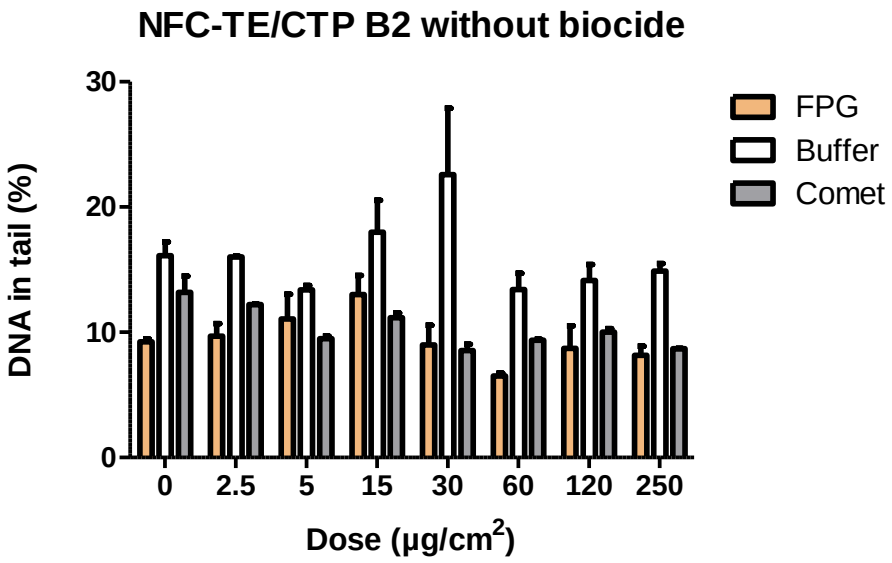


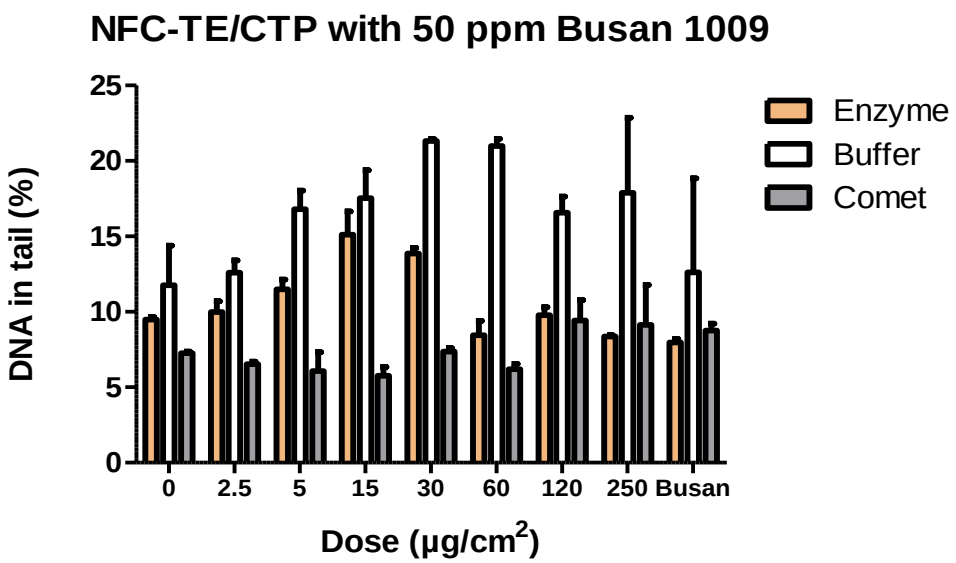


- **Slight Increase in DNA damage in the Buffer and FPG series, but not in ordinary Comet assay**
- **No increase in oxidative DNA damage**

- **Slight increase in DNA damage in the comet assay and Buffer series**
- **No increase in oxidative DNA damage**

DNA damage by NFC-TE/CTP B2 ± biocide

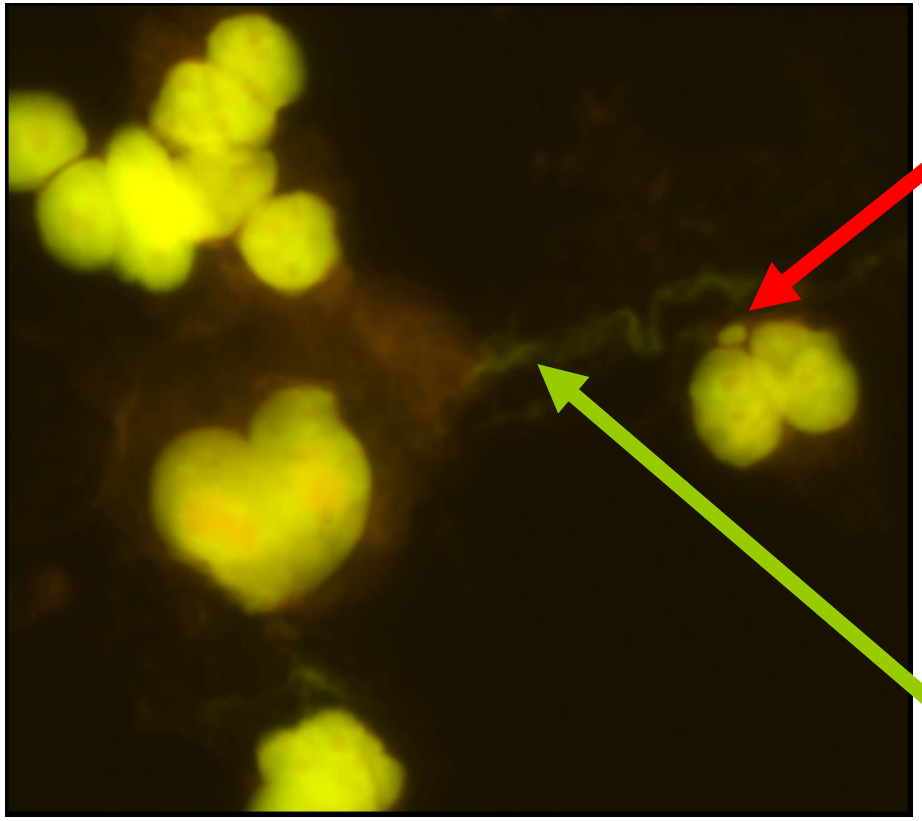




- **No increase in DNA damage**
- **No increase in oxidative DNA damage**

- **Increase in DNA damage in the Buffer series, but not in ordinary Comet assay**
- **No increase in oxidative DNA damage**

Micronucleus assay with NFCs



A micronucleus

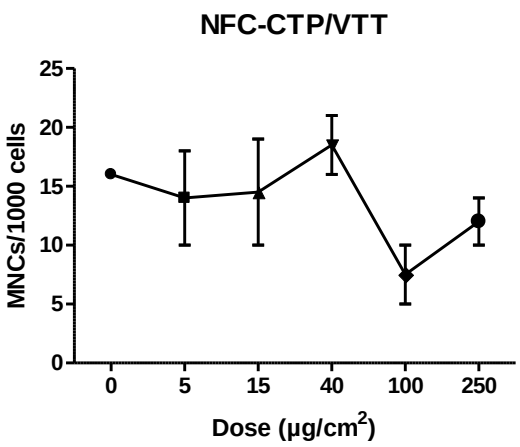
- Micronuclei reflect structural and numerical alterations of chromosomes
- **Acridine orange staining:** nuclei (DNA) are green, cytoplasm (RNA) is red
- Analysis in fluorescence microscope

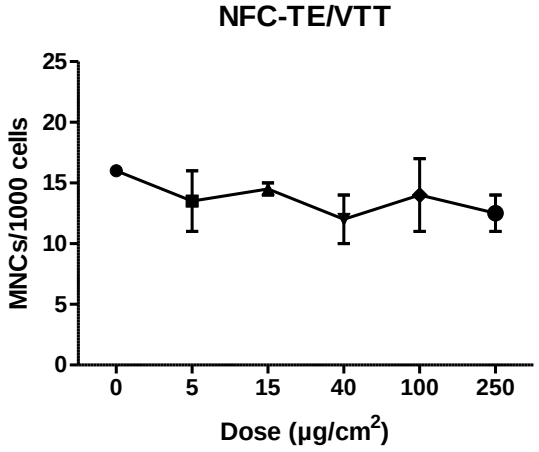
NFC

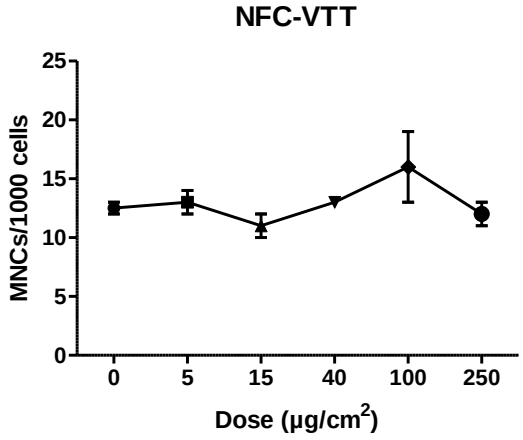
Human bronchial epithelial BEAS 2B cells in fluorescence microscope after treatment with NFC-VTT (250 µg/cm^2).

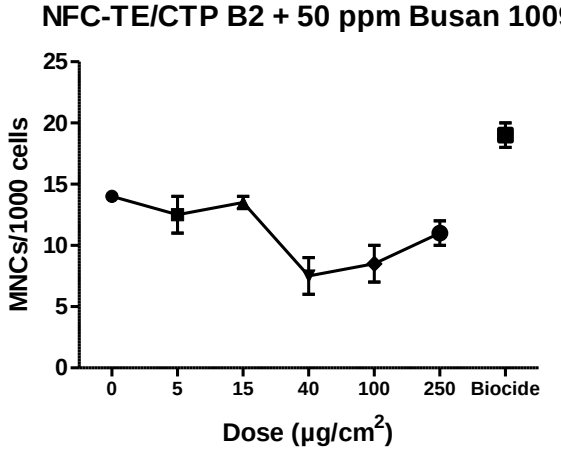
Photo: Kati Hannukainen and Hilkka Järventaus, FIOH

No induction of micronuclei by NFCs in BEAS 2B cells









48-h treatment

- No effect on cell cycle length either (data not shown)

Hannukainen et al., in preparation

Nematode toxicity assay *in vivo*

- Transgenic line (P_{dat-1}::GFP) of ***Caenorhabditis elegans*** expressing
- Green Fluorescent Protein in its dopaminergic neurons
- Bright fluorescence of ganglia indicates functional nervous system
- NFC-TE/CTP dose 0.5 mg/ml (biocide)
- Single walled carbon nanotubes used as fibre control, DMSO (5%) as a positive
- control
- 24-h follow-up for viability, behavior and reproduction
- **The NFCs were not toxic to C. elegans**

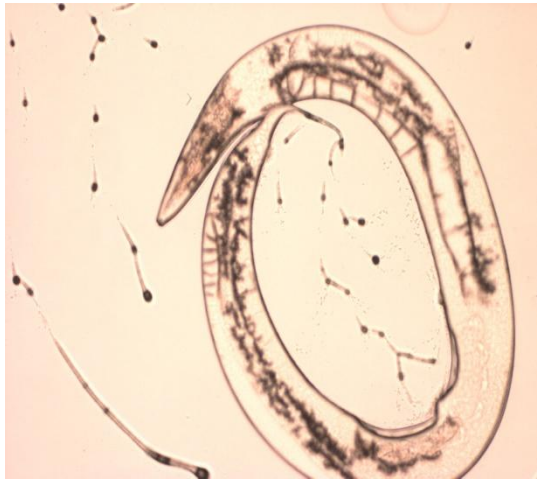


Photo: Prof. Garry Wong,
University of Eastern Finland

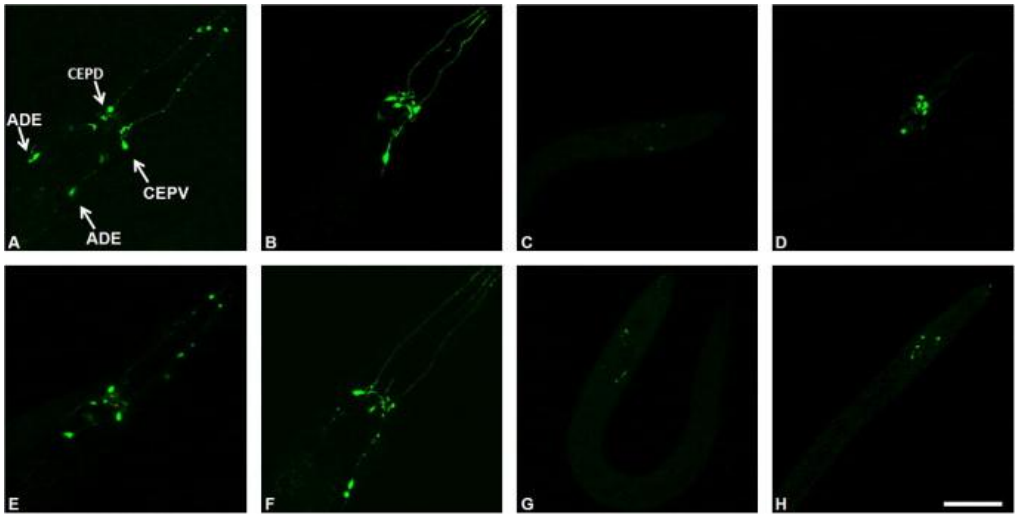
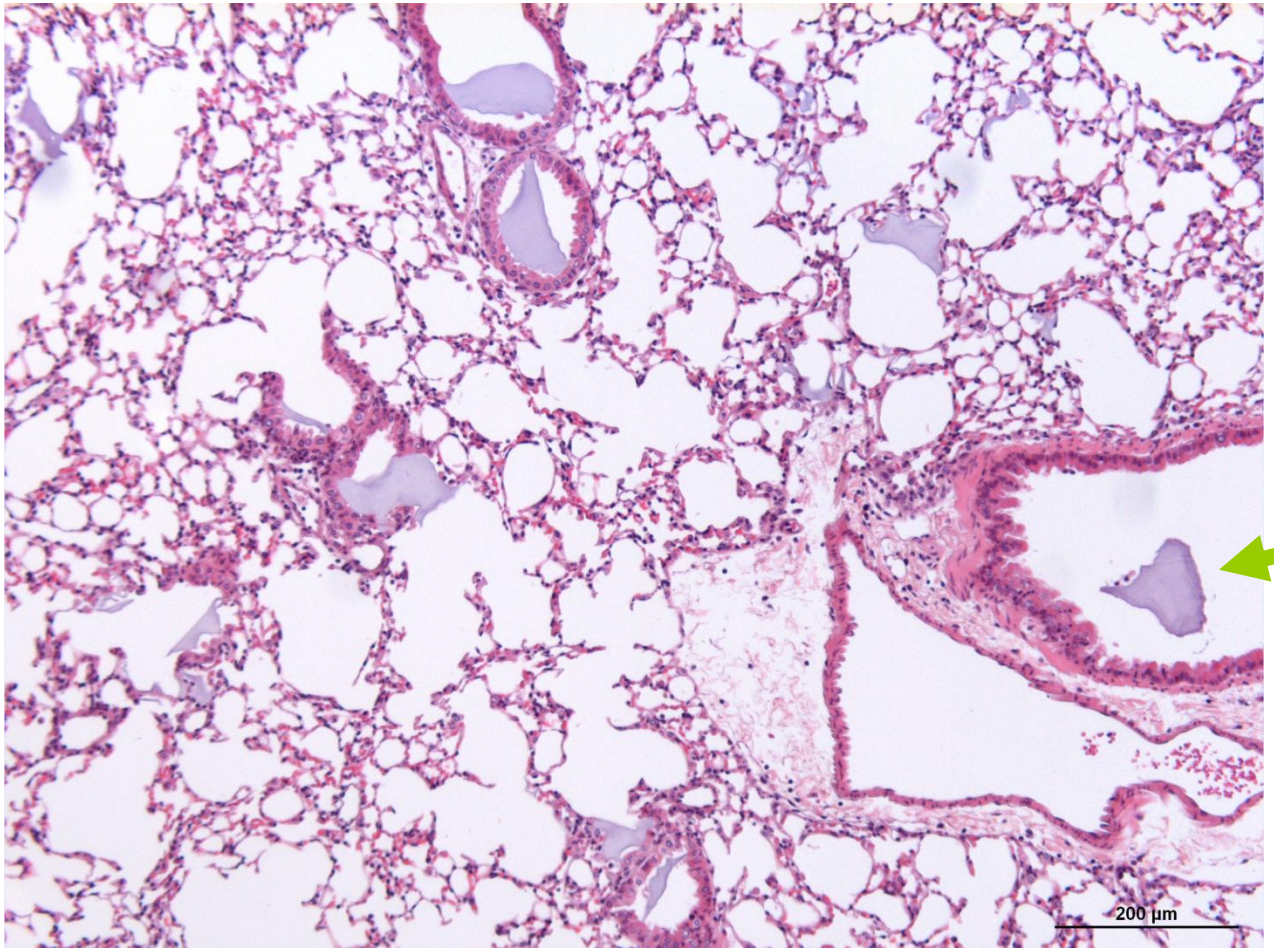


Photo: Jadiya et al. 2011

In vivo study in mice

- Female C57BL/6 mice
- NFC-TE/CTP B1 tested
- Exposure by pharyngeal aspiration
- Single dose: 20, 40, 80 and 200 µg/mouse (in PBS)
- Biocide Busan 1009 tested separately at the same dose as in the NFC
- Negative and positive controls
- Samples collected 16 h after dosing (acute effect)
    - Bronchoalveolar lavage fluid
    - Lungs
    - Blood
- Analysis of pulmonary inflammation and DNA damage

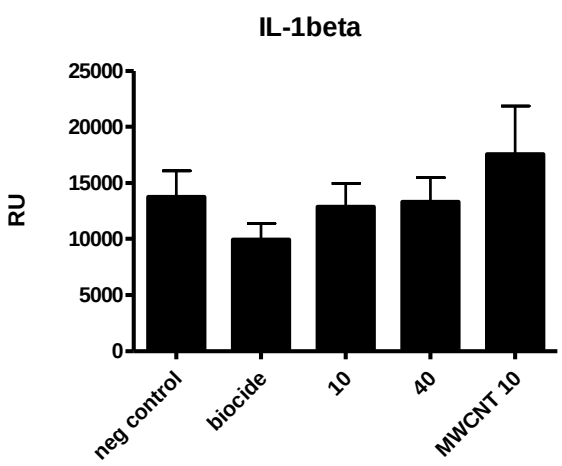
NFC-TE/CTP B2 in mouse lungs

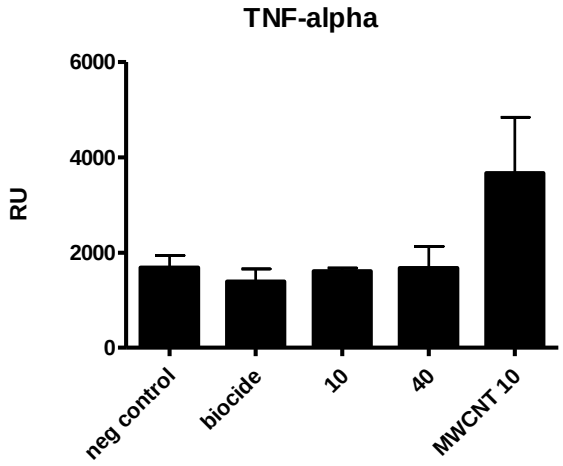


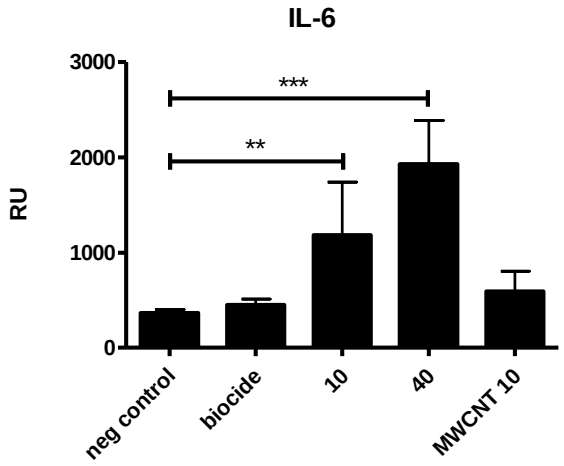
NFC

- In histological samples of the lungs, NFC was seen in or near bronchioles
- No dramatic tissue changes were observed

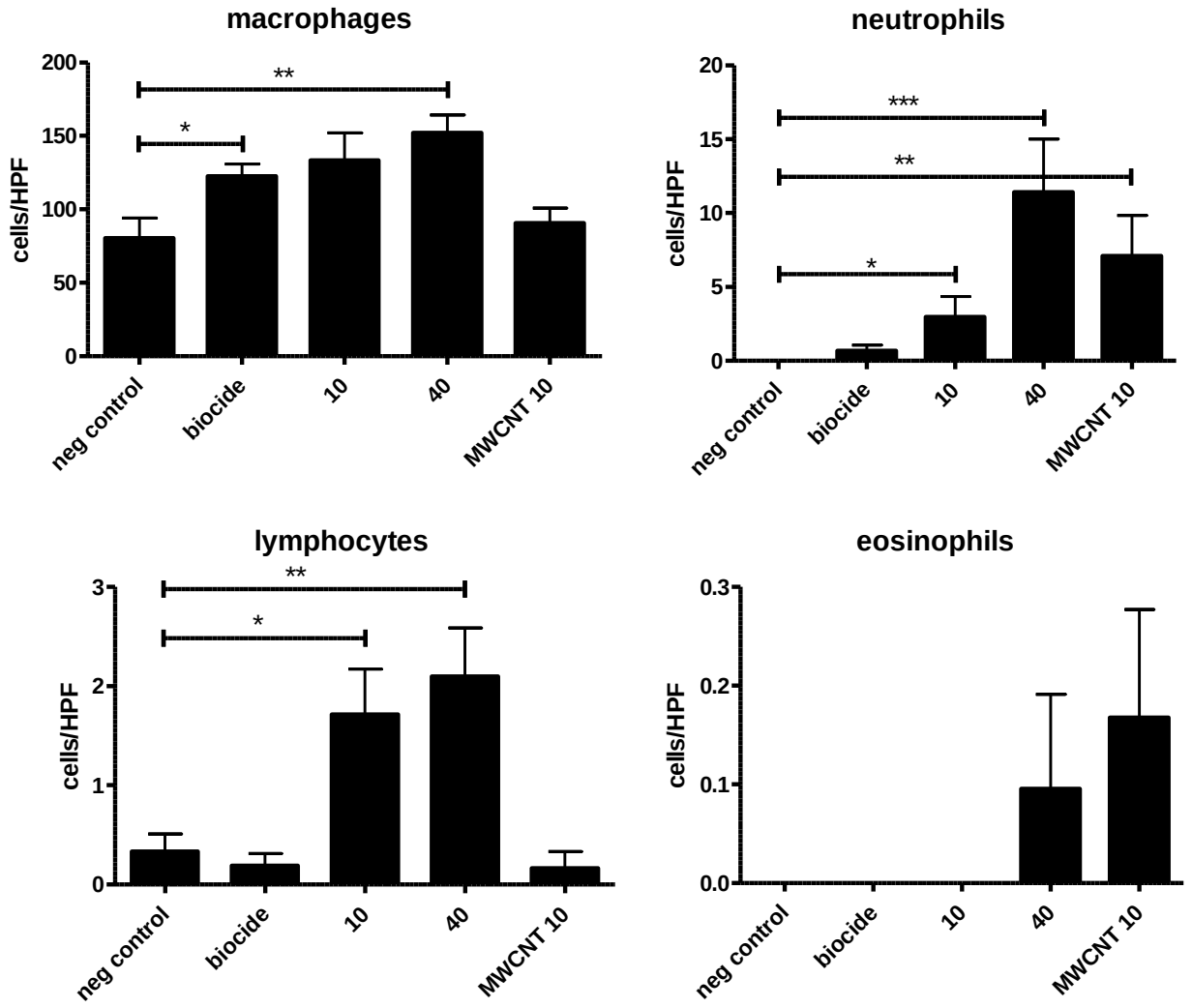
NFC-TE/CTP B2 induced mRNA of pro-inflammatory cytokine IL-6



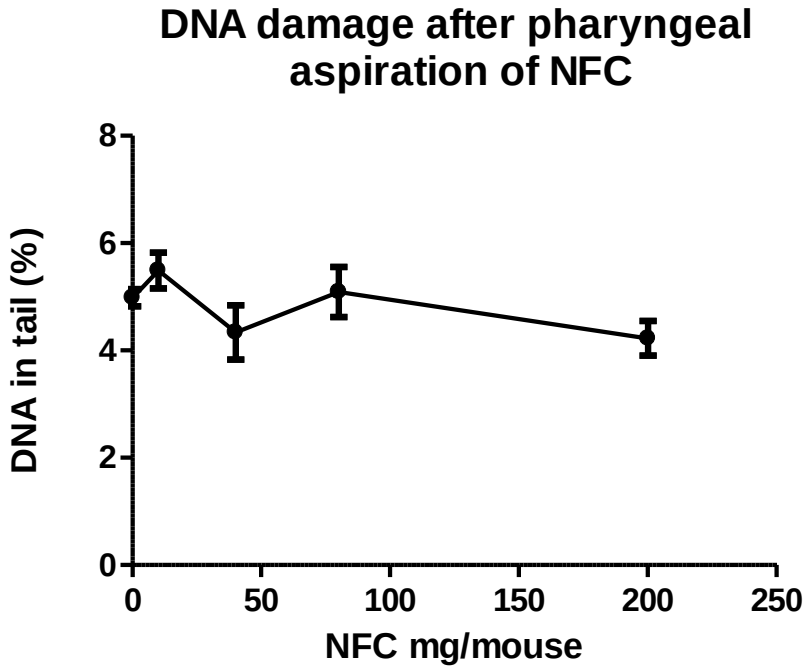


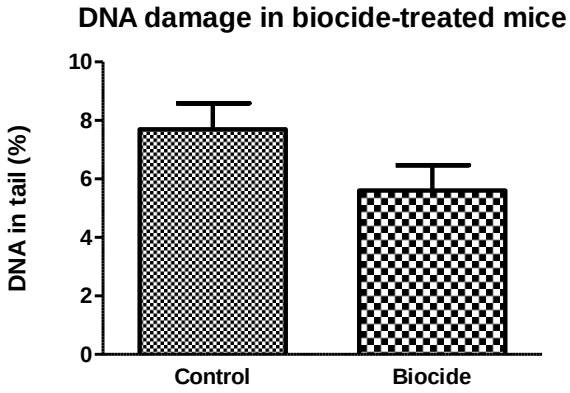


NFC-TE/CTP B2 induced influx of inflammatory cells to mouse lungs



No DNA damage in mouse bronchoalveolar lavage (BAL) cells after pharyngeal aspiration of NFC-TE/CTP B2





Hannukainen *et al*. in preparation

Conclusions – toxicity of NFCs

In vitro

- **Low or no cytotoxicity**
- **Slight induction of proinflammatory cytokines** in macrophages with or without LPS priming *in vitro*
- **Slight DNA damage** in human bronchial epithelial cells – similarly to a number of other "inert" nanomaterials
- **Marginal induction of oxidative DNA damage** *in vitro* (some NFCs)
- **No increase in chromosome damage (micronuclei)**

***In vivo* (NFC-TE/CTP B2, pharyngeal aspiration, mice)**

- **No DNA damage** in bronchoalveolar lavage cells
- **Pulmonary inflammation**

→ Possibly due to the particulate / bacteria in the NFC

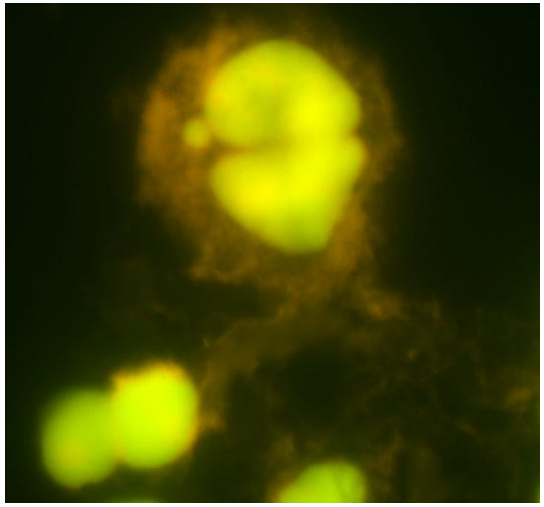
Toxicology research group

FIOH, Helsinki

Harri Alenius
Julia Catalán
Kati Hannukainen
Marit Ilves
Hannu Norppa
Jaana Palomäki
Elina Rydman
Kai Savolainen
Virpi Väänänen

BioSafe, Kuopio

Ulla Honkalampi
Stefanie Kasurinen
Terhi Myöhänen
Atte von Wright



Acknowledgements

- The research leading to these results has received funding from the European Community's Seventh Framework Programme under grant agreement nº 228802.