DELIVERABLE REPORT

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0 List of abbreviations

BMR: bench mark response

CED: Critical Effect Dose

CEDL: Critical Effect Dose Low

CEDU: Critical Effect Dose Upper (high)

CES: critical effect size

DIMS: Direct infusion mass spectrometry

KNN: k-nearest neighbours

MNM: manufactured nanomaterial

PQN: probabilistic quotient normalisation

QC: quality control

RINs: RNA integrity numbers

SIM: selected ion monitoring

1. Introduction

As introduced in the NanoMILE Deliverable Reports 8.1 and 8.2, the Systems Biology work package (WP8) in this project builds on important discoveries and technical advances of the past decade, particularly (a) the ability to discover both genes and metabolites altered in response to a manufactured nanomaterial (MNM) challenge (Taylor et al., 2016); (b) the availability of genomes for model species and humans; and (c) studies that have underscored the similarity across living organisms of biological processes that are highly relevant to human health (Peterson et al., 2008). An important aspect of the research summarised in WP8, therefore, is the exploitation of a chosen set of non-mammalian, 3R-compliant biomedical and environmental model species, coupled with human cell cultures (specifically A549 cells), to attempt to identify the core, evolutionarily conserved, biological pathways and molecular events indicative of the toxicity induced by selected MNMs. This deliverable report, D8.4, describes the results and omics dataset obtained with the human epithelial lung carcinoma cell line A549 after exposure to various MNMs used in the NanoMILE project (specifically Ceria MNMs including the Zr-doped series designed to alter the redox activity, Zinc oxide MNMs with different coatings, and silver MNMs, all with ionic and bulk controls were available and appropriate.

2. Objectives

The overall objective for NanoMILE WP8 is to complement standard toxicological approaches with a carefully selected range of systems biology based studies (or “omics”) to support understanding and comparison of mechanisms of MNMs activity across several species of increasing complexity.

More specifically, the objectives are:

- To measure the potentially harmful effects of multiple MNMs of differing physico-chemical properties using transcriptomics (gene expression profiling), metabolomics (profiling of polar endogenous metabolites) and lipidomics (profiling of endogenous lipids).
- To characterise classic toxicological (or phenotypic) outcomes in response to MNM exposure alongside molecular measurements, including effects on growth, reproductive output, morphological defects and mortality rates, and equivalent end-points in cells such as proliferation, cytotoxicity, genotoxicity etc.
- To employ computational modelling to identify signatures within the 'omics datasets that represent adverse outcome pathways (AOPs), i.e. mechanistically based molecular biomarker signatures, which will be implemented into diagnostic screening assays to identify and characterise the impacts of MNMs on environmental and human health (in WP4).
- To identify both species-specific and evolutionarily conserved molecular responses within the four species and cell line investigated.

Together, these objectives will facilitate NanoMILE to:

- Provide biological response data that will form the basis of future risk assessments of MNMs;
- Help to support industry to innovate without using animal testing, instead focusing on a series of non-mammalian 3R-compliant test systems;
- Offer fit-for-purpose and innovative high-throughput experimental and bioinformatic procedures providing cost-effective toxicity information at an accelerated pace while being financially sustainable.

The specific objective of Deliverable 8.4 is to report on the state-of-the-art multi-omics investigations conducted to determine the responses of A549 cells to several selected NanoMILE MNMs. Of note is the close partnership between WP7 and WP8 in this endeavour, with the experimental design, exposure studies and collection of considerable non-omics toxicity data occurring in WP7, and the state-of-the-art "omics" measurements conducted in WP8. As per the DoW, the tasks performed to achieve this deliverable included: (1) conducting exposure experiments in order to determine the most suitable concentrations and time-points for assessment to see effects from the MNMs but not be operating at concentrations inducing acute toxicity; (2) conducting a large-scale exposure experiment at these pre-determined concentrations and time-points to generate samples for the 'omics investigation; (3) generating multi-omics 'Big Data' using RNA Seq gene expression profiling and mass spectrometry based metabolomics and lipidomics; and (4) making this data available to the computational biology NanoMILE team in WP8 for bioinformatics analysis which will be reported separately in D8.5 (Graphical models for each species/cell type) and D8.6 (Biomarkers for assessing MNM impacts: Adverse outcome pathways to serve as biomarkers for assessing MNM impacts).

3. Current state of the art

Metabolomics and lipidomics are concerned with measurement of hundreds to thousands of endogenous low molecular weight polar and non-polar (lipid) metabolites in biological matrices, whose relative abundances can change as a result of the organisms' exposure to MNMs. Measurement of metabolites requires an array of analytical assays due to the breadth of their physico-chemical properties, with most studies using mass spectrometry (MS) followed by data processing and statistical analyses to discover those

metabolite changes worthy of further investigation. While this field has grown significantly over the last decade from a few hundred to several thousand papers published annually, this has not been without challenges, in particular the need for tools and expertise in analytical chemistry and computational biology. This has significantly slowed the uptake of this methodology by the wider community and the number of metabolomics studies in nanotoxicology is low (a Web of Science topic search for "metabolomic* and nanotoxicology" in April 2016 revealed only 8 papers).

Understanding gene regulation in nanotoxicology is essential for gaining a mechanistic understanding of the effects of MNMs on living organisms, a key aim of the NanoMILE project. Traditional methods for measuring gene expression signatures, over the last decade, have been dominated by the use of high density DNA microarrays. More recently, the quality of next generation RNA-Seq data has been vastly improved with standardised protocols that have provided longer paired-end read-lengths, thereby allowing in-depth characterisation of transcriptomes; see for example (Graveley et al., 2011).

Collectively, the NanoMILE partners have considerable expertise in the application of multi-'omic studies to probe the mechanisms of nanotoxicity. For example, UoB researchers have investigated the effects of ZnO MNMs in *Chlamydomonas reinhardtii* (in collaboration with EAWAG) and *Daphnia magna*. In collaboration with UoE, they have discovered that families of endogenous sulfated lipids in *D. magna* are dramatically modulated by ZnO MNM exposure (Taylor et al., in prep.). UoE have applied sequencing and SuperSAGE analyses of the zebrafish transcriptome in response to Ag MNMs, discriminating between particle and silver ion effects (van Aerle et al., 2013). Eawag has analysed silver ion induced alterations in the transcriptome and proteome of *C. reinhardtii* and anchored these changes to specific phenotypes (Pillai et al., 2014). Here, these approaches and expertise are utilised to assess the metabolomics changes in A549 cells in response to exposure to NanoMILE MNMs.

4. Experimental approaches

4.1 Culturing of A549 cells

A549 cells were used for the MNM exposure experiments. A549 is an epithelial lung adenocarcinoma cell line established in 1972 by Giard (Giard et al., 1973). A549 cells were cultured in tissue culture flasks (75 cm^2 and 275 cm^2) in RPMI 1640 medium with Glutamax (Gibco, ThermoFisher Scientific Inc., Landsmeer, the Netherlands) supplemented with 10% Foetal Bovine Serum (FBS, Greiner BioOne Serum, Greiner BioOne BV, Alphen aan de Rijn, the Netherlands) and 1% penicillin/streptomycin (Gibco, 100 IU penicillin per mL, 100 µg streptomycin per mL). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 in air. The adherent cells were harvested by a short incubation with 0.5% EDTA trypsin in Ca/Mg free Dulbecco's Phosphate Buffered Saline (Gibco). Viable cells were counted in a haemocytometer (Bürker-Türk chamber) by the dye exclusion method using trypan blue (0.4% trypan blue).

4.2 Exposure

A549 cells were exposed to the NanoMILE MNMs at a concentration that induced 20% cell cytotoxicity (80% cell survival). The 80% cell survival was chosen as a cut off point for cytotoxic response of the cells as more than 20% cytotoxicity clearly indicates a toxic response. Cell survival (i.e. cytotoxicity) was determined by a colourometric assay using the cell proliferation reagent WST-1 (Roche, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands; reference number 11644807-001). In this assay the stable

tetrazolium salt WST-1 is cleaved by metabolically active cells into a soluble formazan, and the amount of formazan formed correlates to the number of viable cells in the cell culture. Potential for interference of the MNMs with the formazan detection was assessed and ruled out.

In order to determine the EC20 (effective concentration resulting in 20% cytotoxicity) in a 96 tissue culture plate, a dose response study was performed for the MNM. A549 were cultured in tissue culture flasks and 24 hours before exposure harvested and counted. Fifty thousand ($5x10^4$) cells were seeded in wells of a 96 well tissue culture plate in 100 µL supplemented medium. After 24 hours incubation a (semi)confluent monolayer of cells was obtained in the wells. These 24-hours cultured cells were exposed to the various MNMs and cell survival was determined after 24 hours of incubation (WST-1 assay). All exposures to MNMs were performed in triplicate. Dose response relationships were evaluated by PROAST software version 60.1 (http://www.rivm.nl/en/Documents_and_publications/Scientific/Models/PROAST) (Slob 2002).

Based on the results of the dose response studies, a dose was selected for the incubation of the A549 cells in a 6 well tissue culture cluster. In the 6 well tissue culture cluster, $8x10^5$ cells were seeded and cultured for 18 hours to obtain a (semi)confluent monolayer and a cell harvest of approximately $2x10^6$ cells for the omics evaluation after exposure. After growth to a (semi)confluent monolayer the cells were exposed to the EC20 MNM concentrations as determined in the dose response study. For the omics evaluation cells were exposed for 1, 6 or 24 hours in the incubator at 37 ^oC in a humidified atmosphere of 5% CO_2 in air. Following exposure, cells within the 6 wells tissue culture cluster were deep frozen by quenching in liquid nitrogen (-196^oC) and stored at -80^oC before shipment.

4.3 Nanomaterials

The following materials were used:

NanoMile ID NP00193: Cerium oxide CeO_2 A, undoped cerium oxide consisting of 100% CeO_2

NanoMile ID NP00194: Cerium oxide CeO_2 C, ZrO_2 doped cerium oxide consisting of 75% CeO_2 and 25% ZrO_2 (designated CeO.75ZrO.25O2).

NanoMile ID NP00196: Cerium oxide CeO_2 E, ZrO_2 doped cerium oxide consisting of 25% CeO_2 and 75% ZrO_2 (designated CeO.25ZrO.75O2).

NM-212: Nanograin CeO_2 nanoparticle powder, mean particle size 600 nm, primary particles size 33 nm.

NP00281: ZnO 5 µm

NP00282: NM-110, ZnO, uncoated, mean particle size 150 nm, primary particle size 42 nm.

NP00283: NM-111, ZnO coated with triethoxycaprylsilane, mean particle size 140 nm, primary particle size 34 nm.

Zinc chloride.

NP00221: silver nitrate, $AgNO_3$, soluble.

NP00213: Ag micron sized.

NP00214: Ag, Silver NM300K, mean particle size 15 nm, primary particles size 15 nm.

When provided as powder the MNMs were dispersed using the Nanogenotox protocol. In short for a final concentration of 2.56 mg/mL the powder was pre-wetted with 0.5 vol% ethanol and dispersed in water

with 0.05% w/v BSA, and sonicated for 16 minutes on ice using a 400 Watt Branson Sonifier S-450D (Branson Ultrasonics Corp., Danbury, CT, USA). Those MNMs received as suspensions were diluted according to the NanoMILE WP4 SOP.

4.4 Statistical analysis

Cytotoxic effects of the MNMs were evaluated using the bench mark dose (BMD) approach, by fitting a nonlinear regression model to the data of the triplicate measurements obtained in the cytotoxicity assay. The choice of the model for evaluating the BMD (also called CED or critical effect dose) for continuous endpoints follows from a procedure of applying likelihood ratio tests on the members of the following nested family of models.

Model 1: $y = a$

Model 2: $y = a \exp(bx)$

Model 3: $y = a \exp(bx^d)$

Model 4: $y = a(c - (c - 1)\exp(bx))$

Model 5: $y = a(c - (c - 1)\exp(bx^d))$,where y is the response, and x denotes the applied MNM concentration. The parameter a represents the level of response at concentration zero, and b can be considered as a parameter reflecting the potency of the agent. At high doses, models 4 and 5 level off to the value ac, so the parameter c can be interpreted as the maximum relative change compared to the background. Parameter d can be interpreted as the 'steepness' of the curve (i.e., rate of change in response for a percent change in dose).

All these models are nested to each other, except models 3 and 4, which both have 3 parameters. Therefore, these two models cannot be (formally) compared to each other by a likelihood-ratio test.

For the end point measured (cell viability), one of these models was selected by applying the likelihood ratio test to establish whether extension of the model by increasing the number of parameters resulted in a statistically valid improvement of the fit to the dose response data. The selected model was used to estimate the BMD (CED) and the associated 90%-confidence interval (see results). A critical effect size (CES) or bench mark response (BMR) was chosen at a cytotoxicity level of 20%, i.e. 80% cell viability as measured with the WST-1 assay. The confidence interval was determined using a (parametric) boot strap method, as follows. Once a model is selected to describe the dose response data, this fitted model is used as a basis for generating 200 artificial data sets (according to the Experimental design) by Monte Carlo sampling. For each generated data set, the CED is re-estimated. Taking all these CEDs together results in a distribution representing the uncertainty associated with the CED estimate. The 5th and 95th percentiles of this empirical distribution were determined, serving as a two sided 90% confidence interval (c.i.) of the estimated CED (Slob 2002). The results of the BMD experimental design were analyzed using PROAST software version 60.1 (http://www.rivm.nl/en/Library/Scientific/Models/PROAST, RIVM, Bilthoven, The Netherlands).

4.5 Mass spectrometry based polar metabolomics and lipidomics

4.5.1 Extraction of metabolites and lipids

Following the exposures, A549 cells were quickly washed with 2 mL PBS twice at room temperature before the 6 well plates were quenched on liquid nitrogen. These were shipped from RIVM to UoB, and the extraction protocol was continued. Next 400 µL 80% methanol (pre-cooled on dry ice) was added into each well. Cells were scraped down from the bottom of each well on dry ice and were transferred into a 1.8 mL glass vial which was pre-filled into 640 µL pre-cooled chloroform and 416 µL H_2O. Then 400 µL 80% methanol (pre-cooled on dry ice) was added into the well to wash and all contents were transferred into the same glass vial. After adjusting the ratio of methanol: chloroform: water (v/v/v) to 1:1:0.9, each glass vial was vortexed for 30 s three times, at 30 s intervals. The glass vials were then cooled on dry ice for 10 mins before 10 mins of centrifugation at 4000 rpm at -9 °C. After centrifuging, the mixture separated into two phases (upper polar phase and lower non-polar phase). 300 µL aliquots of the polar phase were transferred into clean 1.5mL Eppendorf tubes and then dried in a speed vac concentrator (Thermo Savant, Holbrook, NY) for 4 hr. 300 µL aliquots of the non-polar phase were transferred into clean 1.8 mL glass vials using a Hamilton Syringe and then dried under nitrogen stream for 5 mins. All dried samples were then frozen at -80°C until analysis.

4.5.2 Direct infusion mass spectrometry (DIMS) and data processing

The DIMS analysis method was similar to that reported previously (Southam et al., 2007; Zhang et al., 2015). The dried polar or non-polar extracts were re-suspended in 80 µL 80:20 (v/v) methanol: water (HPLC grade) with 0.25% formic acid (for positive ion mode analysis of polar extracts) or 80 µL 2:1 methanol: chloroform with 5 mM ammonium acetate (for negative ion mode analysis of lipids). After centrifugation at 22000 rcf, 4 °C for 10 min, 10 µL supernatant of each sample was loaded into one well in a 384-well plate and then analysed (in triplicate) using direct infusion mass spectrometry (Q Exactive, Thermo Fisher Scientific, Germany) in positive ion mode (for polar metabolomics) or negative ion mode (for lipidomics), coupled with a Triversa nanoelectrospray ion source (Advion Biosciences, Ithaca, NY, USA).

Mass spectra were recorded utilising the selected ion monitoring (SIM) stitching approach from m/z 50 to 620 (for polar metabolomics) or from m/z 50 to 1020 (for lipidomics) and then processed using custom-written Matlab scripts as previously reported (Kirwan et al., 2014; Southam et al., 2007). In brief, only mass spectral peaks with a signal-to-noise ratio exceeding 3.5 were retained. Mass spectra of the three technical replicates for each sample were filtered into a single peak list (with only those peaks present in ≥ 2 of the 3 spectra retained). Each filtered peak list (one per sample) was then further filtered to retain only those peaks that were present in 80% of all biological samples in the entire dataset, and missing values were imputed using the k-nearest neighbours (KNN) algorithm. The resulting matrices of peak intensity data (termed “DIMS dataset”) were normalised by the probabilistic quotient normalisation (PQN) method and then made available to the computational biology team at UoB (led by Dr Shan He) for data mining. The results of this data mining will be presented in future Deliverables 8.5 and 8.6.

4.6 RNA seq gene expression profiling

Total RNA of A549 cells was extracted using Qiagen’s micro RNeasy Kit (Qiagen, Crawley, UK) according to the manufacturer’s protocol. RNA was quantified with a NanoDrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA), and the integrity of RNA was evaluated with an Agilent 2200 Tapestation (Agilent Technologies, Santa Clara, CA). Only good quality RNA with RNA integrity numbers (RINs) greater than 7.0 were used for subsequent gene expression analyses or RNA-seq experiments.

All RNA libraries were produced using the Biomek FxP (Beckman Coulter A31842) with Ultra Directional RNA Library Prep Kit (New England Biolab E7420L) and NEBnext Multiplex Oligos for Illumina Dual Index Primers (New England Biolabs E7600S), using protocols provided by the manufacturer and 500ng of total RNA. Constructed libraries were assessed for quality using the Tapestation 2200 (Agilent G2964AA) with High Sensitivity D1000 DNA screentape (5067-5584), and quantified using Kapa Library Quantification Kit (Kapa Biosystems KK4824) on an AriaMx Realtime PCR System (Agilent G8830A). Multiplex library clustering and sequencing was performed on the HiSeq2500 with HiSeq Rapid Cluster Kit v2 (Illumina GD-402-4002) at 12pM library concentration with 10% PhiX Control v3 spiked in (Illumina FC-110-3001). The sequencing run was carried out using HiSeq Rapid SBS Kit v2 (Illumina FC-402-4021). Bcl results files were converted to fastq using bcl2fastq Conversion Software v1.8.4 (Illumina).

5. Results and Discussion

WP8 comprises the collection and deep analysis of omics "Big Data" associated with the biological responses of four model systems (*Daphnia*, *Chlamydomonas*, zebra fish embryos and A549 human cell line) to selected MNMs (silver-based, zinc oxide based and ceria based). Here, we report on the nanotoxicology studies of the A549 cells. The core of Deliverable 8.4 is the large, high-dimensional, multi-omics dataset that has been fully processed and which is now available for statistical analysis and data mining (see below).

5.1 Non-omics results

Dose response studies were performed in 96-well tissue culture cluster and evaluated by PROAST software. The software calculates the best possible fit using all data. Based on the curve fitting a 90% confidence interval is calculated as the critical effective dose low (lower end of 90% confidence interval, CEDL) and upper (CEDU, highest end of 90% confidence interval). The confidence interval indicates the reliability of the data used for the calculations. The results are calculated by using two models, namely an exponential model and the Hill model. The mean of both models is used as EC20 indicating the dose inducing 20% cytotoxicity after 24 hours of incubation with the MNMs. Determination of the EC20 values for Ag nanoparticles (Ag-NP) and silver nitrate ($AgNO_3$) are presented in Figure 1, and EC20 values for ZnCl, bulk ZnO, and ZnO MNMs NM-110 and NM-111 are presented in Figure 2. For the four Ceria (nano)materials and micron sized Ag no cytotoxic dose response was obtained (data not shown).

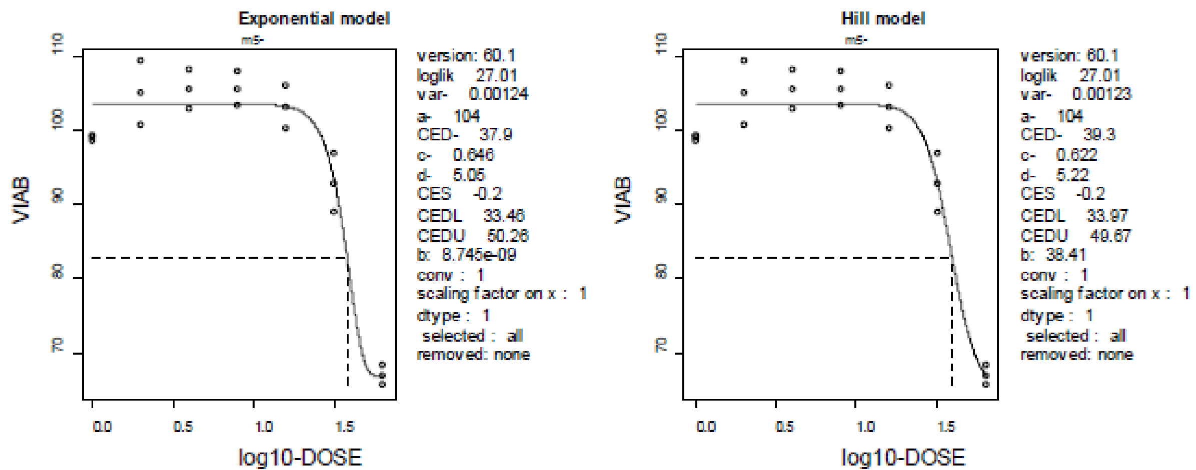

Figure 1A Dose response of survival of A549 cells after 24 hours incubation with silver (Ag, NM300K) nanoparticles size 20 nm. CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

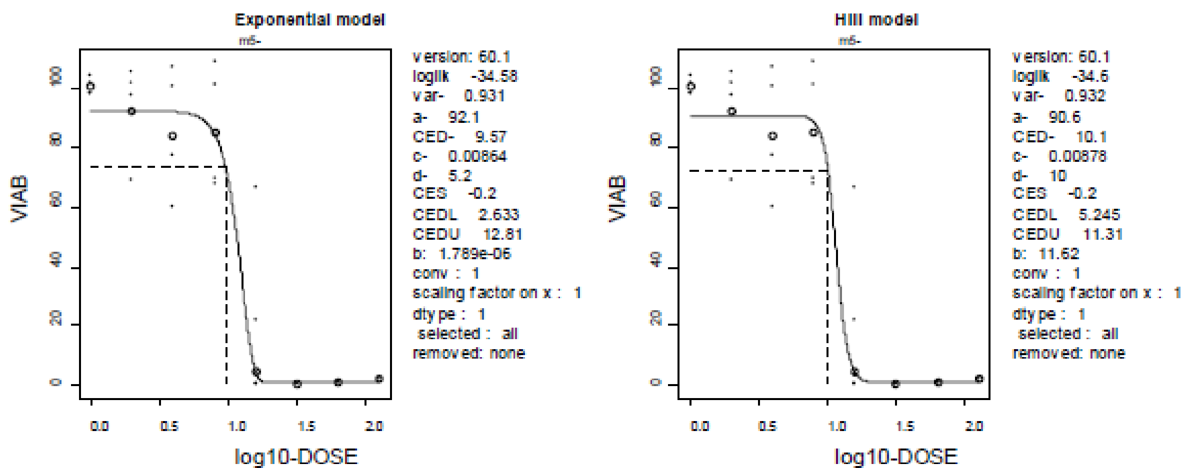

Figure 1B. Dose response of survival of A549 cells after 24 hours incubation with soluble silver nitrate, $AgNO_3$. CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

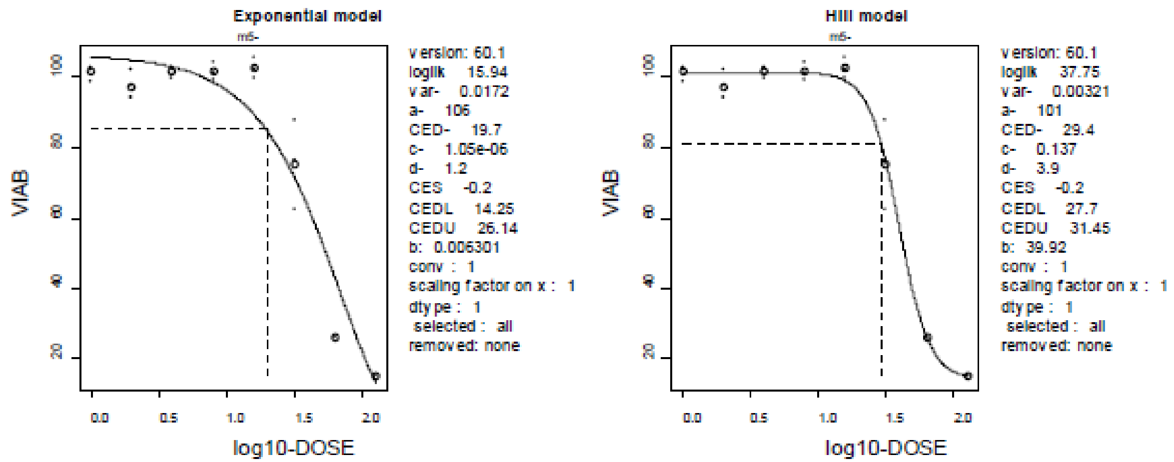

Figure 2A. Dose response of survival of A549 cells after 24 hours incubation with soluble zinc chloride (ZnCl). CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

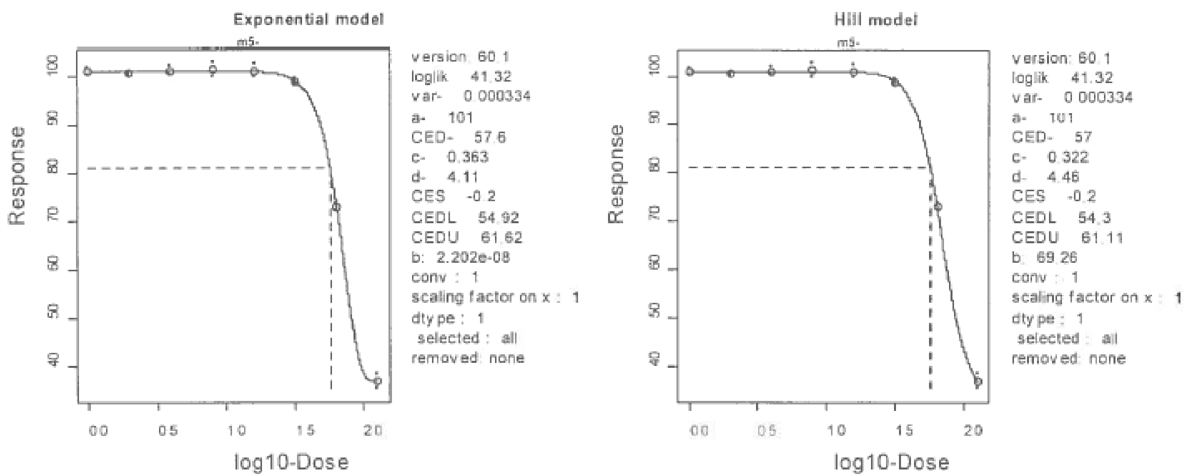

Figure 2B. Dose response of survival of A549 cells after 24 hours incubation with bulk Zinc Oxide (ZnO) particles size of 5 µm. CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

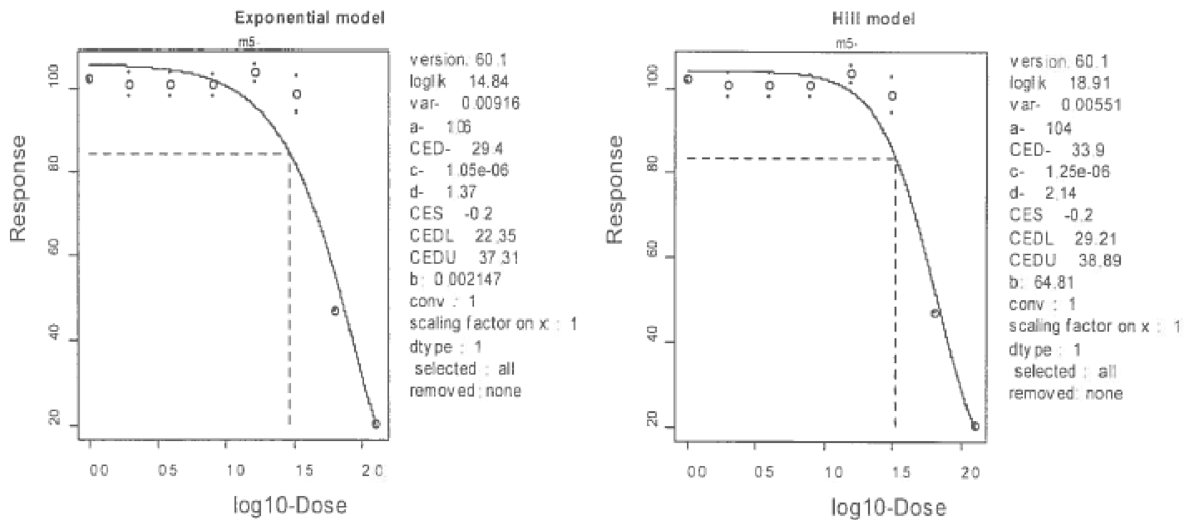

Figure 2C. Dose response of survival of A549 cells after 24 hours incubation with NM-110 Zinc Oxide (ZnO), uncoated, mean particle size 150 nm, primary particle size 42 nm. CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

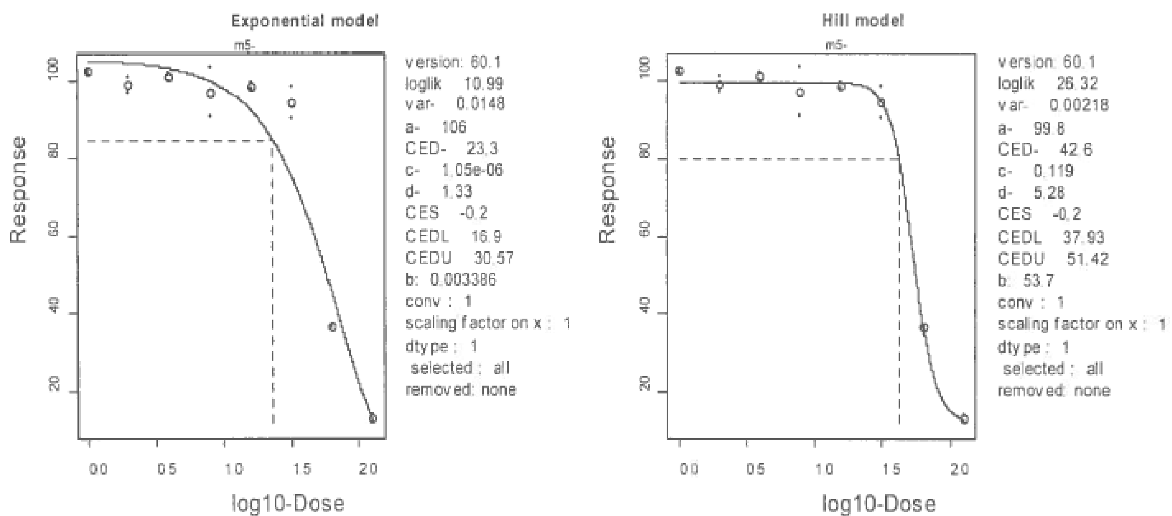

Figure 2D. Dose response of survival of A549 cells after 24 hours incubation with NM-111 Zinc Oxide (ZnO), coated with triethoxycaprylsilane, mean particle size 140 nm, primary particle size 34 nm. CED, critical effective dose for 20% cytotoxicity. CEDL, critical effective dose low, CEDU, critical effective dose high.

Table 1 Overview of EC20 and confidence intervals for dose calculation.

NanoMile ID	Test compound	EC20 Exponential model µg/mL	Confidence interval µg/mL	EC20 Hill model model µg/mL	Confidence interval µg/mL	Selected Dose µg/mL
NP00193	CeO_2 (undoped) CeO_2 A	n.d.		n.d.		128
NP00194	CeO_2 (CeO.75ZrO.25O2) CeO C	n.d.		n.d.		128
NP00196	CeO_2 (CeO.25ZrO.75O2) CeO E	n.d.		n.d.		128
	NM-212, nanograin CeO_2	n.d.		n.d.		128
NP00281	ZnO, 5 µm	57.6	54.9-61.6	57	54.3-61.1	57.3
NP00282	ZnO NM-110	29.4	22.4-37.3	33.9	29.2-38.9	31.6
NP00282	ZnO NM-111	23.3	16.9-30.6	42.6	37.9-51.4	32.9
-	ZnCl	19.7	14.2– 26.1	29.4	27.7-31.4	24.5
NP00221	$AgNO_3$	9.6	2.6 – 12.8	10.1	5.2 – 11.3	9.8
NP00213	Ag micron sized	n.d.		n.d.		128
NP00214	Ag NM300K	37.9	33.5 – 50.1	39.3	34.0-49.7	38.6

The EC20 doses were estimated using the PROAST evaluation program. N.d. dose could not be determined so highest dose (i.e. 128 µg/mL) was used for the studies.

Based on the EC20 dose calculations, A549 cells cultured in 6 wells tissue culture clusters were exposed to the MNM doses presented in Table 1. Additionally, control wells were included at each time point for cell viability, actual cytotoxicity of the MNMs in the 6 well tissue culture plates, and for measuring possible MNM interference with the read out system of the viability assay. An effect of 20% cytotoxicity at t=24 hours was chosen as with a higher cytotoxic effect (e.g. 50% cytotoxicity at 24 hours exposure) the abundant presence of dead cells may potentially obscure the omics results. The cells were harvested at t=1, t=6 and t=24 hours to monitor changes in the omics pattern at a dose inducing approximately 20% cytotoxicity. For some of the MNMs investigated no clear dose response was established (see Table 1), so the highest exposure dose used in the cytotoxicity assay was used for the exposures in the 6 well plates being 128 µg/mL. Using the doses as indicated in Table 1 the controls on the cytotoxicity in the 6 well plates showed a higher cytotoxicity than measured in the 96 well plates. Therefore some doses for the exposure in the 6 well plates were adapted and applied as presented in Table 2.

Table 2 Cell viability of samples for omics evaluation.

NanoMile ID	Test compound	Dose (µg/mL)	Cell viability (%)
NP00193	CeO_2 (undoped) CeO A	128	88
NP00194	CeO_2 (CeO.75ZrO.25O2) CeO C	128	89
NP00196	CeO_2 (CeO.25ZrO.75O2) CeO E	128	89
	NM-212, nanograin CeO_2	128	87
NP00281	ZnO, 5 µm	**30**	82
NP00282	ZnO NM-110	**15**	94
NP00282	ZnO NM-111	**10**	89
-	ZnCl	24.6	67
NP00221	$AgNO_3$	**8**	83
NP00213	Ag micron sized	128	95
NP00214	Ag NM300K	38.6	79

Exposure doses indicated **in bold** for the A549 cells that were adapted for incubation in the 6 wells tissue culture cluster.

5.2 Omics results

The UoB team have successfully completed the collection of the metabolomics and lipidomics raw data from the various MNM exposure studies in A549 cells. The signal processing to convert the raw 'omics data into a format compatible with a range of statistical analyses methods has also been completed. This is a complex process that involves multiple steps and extensive computational resources. The challenges of processing such data are exacerbated by the very small biomass (of human cells) available per sample and hence many of the signals lie close to the signal to noise limit of the instrumentation. Figure 3 indicates the workflow used for the processing of the DIMS metabolomics data.

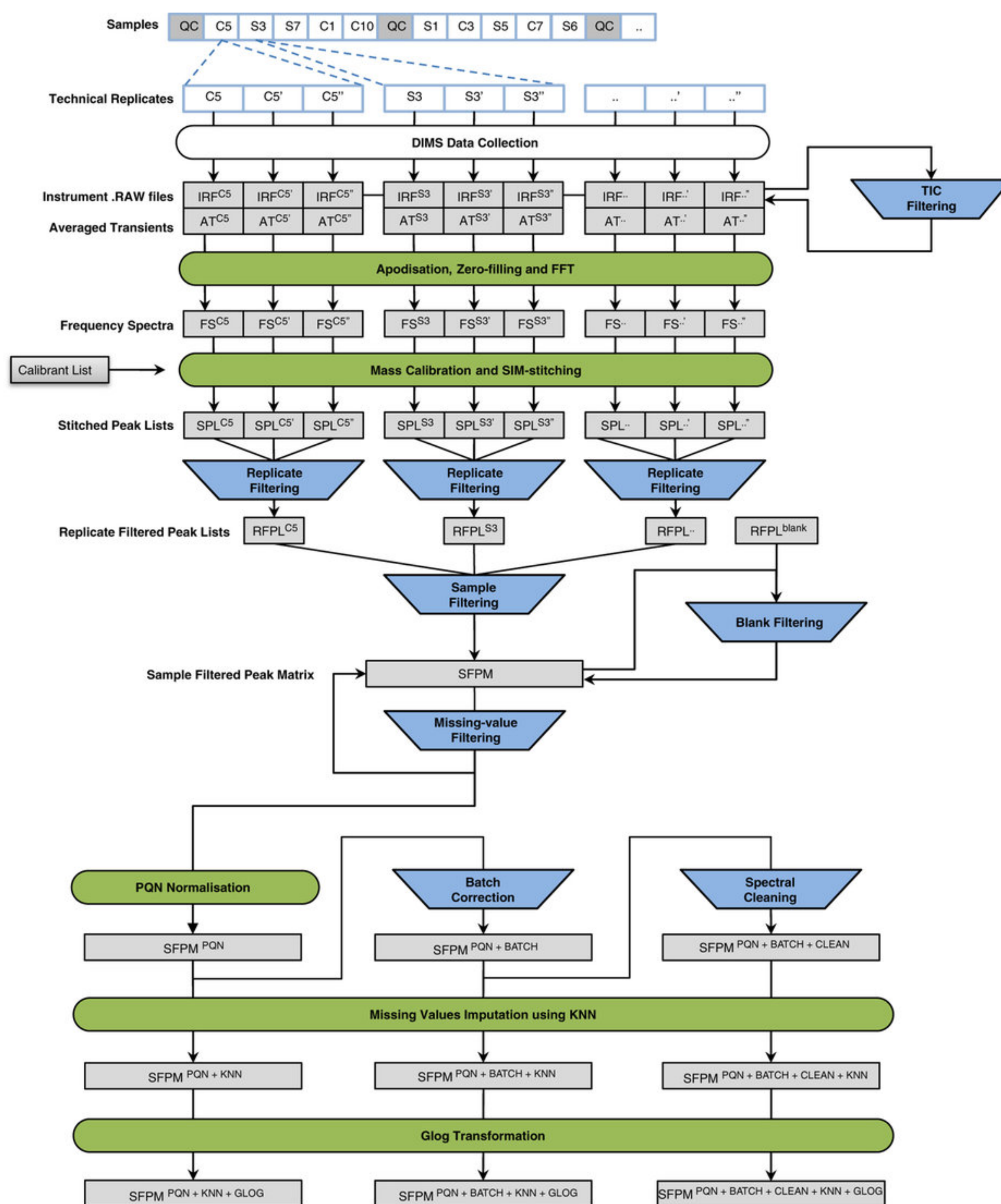

Figure 3 Data processing workflow for direct infusion mass spectrometry-based metabolomics dataset (reported in Kirwan et al., 2014). The workflow shows the processing of generic sample types, biological controls (C) and treated biological samples (S), and quality control samples (QC). Each metabolomics study conducted on MNMs contained all three sample types. All software presented in this figure and used within this deliverable was developed in-house.

Deliverable 8.4 thus comprises a 'dataset report', specifically describing the GigaBite-scale raw 'omics data. The next step in WP8, specifically to support the future Deliverables D8.5 (Computational models for each

species/cell type) and D8.6 (Biomarkers for assessing MNM impacts), will be the computational evaluation and data mining at the University of Birmingham (processed data are not shown in this Deliverable 8.4).

6. Conclusions and recommendations

The collection of RNA Seq transcriptomics data as well as mass spectrometry metabolomics and lipidomics data of A549 cells exposed to all WP8-selected NanoMILE MNM (Ag-MNM, ZnO MNM and CeO MNMs, sa well as relevant bulk and ionic control materials, as shown in Table 2) has been completed. The data and metadata has been made available for computational biology and data mining by the NanoMILE team at the University of Birmingham, to contribute to delivering further Deliverables from WP8.

7. References

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