DELIVERABLE REPORT

Deliverable:	D8.2: Omics datasets - Daphnia
Work Package:	WP8: Systems biology approaches to reveal mechanisms of MNM activity
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Project full title: "Engineered nanomaterial mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology"

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0. List of abbreviations

DIMS: Direct infusion mass spectrometry

DIW: Deionised water

FDR: false discovery rate

KNN: k-nearest neighbours

MNM: manufactured nanomaterial

PCA: principal component analysis

PQN: probabilistic quotient normalisation

QC: quality control

SDS: Sodium dodecyl sulfate

SIM: selected ion monitoring

ZnO: Zinc oxide

1. Introduction

EU policy makers have taken the bold step of legislating toxicity reporting for all chemicals (with nanomaterials considered as alternative forms of chemicals) sold in Europe (e.g. REACH legislation) and have broadened the types of scientific evidence useful for safety assessment and regulation. These laws will ensure more and better information on substances, subsequently reducing risks that threaten the health of citizens, non-human species and ecosystems. It is also intended to dramatically reduce uncertainty in decisions taken to regulate (or not) the use of chemicals, thereby avoiding unwarranted over-regulation of substances that impose a financial drain on industry. Now, the burden of providing substance safety data has fallen on industry that critically needs scientific input to address the huge practical constraints at determining health risks. Using current practices, safety testing is estimated to cost £7 billion over 10 years, yet contributing scarce new knowledge towards the development of "green" chemistry. There are additionally considerable concern that nanomaterials require additional considerations, which is resulting in amendments to the Annexes to REACH with specific additional provisions for nanomaterials, due to be announced in 2016 (postponed from 2015 due to challenges to reach consensus).

The Systems Biology work package (WP8) in the NanoMILE project builds on important discoveries and technical advances of the past decade, particularly (a) the ability to rapidly identify genes and metabolites altered in response to a nanomaterial challenge (Taylor et al., 2016); (b) the availability of genomes for model species such as the freshwater invertebrate *Daphnia*, unicellular algae *Chlamydomonas*, and zebrafish (Colbourne et al., 2011); 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 this deliverable, therefore, is the exploitation of a chosen

set of non-mammalian, 3R-compliant biomedical and ecological model species, coupled with human cell cultures, to attempt to identify the core, evolutionarily conserved, biological pathways and molecular events indicative of the toxicity induced by selected manufactured nanomaterials (MNMs).

2. Objectives

The overall objective for NanoMILE WP8 is to complement standard toxicological approaches with a carefully selected range of systems biology based studies to support the understanding and comparisons of mechanisms of MNMs activity across several species of increasing complexity. More specifically, the objectives are:

- To measure the potentially harmful effects of multiple nanomaterials of differing physico-chemical properties using a suite of 'omics approaches, including transcriptomics, metabolomics and lipidomics.
- To characterise classic toxicological (or phenotypic) outcomes to MNM exposure alongside the molecular measurements, including effects on growth, reproductive output, morphological defects and mortality rates.
- 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 nanomaterials on environmental and human health (in WP4).
- To identify both species-specific and evolutionarily conserved molecular responses within the 4 species and cell line investigated.

Together, these objectives will facilitate NanoMILE to:

- Provide biological response data that will form the basis for current and future risk assessments of manufactured nanomaterials;
- 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 8.2 is to report on the state-of-the-art multi-omics investigations conducted to determine the responses of the model organism *Daphnia* to the selected NanoMILE MNMs. As per the DoW, the tasks performed to achieve the 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 in a regime of 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 (in total 200 samples analysed using all three 'omics approaches to generate ca. 100 Gigabytes of data); and (4) to make this data available to the computational biology NanoMILE team in WP8 led by Dr Shan He (UoB).

3. Current state of the art

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)).

Metabolomics is 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 approaches due to the breadth of their physico-chemical properties, with most studies using either nuclear magnetic resonance (NMR) spectroscopy or mass spectrometry (MS), followed by data processing and statistical analyses to discover those metabolite changes worthy of further investigation. When sample biomass is limiting, as is often the case for model organism and cell based studies, mass spectrometry is used predominantly due to its higher analytical sensitivity. 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" on 22 April 2016 revealed only 8 papers).

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* 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).

4. Experimental approaches

4.1 Daphnia culturing

D. magna were cultured in OECD recommended ISO test media (Table 1, pH=7.6-7.7) at a density of 80 animals per 4,000 mL media (OECD-recommended ISO test media for exposures, including 0.002 mg l–1 sodium selenite) in a 16 h:8 h light:dark photoperiod and temperature of 20 ± 1°C (Taylor et al., 2008). Media was renewed twice weekly and supplemented with an organic seaweed extract: Marinure (Wilfrid-Smith Limited, Oakley Hay, UK). Cultures were fed on suspensions of unicellular green alga, *Chlorella vulgaris*, and supplemented by a daily amount of dried bakers yeast (Sigma–Aldrich, UK). All cultures were initiated using third or fourth brood neonates aged <24 h old.

Table 1. Chemical composition of OECD ISO media used for *D. magna* culturing and chemical exposures.

Compound	Formula	Final concentration (mg/L)
Calcium chloride	$CaCl_2.2H_2O$	294.00
Magnesium sulfate	$MgSO_4.7H_2O$	123.25
Sodium bicarbonate	$NaHCO_3$	64.75
Potassium chloride	KCl	5.75
Sodium selenite	Na_2SeO_3	0.002

4.2 Preparation of dispersion stock of ZnO MNMs

Four types of MNMs were used in this study, including uncoated ZnO nanomaterial NM110 and coated with triethoxycaprylylsilane ZnO nanomaterial NM111, NanoSun ZnO nanomaterial and corresponding SDS coated NanoSun nanomaterial (Table 2). The detailed multi-omics investigation of ZnO MNMs follows from the studies in earlier work packages, in particular WP4, WP6 and WP7. These work packages revealed that ZnO MNMs, unlike many of the other MNMs investigated in NanoMILE, induced measureable toxicity and therefore were prioritised for deeper mechanistic investigation. Two of these nanomaterials are 'core' NanoMILE MNMs, widely studied across several work packages. In this component of WP8, a second ZnO nanomaterial was also included, NanoSun and SDS coated NanoSun. The justification for the inclusion of this additional particle follows from the fascinating earlier research by Taylor et al. (in prep.), who through using an unbiased metabolomics approach discovered that this MNM can perturb the chemical messenger (kairomone) signaling between *Daphnia magna* and the unicellular green algae upon which it preys. This novel toxicity mechanism is being invesitgated further through UK Natural Environment Research Council funding (metabolite analyses). Here, we have included NanoSun to investigate its effects on gene expression in Daphnia, and to investigate whether NM110 and NM111 can induced a similar moelcular response as NanoSun.

Table 2. Coatings and nominal unit size of ZnO MNMs and bulk studied in this project.

Name	Coatings	Nominal unit size (nm)
ZnO NP NM-110	Uncoated	~150
ZnO NP NM-111	Coated with triethoxycaprylylsilane	~140
NanoSun ZnO NP	Uncoated	~40
SDS_NanoSun ZnO NP	Coated with SDS	~40
ZnO Bulk	-	5000

Stocks of ZnO NM110 and NM111 were prepared according to the standard dispersion protocol (http://www.nanogenotox.eu/files/PDF/web%20nanogenotox%20dispersion%20protocol.pdf) used in WP2. The dispersion stock of NanoSun ZnO and bulk ZnO was described in following the standard operation protocol on nanoparticle dispersion by the National Physics Laboratory (available online:

http://www.nanotechia-prospect.org). In brief, the ZnO MNMs and bulk ZnO powders were made into a paste by adding two drops of de-ionized water (DIW) and stirred with a metal spatula. Using a glass Pasteur pipette, 9-10 drops of DIW were added gradually, while continuing to mix the paste. The remaining DIW required to achieve the required concentration of stock was added to the paste and the suspension was sonicated twice for 10 seconds each using an ultrasonic probe (Cole Parmer 130 Watt Ultrasonic Processor). The 250 mg/L stock of SDS coated NanoSun ZnO nanomaterial was prepared by Dr. Isabella Romer-Roche at UoB.

4.3 Dissolution study of ZnO MNMs in *Daphnia* media

To evaluate the dissolution of ZnO MNMs and bulk ZnO particles in *Daphnia* culture media, the dissolved-fraction of ZnO nanomaterial in Daphnia media was separated by filtration. The stocks of ZnO MNMs, bulk and Zn ion form were added into 200 mL *Daphnia* media in order to achieve the nominal concentrations at 1mg/L of ZnO MNM, bulk and Zn ion form. 5mL Daphnia media from each treatment group were collected for total Zn content measurement immediately after adding stocks of ZnO MNMs, bulk and ion form into media. At the same time, an additional 10 mL of Daphnia media was transferred to Ultra-Clear centrifuge tubes serving as 0 h control. After standing for 6, 24 and 48hrs, 10mL exposed media were transferred to Ultra-Clear centrifuge tubes at each time point. Then the media samples collected in centrifuge tubes were centrifuged for 30 mins at 4,000 rpm (Sigma Laboratory Centrifuge 3-16KL, Sigma Laborzentrifugen GmbH, Germany). The supernatants were digested in a 2% HNO_3, and the Zn^{2+} concentration was quantified by ICP-OES. The percentage of dissolved Zn^{2+} under the different conditions was assessed by comparing to the total Zn amount (in nanomaterial, bulk or ion forms) added to the media. Two replicates were measured at each time point.

4.4 Acute toxicity test (48hrs immobility test)

To determine the appropriate sub-lethal concentration for the omics study, an initial range-finding experiment was performed. 3 days old Daphnia neonates were collected for acute toxicity test (48 h immobility test). 10 Daphnia were exposed to a range of concentrations of ZnO MNMs, bulk and Zn^{2+} into each 250 mL beaker with 200 mL exposure media for 48h. The nominal concentrations of ZnO MNMs and bulk were 0, 0.1, 0.5, 1, 5, and 10 mg/L (n = 3 per concentration), while the nominal concentrations of Zn^{2+} were equivalent to 0, 0.03, 0.1, 0.33, 1, 5 and 10 mg/L (n = 3 per concentration). After 48 h exposure, the number of alive animals was recorded and the survival rates were calculated as well.

4.5 Internal Zn load of Daphnia

To assess the uptake of ZnO MNMs, bulk and ionic Zn forms by Daphnia, the internal Zn body loads of Daphnia (n=3, each replicate included 10 *Daphnia* as a sample pool) were measured. 10 three-day old *D. magna* were exposed to 1mg/L of each of the ZnO MNMs, 1mg/L ZnO bulk, 0.3 and 1mg/L Zn ionic form for 2, 6, 24 and 48 h. After exposure, Daphnia were harvested and transferred into clean glass vials (pre nitric acid washed). A group of Daphnia without treatment with any Zn ion form or MNMs have also been collected as control samples. Then Daphnia were digested directly with 100 µL aqua regia (mixing one volume of 70% nitric acid (trace metal grade) to three parts 35% hydrochloric acid (trace metal grade)).

Once fully digested, the samples were diluted (gravimetrically) to 1% acid, i.e. if digested in 100µl add 9.9mL MilliQ water, and were quantified by ICP-MS (performed by Dr. Smitha Pillai at EAWAG, Switzerland).

4.6 Exposure study to generate samples for multi-omics investigation

To measure the omics responses of Daphnia exposed to ZnO MNMs, 15 three-day old Daphnia neonates per replicate were exposed to either 1 mg/L ZnO MNMs, bulk or ionic form in 200 mL media for 6, 24, 48 hours (Table 3). This number of animals per sample is based on the minimum biomass required for a multi-omics study. The rationale for the time points is as follows:

- The earliest molecular changes are likely to occur within a few hours, hence 6 hours is selected as an "early effect" time point.
- The acute toxicity studies that "anchor" the 'omics exposures were conducted using an OECD standard exposure duration of 48 hours, and hence the 48-hour 'omics time point was selected to correspond to this exposure duration.
- One further time point was selected, approximately mid-way between the early and late time points to capture intermediate temporal changes.

The rationale for the exposure concentrations selected is as follows:
(1) no acute toxic effects on Daphnia during exposure,
(2) to allow linking the potential differences of biological responses with different uptake of Zn which may be affected by two physicochemical properties (coatings and sizes) of ZnO MNMs in media, and
(3) to match the concentrations of ZnO MNMs used in the characterizations experiments (i.e. dissolution study) which need to use same starting concentration.

Therefore, 1mg/L ZnO MNMs (including ZnO NM110, ZnO NM111, NanoSun ZnO and SDS coated NanoSun ZnO), 1mg/L ZnO bulk and 1mg/L Zn ion form (supplying by $ZnCl_2$) were selected for omics exposure study. Each time-concentration point included 8 biological replicates. After exposure, Daphnia were collected into precellys tubes and rapidly quenched by liquid nitrogen and stored at -80°C until further extraction.

Table 3. The experimental design of exposure experiment for multi-omics study of *Daphnia*.

Groups	Exposure Conc. (mg/L)	Exposure time (h)	Replicates for Transcriptomics	Replicates for Metabolomics/ Lipidomics
ZnO NP NM-110	1	6, 24, 48	4	8
ZnO NP NM-111	1	6, 24, 48	4	8
NanoSun ZnO NP	1	6, 24, 48	4	8
SDS_NanoSun ZnO NP	1	6, 24, 48	4	8
ZnO Bulk	1	6, 24, 48	4	8
Zn^{2+}	1	6, 24, 48	4	8
Unexposed Control	-	0, 6, 24, 48	4	8

An array of omics responses have been measured, including gene expression profiling (using RNA Seq), metabolic changes (using mass spectrometry metabolomics) and lipid changes (using mass spectrometry lipidomics), as well as traditional phenotypic (apical) endpoints, as described below.

4.7 Mass spectrometry based polar metabolomics and lipidomics

4.7.1 Extraction of Metabolites

For extraction of Daphnia metabolites, Daphnia were firstly quenched in a precellys tube after exposure. 900 µL 80% methanol was added to each precellys tube on dry ice. Then the samples in tubes were homogenised in a Precellys 24 homogeniser for 2 x 10s bursts at 6400 rpm. 300 µL of aliquot was transferred into an Eppendorf tube for RNA extraction. The rest of homogenised mixture was transferred into a clean 1.8mL glass vial using a Pasteur pipette for metabolites extraction. Another 300 µL 80% methanol, 640 µL chloroform and 320 µL water were added each vial and vortex vials on full power for 30 seconds then leave on ice for 10 mins. After centrifugation (4000 rpm at 4ºC for 10 mins), the mixture separated into two phases (upper polar phase and lower non-polar phase). 400 µL aliquots of the polar phase were transferred into clean 1.5 mL Eppendorf tubes and then dried in a speed vac concentrator (Thermo Savant, Holbrook, NY) for 4 hrs. 400 µL aliquots of the non-polar phase were transferred into clean 1.8 mL glass vials and then dried under a stream of nitrogen to minimise oxidation. All dried samples were then stored at -80°C until analysis.

4.7.2 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 methonal: 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 Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometry in positive ion mode (for polar metabolomics) or negative ion mode (for lipidomics) (Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermo Fisher Scientific, Germany, 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 (PQN) method prior to statistical analyses.

For univariate statistical analysis, one way ANOVA (or t-test) with Benjamini-Hochberg correction was conducted on the peaks in the normalised DIMS datasets to determine whether they changed intensity significantly between control and treatment groups (at a false discovery rate (FDR) < 10% to correct for

multiple hypothesis testing). For multivariate statistical analysis, the normalized DIMS datasets were generalized log transformed and principal component analysis (PCA) performed using PLS_Toolbox (Eigenvector Research, Wenatchee, USA) in MatLab (version 7, the Math-Works, Natick, MA, USA). ANOVA (or t-test) with a Tukey-Kramer's post-hoc test was conducted on the PC scores for the top four PCs from each model (with an FDR of <10%) to evaluate the statistical significance of the treatments on the basis of the overall metabolic profiles.

4.8 RNA seq gene expression profiling

Total RNA of Daphnia were 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 a 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 provided protocols 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 upon 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 specific progress for nanotoxicology studies of the crustacean *Daphnia.*

Figure 1 shows the range finding study and compares the acute toxicity of the various ZnO nanomaterials, bulk and Zn ions towards *Daphnia magna*. This was coupled with a large scale ZnO MNM exposure study led by UoB, including characterisation of the dissolution of the materials used and measuring the Zn load within the *Daphnia*. The characterisation of the dissolution and metal uptake has been essential for designing the large-scale, and therefore relatively expensive, biological response study, and the data are shown in Figure 2 which compares the dissolution of ZnO MNMs and bulk after added into Daphnia culture media for 6, 24 and 48hrs. Figure 3 shows the corresponding internal Zn load in Daphnia magna after exposure to ZnO MNMs, ZnO bulk and Zn ions determined from the ICP-OES analysis of tissue.

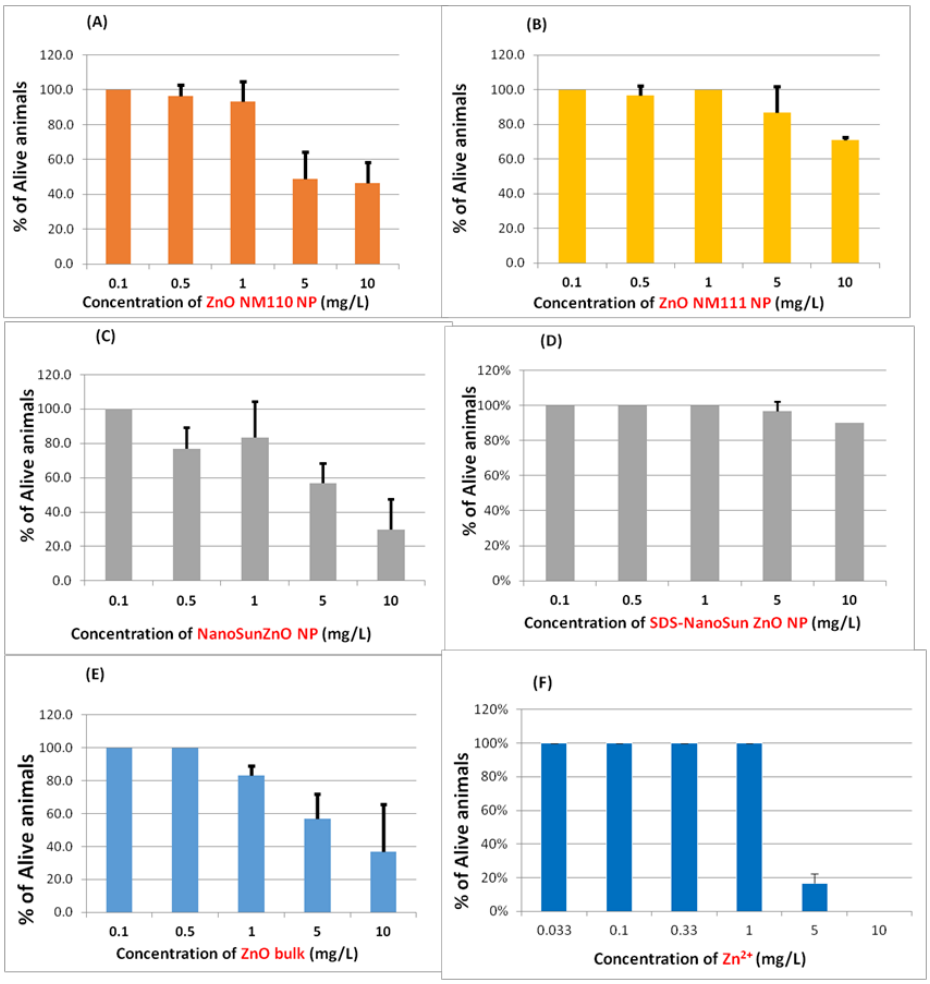

Figure 1. Acute toxicity screening test of ZnO nanomaterial, bulk and Zn ions in *Daphnia magna*. The survival rate of *Daphnia magna* were assessed after 48 hrs exposure to different concentrations of ZnO NM110 (A), ZnO NM111 (B), NanoSun ZnO (C), SDS coated NanoSun ZnO (D), ZnO bulk (E) and Zn ion (F) (each n=3). The results of EC_{50} values estimated by dose-response curves in different conditions suggested the ZnO MNMs with coatings were less toxic than their corresponding uncoated ZnO MNMs to *Daphnia*.

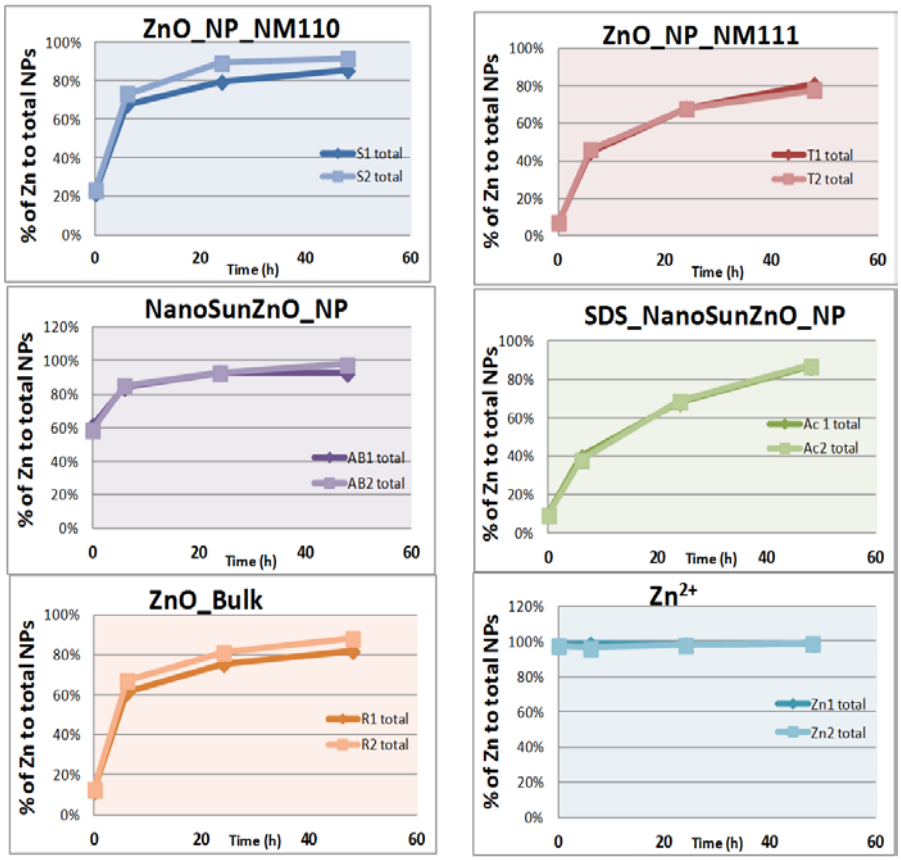

Figure 2. Dissolution of ZnO MNMs and bulk after added into Daphnia culture media for 6, 24 and 48hrs. The concentration of dissolved Zn was measured by ICP-OES after 1mg/L ZnO MNMs, 1mg/L ZnO bulk and 1mg/L Zn ion form added into *Daphnia* media for 6, 24 and 48hrs (n=2). The percentage of dissolved Zn in different conditions was estimated by comparing to the total Zn amount (in nanomaterial, bulk or ion forms) added into the media. Two replicates were measure at each time point. The results of dissolution of ZnO MNMs in *Daphnia* media suggested that without coatings, ZnO NM110 and NanoSun ZnO can dissolve rapidly in the media (i.e. after 6 hrs the dissolution reached ca.70% and 85%, respectively). The coated ZnO NM111 and SDS coated NanoSun ZnO dissolved more slowly than the uncoated materials, however they still dissolved relatively rapidly with more than 80% dissolved after 48 hrs in the *Daphnia* media.

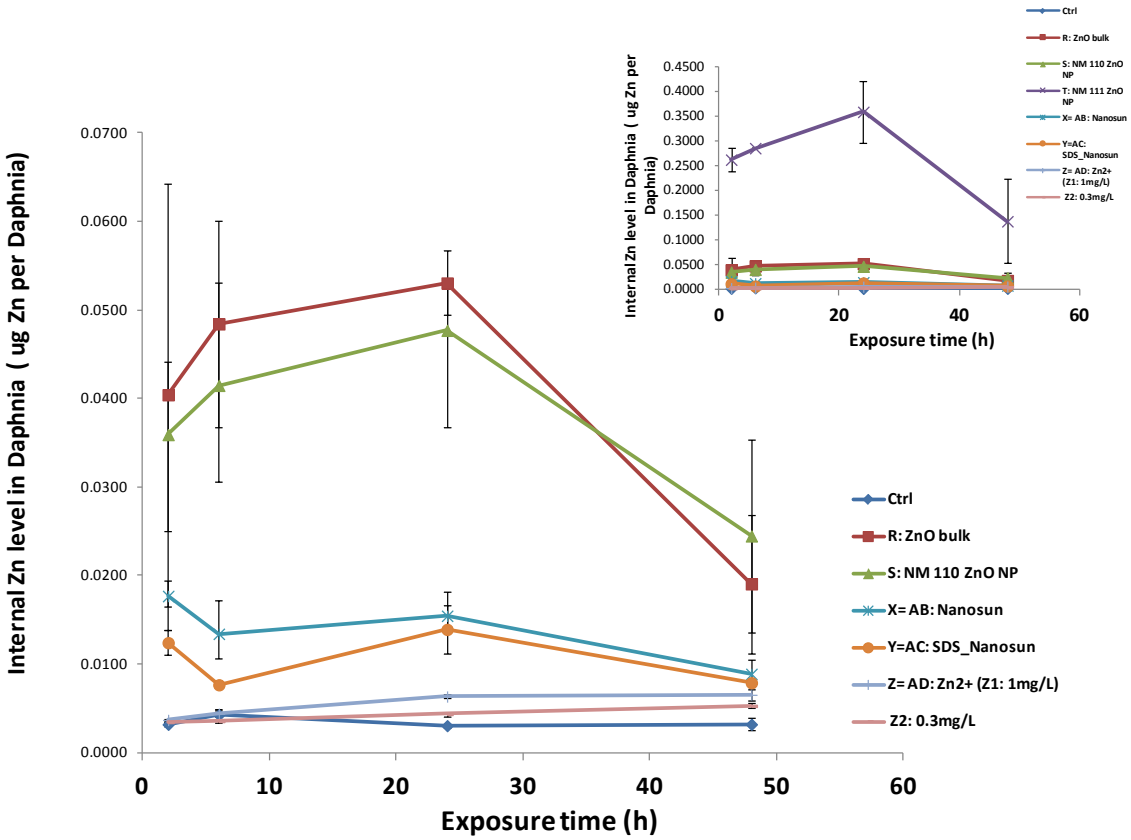

Figure 3. The internal Zn load in Daphnia magna after exposure to ZnO MNMs, ZnO bulk and Zn ion. *Daphnia magna* were exposed 1mg/L ZnO MNMs, 1mg/L ZnO bulk, 0.3 and 1mg/L Zn ion form for 2, 6, 24 and 48hrs. The internal Zn load in Daphnia after exposure were quantified by ICP-MS (n=3, each replicate including 10 *Daphnia* as a sample pool). The results of internal Zn load of Daphnia indicated that the uptake of ZnO MNMs or ZnO bulk was significantly higher than the Zn ion form. The size and the coating of the ZnO nanomaterial might have an effect on uptake into the *Daphnia* body as the values of the internal Zn load in the NM111 group was significantly higher than the NM110 group; furthermore the larger sized MNMs (NM110 and NM111) were associated with a larger internal load than for the small sized MNMs (NanoSun and SDS coated NanoSun).

UoB led the RNA Seq based gene expression profiling measurements of the *Daphnia* exposed to ZnO MNMs. The sequencing runs were performed on an Illumina Hiseq2500 platform, as described above, yielding >5 million 100bp reads per sample. The raw Bcl results files have been converted to fastq format using bcl2fastq Conversion Software v1.8.4 (Illumina). Further quality checking and subsequent bioinformatic analyses are on the way. Here, the results of the quantification and the quality assessments of the RNA samples used for the RNA Seq are reported (Figures 4 & 5)

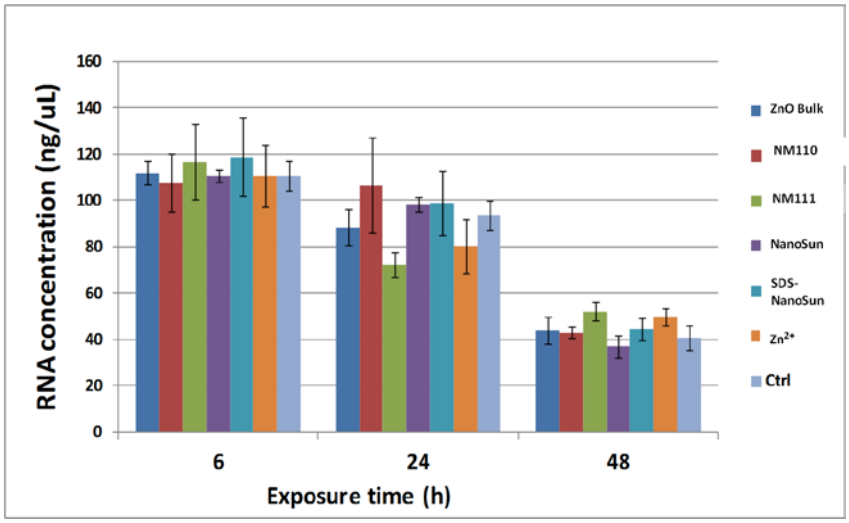

Figure 4. RNA concentrations of Daphnia exposed to ZnO MNMs, ZnO bulk and Zn ion form for 6, 24 and 48 hrs (n=4). This plot indicates a decreasing amount of RNA per experimental replicate. Importantly, at each time point, the concentration of RNA is similar across all the treatment groups including the controls. This indicates that no acute toxicity of the *Daphnia* occurred during the investigation.

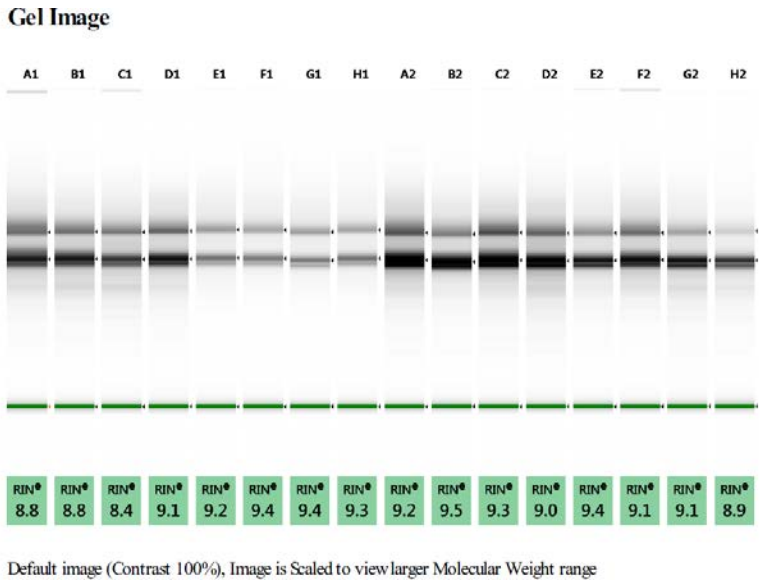

Figure 5. An example of results from the RNA quality evaluation by Agilent 2200 Tapestation which shows the majority of Daphnia RNA samples are of good integrity (RINe >7.0) for further RNA-seq.

In addition, we have successfully completed the collection of the metabolomics and lipidomics raw data from the *Daphnia* ZnO MNM exposure study. 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 processing steps and extensive computational resources. The challenges of processing such data are exacerbated by the very small biomass (of *Daphnia*) available per sample and hence many of the signals lie close to the signal to noise limit of the instrumentation. Figure 6 indicates the workflow used for the processing of the DIMS metabolomics data.

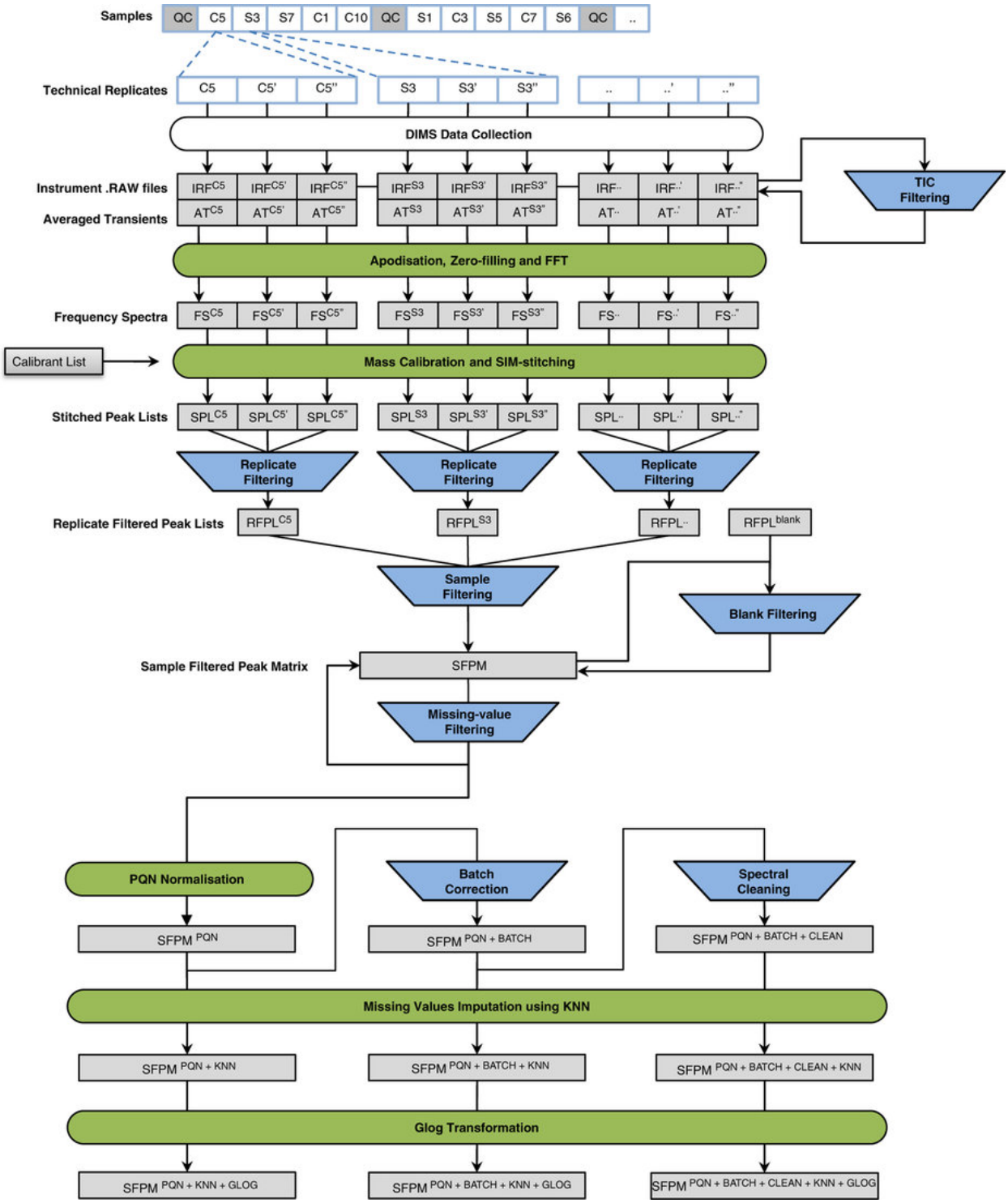

Figure 6. Data processing workflow for direct infusion mass spectrometry-based metabolomics dataset (reported in Kirwan et al., 2014). The workflow shows the processing of sample types C and S, and quality control samples QC. All software is developed in-house.

Deliverable 8.2 comprises a 'dataset report', specifically for the 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 conducted by the newly appointed computational biologist. Dr Albert Zhou joined Dr Shan He's research team and the NanoMILE project team at UoB on 1st

April 2016 to undertake this work. However, Dr Jinkang Zhang has undertaken some preliminary data mining of the new 'omics datasets, presented in Figure 7.

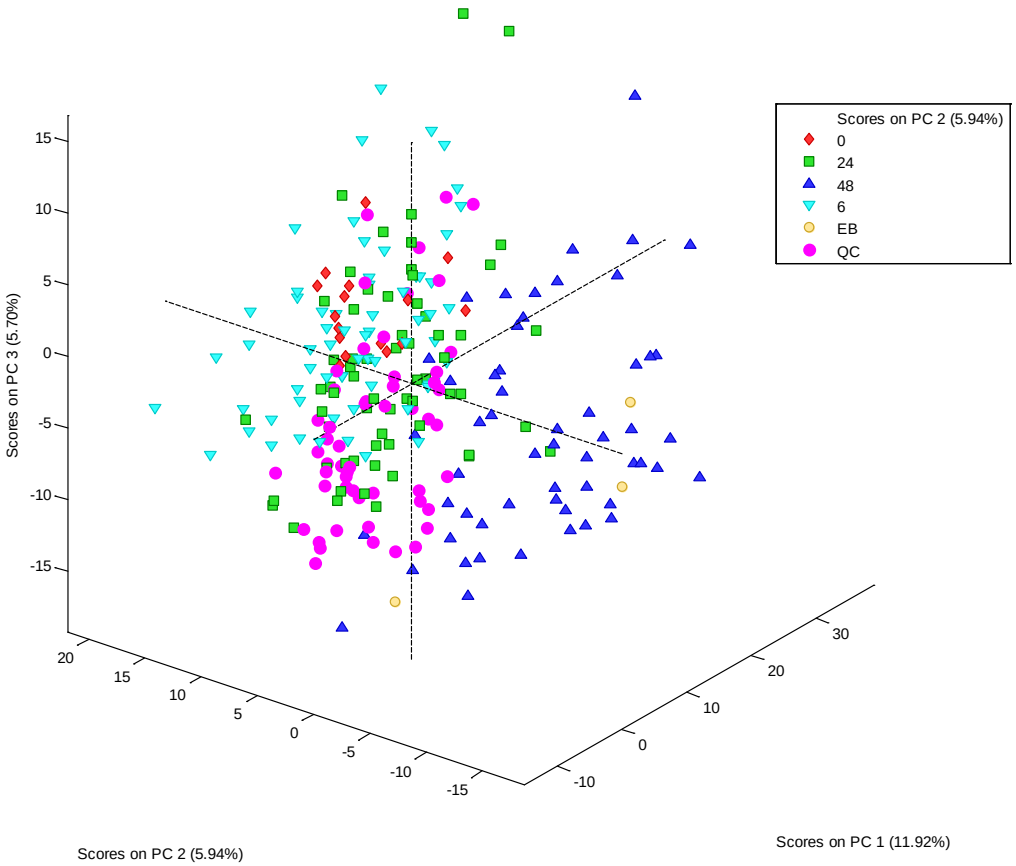

Figure 7. PCA plot of lipidomic profile data of *Daphnia* exposed to ZnO MNMs, ZnO bulk and Zn ion form for 6, 24 and 48 hrs (n=8). The majority of QCs (pink spots) are located in the central of the plot which means the DIMS measurement was successful. Further detailed statistical analyses and data mining are ongoing at the moment.

6. Conclusions and recommendations

Deliverable 8.2 comprises a 'dataset report', specifically for the raw 'omics data. Following the range-finding experiment which also assessed internal Zn load, Daphnia magna 3-day old neonates were exposed 1 mg/L ZnO MNMs (uncoated ZnO NM110, triethoxycaprylylsilane coated ZnO NM111, NanoSun ZnO and a corresponding SDS coated NanoSun), bulk or ionic form in 200 mL media for 6, 24, 48. The collection of RNA Seq transcriptomics data, mass spectrometry metabolomics and lipidomics data, and phenotypic (apical) measurements of MNM induced toxicity to *Daphnia* has been completed for the large-scale ZnO MNM study. The data and metadata has been made available to Dr Shan He, the computational biology lead within the NanoMILE team at Birmingham, for deeper analysis and to contribute to delivering further Deliverables, specifically Deliverables D8.5 (Computational models for each species/cell type) and D8.6 (Biomarkers for assessing MNM impacts), which will be reported on later in the project.

7. References

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