

Establishing the impact of metal oxide nanoparticle size and solubility on lung epithelial cell toxicity

by

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Abstract

Metal oxide nanoparticles (MONPs) are one of the most commonly used types of engineered nanomaterials (manufactured substances with at least one dimension in the 1-100 nm range), and they are used in a wide variety of applications in several industries. Many studies have observed cytotoxicity, genotoxicity, oxidative stress, and pulmonary inflammation induced by MONPs in *in vitro* and *in vivo* models. The overall objective of this study was to investigate and compare the toxicities of metal oxide nanoparticles (MONPs) of different chemical composition, size, and solubility to provide insight into the importance of physicochemical properties on MONP induced toxicity. FE1 mouse lung epithelial cells were exposed to manganese dioxide (MnO_2) and iron (III) oxide (Fe_2O_3) nanoparticles (NPs), microparticles (MPs), and soluble manganese sulfate (MnSO_4). Cell viability was assessed after 24h and 48h using the trypan blue exclusion assay while DNA damage was assessed using the high throughput CometChip® platform after 2h and 4h of exposure. Chromosomal damage was examined using the microflow assay at 40h. Changes in gene expression after 24h and 48h were examined using microarray to identify perturbations in molecular pathways that are associated with the manifestation of toxicological responses. The NPs in general appeared to be slightly more cytotoxic and genotoxic than the MPs and produced a larger transcriptomic response. MnO_2 NPs were more potent than Fe_2O_3 NPs with respect to genotoxicity and transcriptional perturbations, although they both induced low levels of cytotoxicity. Dissolved manganese ions from MnSO_4 (at concentrations close to what would be released by the MnO_2 NP treatments) did not induce cytotoxicity or genotoxicity but did induce transcriptional alterations. The molecular pathways perturbed by MnO_2 NPs, MnO_2 MPs, and MnSO_4 were primarily related to inflammation, immune response, and DNA damage/cell cycle while Fe_2O_3 NPs and Fe_2O_3 MPs primarily affected pathways involving inflammation, immune response, and cellular/tissue

development. These results suggest that size, solubility, and chemical composition influence their potential to induce toxicity. The information produced will address data gaps identified by risk assessors and aid in the development of read-across strategies for the assessment of other MONPs.

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Statement of Attribution

- Dongmei Wu performed the Dynamic Light Scattering analysis and microflow experiments.
- Andrey Boyadzhiev generated the Copper II Oxide (CuO) NP, MP, and Copper (II) chloride (CuCl₂) transcriptomics data.
- Andrew Williams performed the statistical analysis of the microarray data.
- Callum Christ performed the trypan blue assay, comet assay, and generated microarray data for the Manganese (IV) oxide (MnO₂) particles, Iron (II) oxide (Fe₂O₃) particles, and Manganese sulfate (MnSO₄). The data mentioned above was analyzed and interpreted by Callum.

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Introduction

Nanomaterials: Definitions and Types

Nanomaterials (NMs) are generally defined as natural or manufactured substances containing particles in an unbound or aggregated/agglomerated state with at least one dimension in the 1-100 nm range (Boverhof *et al.*, 2015). Health Canada defines NMs as any manufactured substance, product, component material, ingredient, device, or structure that has at least one dimension within the nanoscale (1-100 nm), an internal or surface structure at the nanoscale, or any material that exhibits nanoscale properties/phenomena despite being smaller or larger than the nanoscale in all dimensions (Health Canada, 2011). NMs can be classified into 3 broad groups: natural, incidental, and engineered (Hochella *et al.*, 2019). Natural NMs refer to any NM that is produced in the absence of human activity/anthropogenic processes (Ex: NMs originating from mineral dust in volcanic emissions); incidental NMs are produced unintentionally by human activity/anthropogenic processes (Ex: carbon nanotubes found in diesel exhaust); and engineered NMs (ENMs) are intentionally produced for a specific function (Ex: TiO₂ nanoparticles produced for use in cosmetics such as sunscreen) (Hochella *et al.*, 2019; Jung *et al.*, 2013; Dréno *et al.*, 2019).

Exposure to natural and incidental NMs have always been a health concern to humans (Hochella *et al.*, 2019; Oberdorster *et al.*, 2005). While air pollution is often a result of human activity, natural events also produce a large amount of the airborne nanoparticulate matter worldwide. Sources of natural NMs include the particulate products of incomplete combustion from forest fires (such as fullerenes, graphene, and carbon nanotubes), volcanic ash (carbon and heavy metals), dust storms, ocean Spray (sea salt aerosols), the weathering of metal/anion containing rocks, and viruses (Barhoum *et al.*, 2022; Buzea *et al.*, 2007). Exposure to airborne

dust particles, which can also carry harmful things like pollutants, bacteria, viruses, and allergens, may lead to health problems including obstructive airway diseases (asthma, bronchitis, chronic obstructive pulmonary disease [COPD]), lung fibrosis (sand/dust storm associated silicosis), lower respiratory tract infections, and cardiovascular diseases (Gross *et al.*, 2018). There is a growing body of evidence showing that exposure to particulate matter from wildfires causes a variety of adverse health outcomes and increases the risk of respiratory and cardiovascular morbidity (Cleland *et al.*, 2021). Epidemiological evidence indicates that respiratory and cardiovascular hospital and emergency department admissions increase following short-term exposure to smoke (Cleland *et al.*, 2021). Adverse respiratory symptoms occur after exposure to volcanic ash and an increased mortality from respiratory diseases such as COPD and lung cancer is associated with long-term exposure to ash fall (Hlodversdottir *et al.*, 2016).

Sources of incidental NMs include combustion by-products from cooking food, vehicle engines, welding, refining, and smelting (Buzea *et al.*, 2007). Diesel and engine exhaust are the primary source of atmospheric nano and micro sized particulate matter in urban areas (Buzea *et al.*, 2007); diesel exhaust also contains high levels of polycyclic aromatic hydrocarbons (Penn *et al.*, 2005). The inhalation of ultrafine ambient particulate matter is associated with increases in cardiovascular and respiratory morbidity/mortality. Adverse respiratory outcomes such as lung inflammation and lung remodeling are observed in workers exposed to welding fumes (which contain nano sized Fe, Mn, Cr oxide particles) (Andujar *et al.*, 2014). Silicosis and coal worker's pneumoconiosis are also lung diseases primarily related to occupational exposure to silica and coal mine dust (Castranova and Vallyathan, 2000). Overall, it is clearly established that several types of incidental NMs induce adverse health outcomes in humans.

The relatively recent and rapidly developing field of nanotechnology is a growing source of exposure to ENMs, which presents an additional health concern for humans. ENMs have physicochemical properties that differ from their respective bulk (>100 nm) materials. They are increasingly used in a wide variety of applications in several industries due to their unique physical, thermal, optical, and biological properties (Nel *et al.*, 2006; Kessler, 2011). ENMs are commonly used in biomedical applications (bioimaging, biosensing, drug delivery), electronics (data storage, batteries, displays), cosmetics, sporting goods, antimicrobial coatings, and pollution remediation (Buzea *et al.*, 2007). The global nanotechnology market was valued at \$75.80 billion USD in 2020 and is projected to reach \$290.93 billion USD by 2025 (Emergen Research, 2021). Due to the rise in production and use of ENMs in commercial products over recent years, the need for regulation and testing of their toxicity has become increasingly important.

Engineered Nanomaterial Properties

The unique properties of ENMs are attributed to their size, surface area, surface properties, shape, chemical composition, solubility, and aggregation (Nel *et al.*, 2006). Each property directly impacts the toxicity of ENMs in biological systems.

The small size and corresponding larger surface area of ENMs results in a greater ratio of surface atoms/molecules to total atoms/molecules than their respective bulk material (Oberdorster *et al.*, 2005). As a result, ENMs exhibit greater biological activity than their bulk material per given mass. Their small size also enables these particles to translocate into organs/tissues at a higher rate than larger particles. For example, smaller (10 nm) iridium-192 nanoparticles (NPs) inhaled by rats had a higher rate of translocation into the liver and kidney

than larger (75 nm) iridium-192 NPs (Buckley *et al.*, 2017). Huo *et al.* (2014) showed that smaller gold (Au) NPs (<6 nm) enter the nucleus of MCF-7 (human breast cancer epithelial) cells, while larger Au NPs (10 and 16 nm) were confined to the cytoplasm, which implies that smaller NPs exert a greater toxic potential than larger particles. Smaller Au NPs (5 and 10 nm) also induce a greater amount of cytotoxicity than larger particles (80nm) in A549 (human lung adenocarcinoma type-II alveolar epithelial) cells, and reactive oxygen species (ROS) generation is size and concentration dependent (Ibrahim *et al.*, 2023). Halappanavar and colleagues (2015) examined the pulmonary transcriptional profiles of mice exposed via single intratracheal instillation to TiO₂ NPs of differing size. They found that larger TiO₂ NPs (38nm) exhibited far fewer differentially expressed genes than smaller TiO₂ NPs (10nm) 24h post-exposure.

ENMs also have a wide variety of shapes, including spheres, fibres, tubes, and planes (Oberdorster *et al.*, 2005). A study comparing the toxicity of hydroxyapatite NPs of different shapes (needle, plate, rod, and sphere) showed that the needle and plate shaped NPs induced more cytotoxicity than the rods and spheres in BEAS-2B (human bronchial epithelial) and RAW264.7 (murine macrophage) cells (Zhao *et al.*, 2013). The shape of a particle can also affect its deposition kinetics and cellular uptake mechanisms (like the ability to be phagocytosed – a common mechanism of particle uptake - by alveolar macrophages) (Buzea *et al.*, 2007; Champion and Mitragotri, 2006). Zhao *et al.* (2013) also observed a higher degree of particle-cell association and increased cellular uptake in the rod-shaped hydroxyapatite NPs than the needle shaped NPs, despite the needle shaped NPs showing greater levels of cytotoxicity.

Chemical composition is also a factor that affects ENM toxicity, as it can influence the mechanism by which toxicity is induced. A comparative study on the cytotoxicity, oxidative stress, and genotoxicity induced by carbon black (CB) NPs, carbon nanotubes (CNTs) NPs, silica

(SiO₂) NPs, zinc oxide (ZnO) NPs in primary mouse embryo fibroblasts showed that the difference in toxicity between ZnO (induced lots of viability reduction and oxidative damage) and SiO₂ (little reduction in viability and oxidative damage) could be attributed to their chemical composition as both particles had similar crystal shape and particle size (Yang *et al.* 2009).

The crystal structure of NPs has an impact on toxicity as certain arrangements of atoms with the same chemical composition may have higher intrinsic ROS producing capabilities than others (Jiang *et al.*, 2008). For example, the anatase crystal structure of titanium dioxide (TiO₂) induced a greater amount of cytotoxicity than the rutile structure in HDF (human dermal fibroblast) cells and A549 cells in addition to greater ROS generating capabilities (anatase is the more photoactive crystal structure of TiO₂) (Sayes *et al.*, 2006). The overlap of band gap energies of certain insoluble metal oxide nanoparticles (MONPs) such as cobalt (II, III) oxide (Co₃O₄), chromium (III) oxide (Cr₂O₃), nickel (III) oxide (Ni₂O₃), manganese (III) oxide (Mn₂O₃) and cobalt (II) oxide (CoO) with cellular redox potential are correlated to their ability to produce ROS, oxidative stress, and inflammation (Zhang *et al.*, 2012).

Many metals and metal oxide ENMs dissolve in water and solutions including biological and environmental matrices, although the extent of dissolution depends on the chemical composition of the ENM as well as properties of the liquid (such as pH and temperature). The presence of biomolecules (chlorides, proteins, polysaccharides etc.) in the liquid can enhance dissolution if they are able to form soluble complexes with the released ions or hinder dissolution if less soluble complexes are formed (Utembe *et al.*, 2015). Certain ENMs are taken up by cells and dissolve once they are in acidic cell compartments, which may lead to adverse outcomes associated with the release of toxic ions (Nordberg and Costa, 2022). This is referred to as the Trojan horse-type uptake mechanism and it applies to several metal oxide ENMs including zinc,

iron, manganese, cobalt, copper, and nickel oxide (Stark, 2011; Nordberg and Costa, 2022).

Poorly soluble ENMs can accumulate in the lungs, overwhelm the clearance capacity of alveolar macrophages, and cause lung tissue injury (Buzea *et al.*, 2007). The formation of aggregates and agglomerates is generally thought to reduce the hazard potential of ENMs as it reduces surface area and number of particle-cell interactions (Liu *et al.*, 2022). Béteky and colleagues (2019) treated A549 and MRC-5 (human fetal lung fibroblast cells) cells with silver NPs in different levels of aggregation and observed a decrease in toxicity (measured using cell viability) as the grade of aggregation increased.

Although more than two decades of research have advanced our understanding of the health effects of ENMs, the research community has yet to arrive at consensus on the specific ENM properties that cause toxicity. Thus, when studying ENMs, the characterization and understanding of physicochemical properties and interpretation of results in the context of ENM properties investigated is crucial, as they influence the nature and magnitude of toxicity.

Inhalation as Route of Exposure

ENM exposure is most likely to occur in the workplace, which includes research labs, companies, and facilities where ENMs are synthesised, used, processed, disposed, and recycled (Yokel and Macphail, 2011). Common routes of exposure to ENMs include inhalation, oral, and dermal; however, inhalation is the predominant route for occupational exposure due to the presence of airborne particles in the workplace. Following inhalation of particulate material, larger particles often deposit in the upper respiratory tract (nasopharyngeal region) while smaller particles deposit in the lower respiratory tract (tracheobronchial region) and can reach the alveolar regions of the lung (De Matteis, 2017). The larger particles in the upper respiratory tract

can be cleared from the lung by mucociliary action (fast particle clearance) and coughing while the smaller particles deep in the lungs are cleared less quickly (Blank *et al.*, 2009). In the alveolar regions, deposited particles are primarily cleared by macrophage phagocytosis (Buzea *et al.*, 2007). Alveolar macrophages take up particles and migrate from the alveoli to the mucociliary escalator or across the alveolar epithelium to the lymph nodes (Buzea *et al.*, 2007). The longer it takes particles to clear, the more likely adverse health outcomes become due to extended particle-tissue and particle-cell interactions (Blank *et al.*, 2009). However, small ENMs can avoid clearance and penetrate the alveolar epithelium to reach the interstitial space and circulate to other parts of the body via the blood or lymphatic system (Buzea *et al.*, 2007). The stability of the intracellular environment is maintained by the cell membrane, which is a bilayer primarily composed of phospholipids that controls the passage of substances into and out of the cell (Liu and Tang, 2020). The primary method by which ENMs enter the cell is through endocytosis, which features a change in membrane structure to internalize the particles. The main pathways of ENM uptake via endocytosis include phagocytosis, macropinocytosis, clathrin-dependent endocytosis, and caveolin-dependent endocytosis (Liu and Tang, 2020). Properties such as shape, size, and coating have an impact on the type of endocytic pathway used for the uptake of a specific particle. Adverse health outcomes associated with ENM inhalation include tissue inflammation, epithelial cell proliferation, fibrosis, emphysema, and tumour formation (Buzea *et al.*, 2007). Studies have found that occupational inhalation exposure to certain incidental NMs (asbestos, coal mine dust/silica, metals) results in increased rates of lung carcinoma, lung fibrosis, and pneumoconiosis/silicosis among workers (Castranova and Vallyathan, 2000; Assad *et al.*, 2018; Mossman *et al.*, 2011; Halappanavar *et al.*, 2020).

Toxicity of Metal Oxide NPs

Metal oxide nanoparticles (MONPs) are one of the most widely used types of ENM and are commonly used in applications such as paints, cosmetics, sunscreen, electronics, antibacterial products, and environmental remediation (Sengul and Asmatulu, 2020). The severity of the toxic effects largely depends on the physicochemical properties of the MONP, which include size, surface area, surface properties, shape, chemical composition, solubility, and aggregation. The primary route of exposure to MONPs is via inhalation during their production and use in the workplace. Given that engineered MONPs have been widely used for a relatively short time, their adverse health effects have been documented in a limited number of epidemiological studies (Pietrojusti *et al.*, 2018). Epidemiological studies focusing specifically on engineered MONPs detect increases in markers for oxidative stress and elevated biomarkers for inflammation for a fairly small number of engineered MONPs (such as TiO₂ and indium tin oxide) (Pelclova *et al.*, 2017; Liu *et al.*, 2012; Wu *et al.*, 2014).

Many studies demonstrate cytotoxicity, genotoxicity, oxidative stress, and pulmonary inflammation induced by MONPs in *in vivo* models. For example, Cho *et al.* (2010) observed lung inflammation in rats after treatment with cerium dioxide (CeO₂), nickel oxide (NiO), zinc oxide (ZnO), and copper oxide (CuO) NPs by intratracheal instillation. The inflammation was characterized by different types of inflammatory cells and inflammatory mediators, as well as variation in the time course of effects and levels of cytotoxicity observed for each MONP, indicating that each particle type may have its own unique “inflammatory footprint”. A similar study investigated inflammation and genotoxicity induced by MONPs (TiO₂), aluminium oxide (Al₂O₃), cerium oxide (CeO₂), and two types of zinc oxide (ZnO) NPs in mice after aerosol inhalation (Larsen *et al.*, 2016). ZnO and (to a lesser extent) CeO₂ NPs caused inflammation

while only TiO₂ NPs induced a significant amount of DNA strand breaks in the lung tissue. Similar adverse outcomes are observed *in vitro*. The genotoxicity of CuO, TiO₂, iron (III) oxide (Fe₂O₃), iron (II, III) oxide (Fe₃O₄) NPs and MPs was evaluated using the comet assay in A549 cells (40 µg/cm² exposure for 4h) (Karlsson *et al.* 2009). The CuO and TiO₂ NPs induced significantly more DNA damage (in the form of single strand breaks and alkali labile sites) than the control, while the Fe₂O₃ and Fe₃O₄ NPs did not produce significant amounts of DNA damage. CuO, TiO₂, Fe₂O₃, and Fe₃O₄ MPs all induce a significant increase in DNA damage relative to the control. CuO NPs were significantly more potent in damaging DNA than their MP counterparts, while TiO₂ MPs and Fe₂O₃ MPs were more potent than their corresponding NPs. These studies provide strong evidence that many types of MONP induce toxicity *in vivo* and *in vitro*.

Genotoxicity is a concern as damage and alterations to genetic material may lead to mutations, cancer, and heritable genetic disease. MONPs induce primary genotoxicity by interacting with DNA directly or indirectly through the production of NP-induced ROS or toxic ion-induced ROS after NP dissolution (Kohl *et al.*, 2020). Secondary or indirect genotoxicity, which is considered the main mechanism of ENM genotoxicity also involves the production of ROS by NP-activated inflammatory cells (phagocytes) (Kohl *et al.*, 2020). Excess ROS caused by MONPs are believed to overwhelm the natural antioxidant defenses of the cell to induce inflammation and cytotoxicity (Nel *et al.*, 2006). ROS can result from redox cycling on the surface of MONPs, via oxidative groups functionalized on MONPs, or can be produced during the process of inflammation following MONP exposure (Li *et al.*, 2010). The toxicity of soluble MONPs is thought to depend on the combination of particles and dissolved ion species. Soluble MONPs (such as CuO and ZnO NPs) release toxic ions in the acidic environment of the

lysosome (Donaldson *et al.*, 2013; Boyadzhiev *et al.*, 2021). The soluble MONPs are deposited as particles under the neutral conditions of the lung lining, but dissolve once they are taken up into the phagolysosome. The accumulation of toxic ions can destabilize the lysosomal membrane and result in oxidative damage to nucleic acids, lipids, and proteins (Strauch *et al.*, 2020). Particle size is also a factor to consider, as NPs dissolve at a faster rate than MPs due to their increased surface area (Boyadzhiev *et al.*, 2021). Transcriptomic studies have been performed to investigate the molecular pathways involved in MONP toxicity. Several studies have used microarrays and RNA sequencing to analyze gene expression and identify characteristic biological responses and pathway perturbations induced by MONPs (Halappanavar *et al.*, 2015; Halappanavar *et al.*, 2019; Costa *et al.*, 2018; Boyadzhiev *et al.*, 2021; Nikota *et al.*, 2016; Rahman *et al.*, 2017). These studies provide strong evidence that many types of MONPs are cytotoxic, genotoxic (DNA strand breaks, mutagenicity, clastogenicity), and perturb gene expression.

Manganese Oxide NPs

Manganese is an essential nutrient that has several functions in biological systems. It is a cofactor in many enzymes and plays roles in development, antioxidant defense, energy production and immune response (Chen *et al.*, 2018). Manganese can exist in a variety of oxidation states ranging from Mn^{3-} to Mn^{7+} , although the only oxidation states commonly found in living tissue are Mn^{2+} , Mn^{3+} , and Mn^{4+} (Avila *et al.*, 2013; Archibald and Tyree, 1987). While Mn^{2+} is the most stable oxidation state, unbound Mn^{3+} easily enters redox reactions and can undergo disproportionation to Mn^{2+} and Mn^{4+} (Chen *et al.*, 2018; Sobańska *et al.*, 2021). Ingestion (dietary consumption), dermal absorption, and inhalation are the main routes of

exposure to manganese (Avila *et al.*, 2013). Following absorption in the GI tract or lungs, manganese enters the blood stream and reaches various organs (highest concentrations are found in the liver, pancreas, bone, kidney, and brain) (Chen *et al.*, 2018). The influx of manganese into cells is regulated in several different manners (including divalent metal transporter 1 [DMT1], Zn-interacting protein 8 and 14 [ZIP8 and ZIP14], transferrin [Tf] receptor-mediated endocytosis, calcium channels and citrate transporters) that depend on the oxidation state of manganese (Mn^{2+} or Mn^{3+}) (Chen *et al.*, 2018; Avila *et al.*, 2013). Manganese is easy to oxidize and forms a variety of poorly soluble oxides with differing crystal structures (Chen *et al.*, 2018; Sobańska *et al.*, 2021; Avramescu *et al.*, 2022).

Occupational inhalation is a major route of exposure of manganese dust that is associated with various lung diseases (Roels *et al.*, 1992). Miners, smelters, welders, and workers involved in manganese oxide manufacturing processes and their applications are particularly susceptible to the inhalation of Manganese-containing aerosols (Frick *et al.*, 2011; Sobańska *et al.*, 2021). Several studies show an increased level of manganese in a variety of biological matrices (saliva, blood, urine, and hair) following occupational exposure to manganese oxide dust (Cowan *et al.*, 2009; Roels *et al.*, 1992; Li *et al.*, 2004).

Many studies have examined the effects of human occupational manganese exposure on neurological function. Elevated levels of manganese are associated with neurological disorders that resemble Parkinson's disease (known as manganism), though some studies also show respiratory effects and increases in the incidence of various cancers. Roels and colleagues (1987) examined neurological symptoms, respiratory symptoms, and a variety of biological parameters in a group of workers exposed to manganese in a manganese oxide and salt producing plant and compared them to a matched control group of workers from a different chemical plant. The

group exposed to manganese had respiratory tract symptoms (such as cough/sputum in cold season and episodes of acute bronchitis) more frequently than the control group, in addition to symptoms related to the central nervous system. A similar study by Roels *et al.* (1992) focused on a group of workers exposed to MnO₂ dust in a battery factory relative to a matched control group of workers in a polymer processing factory where there was no exposure to neurotoxic chemicals and lung irritants. The reported respiratory and neuropsychological symptoms did not differ from the control group, although the exposed group did not perform as well in certain neurological tests compared to the control group. Welders occupationally exposed to welding fumes (which contains several metals including manganese) have a 4.3-fold increase in manganese levels in serum compared to matched controls and present indications of oxidative stress (decrease in superoxide dismutase activity and increase in malondialdehyde levels) (Li *et al.*, 2004). Hobbesland *et al.* (1999) examined cancer incidence among workers in four Norwegian ferromanganese and silicomanganese producing plants, the relative incidence rates (compared to the expected incidence for Norwegian men during the same time period) of several cancers (including respiratory cancers) were positively associated with the duration of furnace work. However, it is difficult to solely attribute all of these negative health symptoms to manganese exposure as workers are often exposed to other substances as well.

Manganese dioxide (MnO₂) NPs are commonly used in electronics (capacitors and batteries), biomedical applications (sensors/contrast agents/drug delivery), and for wastewater/soil treatment as they adsorb heavy metals and other organic pollutants (Dawadi *et al.*, 2020). Given the increasing use of these NPs, it is important that their toxicity is studied while taking into account the effects of their physicochemical properties.

The impact of particle size and solubility on the toxicity induced by MnO₂ NPs has been scarcely studied in lung models/cell lines (neural models/cell lines are more commonly used due to the association between neurological disorders and elevated levels of manganese). Alhadlaq *et al.* (2019) measured cytotoxicity and oxidative stress induced by MnO₂ NPs (5-200 µg/mL) in MCF-7 (human breast cancer epithelial) and HT1080 (human fibrosarcoma epithelial) cell lines. There was a significant concentration-dependent decrease in cell viability (measured using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) and lactate dehydrogenase (LDH) assays) and increase in ROS production, lipid peroxidation, and intracellular H₂O₂ after the 25 µg/mL treatment. Mn oxides are soluble in low pH environments so the impact of Mn ions must be considered when investigating their toxicity (Sobańska *et al.*, 2021). Frick *et al.* (2011) investigated the impact of Mn₃O₄ NP solubility on oxidative stress, cellular uptake, and apoptosis using manganese sulfate (water soluble) and a rat type II alveolar epithelial cell line. Mn₃O₄ NPs induced more ROS production and intracellular oxidized glutathione than manganese sulfate, but similar increases in levels of extracellular oxidized glutathione. Exposure to Mn₃O₄ NPs also resulted in greater intracellular amounts of Mn than manganese sulfate, although they both caused similar increases in apoptosis. The results suggest that the apoptosis induced by Mn₃O₄ NPs and manganese sulfate are triggered by different mechanisms. MnO₂ NPs also induced DNA damage (comet assay) in a human neuronal cell lines at exposure concentrations of 30 µg/mL and 60 µg/mL at both 24h and 48h (Alarifi *et al.*, 2017). Hain *et al.* (2012) observed DNA damage (comet assay) in A549 and BEAS-2B cells after exposure to 20 µg/cm² and 40 µg/cm² of MnO₂ microparticles (MPs) for 24 and 48h. While many of the *in vitro* studies described above show that MnO₂ NPs and other manganese oxide NPs exhibit various forms of toxicity, others do not find this to be the case. For example, Xiao *et al.* (2013) exposed 293

(human embryonic kidney), NP69 (normal nasopharyngeal epithelium), and CNE-2 (human nasopharyngeal carcinoma) cells to Mn₃O₄ NPs at concentrations of up to 10 µM and did not observe any decrease in cell viability (MTT assay) after 24 and 48h. Similarly, Bi *et al.* (2019) and He *et al.* (2020) did not see a significant decrease in cell viability following exposure to MnO₂ nanosheets at concentrations of up to 200 µg/mL for 24h (L929 mouse fibroblast cells) and up to 1 mM for 24h (A375 human melanoma cells), respectively (both measured by the MTT assay). Although there are conflicting results, the weight of evidence supports MnO₂ NP induced toxicity *in vitro*.

Although most studies focus on neurotoxicity, the toxic effects of MnO₂ NP exposure observed *in vivo* also commonly include pulmonary inflammation and genotoxicity. Sárközi *et al.* (2009) treated rats with a MnO₂ nanosuspension via intratracheal instillation (doses of 2.63 and 5.26mg Mn/kg for 3, 6 and 9 weeks) to examine its toxicological effects. The lungs of the rats displayed an increasingly emphysematic exterior with increasing dose and time. The authors suggested that the emphysema on the removed lungs was likely caused by chronic airway inflammation and fibrosis. MnO₂ NPs and MPs have also been shown to affect biochemical parameters (LDH, alanine transaminase, and aspartate transaminase), induce genotoxicity (micronucleus assay, comet assay, and chromosomal aberration assay) and cause tissue damage in the liver, kidney, spleen, and brain in Wistar rats after acute oral exposure (doses of 30, 300, or 1000 mg/kg bodyweight per day for 28 days) (Singh *et al.*, 2013). The MnO₂ NPs were generally more potent than the MPs and induced toxicity at lower doses with respect to the biochemical parameters, genotoxicity, and tissue damage examined. Wistar rats exposed by intraperitoneal injection to a 100 µg/kg suspension of MnO₂ NPs daily for 15 days exhibit effects on the central nervous system (Sadeghi *et al.* 2018). Hippocampal homogenates from exposed

rats showed greater levels of oxidative stress (ROS levels and lipid peroxidation) and altered the appearance of hippocampus tissue (increased number of apoptotic and necrotic cells) relative to the control (unexposed) group.

In contrast, there have also been studies that show no apparent toxicity *in vivo* following exposure to NPs with surface coatings. Zhan and colleagues (2018) treated BALB/c mice with a 20 mg/kg dose of PEG-coated Mn₃O₄ NPs by injection via the tail vein and performed serum biochemistry assays and histological analysis 14 days post injection. No tissue or cellular damage was observed in any of the major organs (lungs, heart, spleen, liver, and kidneys) after comparison to the control group, and biochemical markers for liver and kidney function were either unchanged (alkaline phosphatase, total bilirubin, serum creatinine, and serum urea) or somewhat decreased but within normal range (aspartate aminotransferase and alanine aminotransferase). In another study, 20 mg/kg doses of soybean phospholipid-coated MnO₂ nanosheets administered to Kunming mice by injection into the tail vein caused no histological changes in the heart, liver, spleen, lung, or kidney compared to control mice one month post-exposure (Liu *et al.*, 2018).

Overall, while there is evidence that MnO₂ NPs induce cytotoxicity, genotoxicity, and inflammation, there are some conflicting findings. Moreover, there are a limited number of published studies that contextualize observed effects of MnO₂ NPs to the size and solubility of the NPs.

Iron Oxide NPs

Iron is one of the most abundant metals on earth and plays an essential role in many biochemical processes, such as electron transfer reactions, gene regulation, oxygen binding and

transport, and cell growth/differentiation (Fernández-Bertólez *et al.*, 2022). While iron is an essential mineral, it can also act as a toxicant to cells at sufficiently high concentrations – therefore the concentration of iron ions in cells is tightly regulated to maintain homeostasis. When not bound in functional protein complexes in cells and tissues, free iron can disrupt redox homeostasis and produce ROS (via the Fenton reaction) that can lead to oxidative stress and cellular/tissue damage (Galaris *et al.*, 2019). Iron generally forms compounds in the 2+ (ferrous) and 3+ (ferric) oxidation states and can form iron oxides with several different crystal structures containing ferric and ferrous ion centres (Fernández-Bertólez *et al.*, 2022). Iron (II) and (III) oxides generally display low solubility, except at extreme pH values (Cornell, R. M., & Schwertmann).

Iron oxide NPs have become increasingly popular due to their unique physical, chemical, and magnetic properties as well as their potential for use in a wide variety of applications. They are commonly used for biomedical applications (drug delivery, magnetic hyperthermia, MRI contrasting agent), agriculture/food (nanofertilizers, nanofungicides, nanopesticides, food pigment), environmental remediation/waste water treatment (permeable reactive barriers, membrane filtration, adsorption, pollutant sensors), energy (photovoltaic film coatings, fuel cells, batteries), and construction (nanocoatings, nanocomposites, pigment used in colouring construction materials) (Ali *et al.*, 2016; Voss *et al.*, 2020). Iron oxide NPs are often synthesized with coatings to protect the iron oxide core from corrosion and/or contribute to its physical/chemical functions for specific applications (Ali *et al.*, 2016). The primary routes of exposure to iron oxide NPs are inhalation, ingestion, and dermal uptake (Fernández-Bertólez *et al.*, 2022). Additionally, intravenous injection must also be considered given their use in biomedical applications. The most common unintentional route of exposure to engineered iron

oxide NPs is via inhalation as the particles tend to remain airborne for extended periods of time in occupational settings (Fernández-Bertólez *et al.*, 2022). Particle deposition mainly occurs in the alveolar region of the lung where they may penetrate the epithelium and circulate to other parts of the body via the blood or lymphatic system (Guo *et al.*, 2021). Iron oxide NPs distribute primarily to the lung, liver, testis, spleen, and brain (Kwon *et al.*, 2008; Sutunkova *et al.*, 2016). They can also reach the olfactory region of the brain, likely by translocation from the nasal cavity along the olfactory nerve fibers (Sutunkova *et al.*, 2016; Oberdörster *et al.*, 2004).

Human exposure to iron oxide NPs has been fairly well studied with respect to occupational exposure (miners, foundry workers, metal grinders etc.). Iron oxide dusts often contain quartz and silica, making it difficult to distinguish the effects of iron oxide specifically (Kornberg *et al.*, 2017). The inhalation of iron oxide NPs is associated with an increased risk of adverse respiratory outcomes and lung cancer in some epidemiological studies. For example, Boyd *et al.* (1970) found that English iron miners working underground had a 75% increase in mortality from lung cancer compared to the English national average, whereas surface workers showed no excess in lung cancer mortality. An epidemiological study examining the association between iron fumes/dust and welding fumes with the incidence of lung cancer in Finnish men found a 35% increase (standard incidence ratio: 1.35 CI 1.05 – 1.73) in the risk of lung cancer in the highest exposure category for iron fumes (Siew *et al.*, 2008). Overall, the relative risks for lung cancer increased with increasing cumulative exposure to iron fumes/dust and welding fumes. However, for many retrospective studies, there are a variety of potential concomitant exposures (such as radon, polycyclic aromatic hydrocarbons, and cigarette smoke) that make it difficult to directly attribute an observed increase in health risk to iron oxide specifically. There are also contrasting studies that don't find an association between occupational iron oxide NP

exposure and increased risk of adverse health outcomes (Bourgkard *et al.*, 2009; Moulin *et al.*, 2000; Lewinski *et al.*, 2013).

The toxicity induced by iron oxide NPs has been well studied in lung models/cell lines, although the impact of size and solubility on toxicity has not been particularly well characterized. Many *in vitro* studies have shown that iron oxide NPs exhibit relatively low toxicity, although there are additional studies that have observed higher toxicity. Oliveira *et al.* (2019) observed a concentration dependent increase in cytotoxicity using the LDH assay in A549 alveolar epithelial cells exposed to Fe₂O₃ NPs with a manufacturer reported size of <50 nm and mean diameter of 602.3 nm after Dynamic Light Scattering (DLS) analysis (~5% cytotoxicity after 24h exposure to 50 µg/mL and ~10% cytotoxicity after 24h exposure to 100 µg/mL). There was no concurrent change in the expression of inflammatory cytokines (IL-8 and IL-1β) released by these cells in this study. Karlsson *et al.* (2009) found that Fe₂O₃ and Fe₃O₄ NPs and MPs showed low toxicity in A549 cells (Trypan blue and comet assays) and no clear difference in toxicity between particle sizes (in contrast to CuO, where the NPs were much more potent than the MPs). Könczöl *et al.* (2011) observed concentration-dependent increases in DNA damage (comet assay; 4h exposure) and chromosomal damage (micronucleus assay; 24h exposure) after exposing A549 cells to different sized fractions of Fe₃O₄ NPs/MPs at concentrations up to 100 µg/cm². The smaller sized fractions of Fe₃O₄ NPs/MPs induced more micronuclei than the larger sized fractions, while DNA damage in the comet assay appeared to be less influenced by size (only slightly more DNA damage in smaller sized fractions). Exposure to Fe₃O₄ NPs/MPs (at concentrations up to 200 µg/cm²) induced slight (WST-1 assay) or no (neutral red uptake assay) cytotoxicity after 24h, though there was a significant amount of ROS generation. ROS scavengers were able to reduce the genotoxicity seen in the comet and micronucleus assays,

indicating that ROS play an important role in Fe₃O₄ induced genotoxicity. Ahamed *et al.* (2013) treated A549 cells with Fe₃O₄ NPs (mean diameter 24.83 nm) for 24h to examine their toxicological effects. There was a significant concentration dependent decrease in cell viability (41% cell viability after exposure to 100 µg/mL; measured by MTT assay) and a significant concentration dependent increase in LDH leakage (211% of the control after exposure to 100 µg/mL; LDH assay). The levels of ROS significantly increased in a concentration dependent manner and the levels of GSH (antioxidant) significantly decreased in a concentration dependent manner. There was also a significant concentration dependent increase in the percent of DNA in the comet tail after performing a comet assay (~10% after exposure to 100 µg/mL for 24h) (Ahamed *et al.*, 2013). Enzyme activity and mRNA levels of Caspase-3 and Caspase-9, which play important roles in the intrinsic (mitochondrial) apoptosis pathway, were significantly increased in a concentration-dependent manner after 24h of exposure to Fe₃O₄ NPs (Wu & Bratton, 2013).

In contrast, certain *in vitro* studies have not observed significant increases in toxicity after exposure to iron oxide NPs. Karlsson *et al.* (2008) exposed A549 cells to a variety of MONPs and carbon nanotubes at concentrations of 40 µg/mL and 80 µg/mL. Fe₂O₃ and Fe₃O₄ NPs (29 nm and 20-30 nm, respectively) did not induce significant increases in cytotoxicity relative to controls after exposure for 18h, although the Fe₂O₃ NPs were slightly more cytotoxic than the Fe₃O₄ NPs (measured using percent of non-viable cells; Trypan blue assay). No significant increase in DNA damage was found after exposure to Fe₂O₃ and Fe₃O₄ NPs for 4h (measured by percent DNA in comet tail; Comet assay). Fe₂O₃ and Fe₃O₄ NPs did not increase intracellular ROS levels (measured using an oxidation-sensitive fluoroprobe) and did not induce a number of oxidative lesions that was significantly different from the control after 4h aside from the 80

µg/mL concentration of Fe₃O₄ NPs (measured by level of FPG sites). Overall, the iron oxide NPs were among the least toxic of the particles (TiO₂ NP, CuZnFe₂O₄ NP, ZnO NP, CuO NP, carbon nanotubes) examined. Williams and Zosky (2019) did not see any significant cytotoxicity (LDH assay) after exposing BEAS-2B cells to up to 57 µg/ml of Fe₂O₃ and Fe₃O₄ MPs (200-800 nm) and A549 cells to 19 µg/mL of Fe₂O₃ and Fe₃O₄ MPs for 24h. Although IL-8 and IL-6 levels did not significantly increase after 24h exposure to up to 57 µg/mL of Fe₂O₃ and Fe₃O₄ MPs in BEAS-2B cells, IL-8 levels significantly increased after 24h exposure to 19 µg/mL of Fe₂O₃ MPs in A549 cells. Overall, the weight of evidence suggests that iron oxide NPs are less potent than many other MONPs in terms of cytotoxicity and genotoxicity *in vitro*.

The pulmonary toxicity of iron oxide NPs *in vivo* has been well characterized. Inflammation, oxidative stress, and histopathological changes in lung tissue are commonly observed in animals following exposure to iron oxide NPs. Zhu *et al.* (2008) exposed male Sprague Dawley rats to 0.8 mg/kg bw and 20 mg/kg bw of Fe₂O₃ NPs and MPs (22 nm and 0.280 µm, respectively) via intratracheal instillation and observed an increase in the relative number of neutrophils, lymphocytes, and macrophages in bronchoalveolar lavage fluid (BALF). There was also an increase in glutathione peroxidase activity and decrease in reduced glutathione (GSH) after Fe₂O₃ NP and MP exposure, indicating oxidative stress. Pettibone *et al.* (2008) exposed mice to Fe NPs (with an Fe₂O₃ surface coating) by whole body inhalation in an acute (4h; 7.62 mg/m³ dose) and sub-acute manner (4h per day, 5 days per week for 2 weeks; 3.68 mg/m³ dose). An increase in inflammation was observed immediately following sub-acute exposure (increase in the number of macrophages, neutrophils, and lymphocytes in BALF) although no cytotoxicity was seen (no increase in LDH activity in BALF). Hadrup *et al.* (2020) examined the pulmonary toxicity of αFe₂O₃ NPs, αFe₂O₃ nanorods, and metal-Fe₂O₄ NPs by

exposing C57BL/6 J BomTac mice via intratracheal instillation to a single dose of 14 µg/mouse (0.7 mg/kg bw), 43 µg/mouse (2.1 mg/kg bw) and 128 µg/mouse (6.4 mg/kg bw) for each particle. Fe₂O₃ NPs induced inflammation (increased presence of neutrophils in BALF) and DNA damage (using the comet assay) at the highest dose 3 days postexposure whereas, the Fe₂O₃ nanorods did not induce inflammation but did cause DNA damage at the two highest doses 3 days post-exposure. Srinivas *et al.* (2012) found that several markers of inflammation and oxidative stress increased and lung histopathology was altered (low grade foreign body type granulomatous inflammation observed 14 days post-exposure along with alveolitis 48h and 14 days post exposure) after a high dose (640mg) exposure to Fe₃O₄ NPs (4h of continuous inhalation). In conclusion, there is evidence that iron oxide NPs can induce adverse effects such as cytotoxicity, genotoxicity, and inflammation, although these particles do not appear to be particularly potent relative to other MONPs.

Thus, *in vitro* and *in vivo* models have been used to assess the effects and behaviours of NPs in toxicological studies (Xuan *et al.*, 2023). 2D cell models are often used to evaluate the toxicity of NPs as they are efficient, economical, and allow for the understanding of molecular causes of nanotoxicity; however, they may not account for complexities such as pharmacokinetics, organ toxicity, and clinical conditions. Organoids, which are 3D aggregates of various types of cells that can replicate the physiological and genomic features of tissues/organs, provide a more physiologically relevant model for the toxicological testing of NPs and are becoming more widely used. *In vivo* models (small animals) are more realistic compared to the *in vitro* models and provide an effective understanding of NP properties and disease development patterns, although they are more costly and time consuming. However, animal models are not perfectly consistent with one another or directly indicative of human exposure –

with respect to the inhalation of insoluble TiO₂ NPs, certain animal models (rats and mice) have higher lung burdens, lower particle clearance rates, and more histological lesions than others (such as hamsters) after exposure to equivalent concentrations of NPs (Bermudez *et al.*, 2004). The relevance of rat studies in predicting the hazard of poorly soluble particles to humans has been debated. While it has been found that humans and larger mammals have slower particle clearance than rats, particulate material inhaled by rats and humans has been shown to have different lung distributions, which may account for rats' sensitivity to particle overload (high particle doses accumulate in the interstitium for humans and alveoli/alveolar duct lumen for rats) (Hsieh and Yu, 1998; Hext and Thompson, 2005; Nikula *et al.*, 2001). However, there is insufficient evidence to conclude that rats are not a valid model for predicting poorly soluble inhalation hazards to humans despite some uncertainties (NIOSH, 2011). Thus, the available models to study the effects of NPs are not robust and as a consequence, novel methods that incorporate the physiological complexity of an organism and permit the assessment of effects of exposure to NPs in the context of their different properties, are urgently needed.

Issues and Knowledge Gaps

Toxicological data for many ENMs is scarce relative to their bulk forms (Escorihuela *et al.*, 2018). For MONPs, the influence of size and solubility on toxicity is poorly understood. There is a lack of standardization in the experimental procedures used in nanotoxicology studies and ENM characterization is often inadequate, making it difficult to compare studies and form conclusions regarding the relationships between physicochemical properties and toxicity (Krug, 2014). Although NP solubility has been found to influence toxicity by releasing harmful ions into solution, few studies have systematically compared the toxicity induced by particulate

ENMs to the dissolved fraction in solution (Alkilany *et al.*, 2016). A detailed examination of how different physicochemical properties relate to biological effects, and an understanding of underlying mechanisms would help to address these current knowledge gaps in nanotoxicology. However, due to the large number and wide variety of physicochemical property combinations of ENMs, traditional time-consuming toxicity assessment methods that rely on animal studies and disease-associated endpoints are not practical for this purpose. Thus, novel techniques and strategies are necessary to characterize underlying mechanisms of action and assess/predict the toxicity induced by ENMs in a higher throughput manner.

High Throughput and High-Content Techniques

High throughput *in vitro* techniques allow for the toxicological testing of large numbers of chemical substances at the same time reducing the time and efforts needed for their assessment. Some traditional genotoxicity assays have been modified to increase throughput for this purpose, including the alkaline comet and flow cytometry micronucleus assays. The alkaline comet assay detects DNA strand breaks and alkaline-labile sites in individual eukaryotic cells after exposure to genotoxicants (Karbachi *et al.*, 2019). The traditional comet assay involves exposing cells to a substance of interest followed by mixing single cells with low melting agarose and lysing them. The nucleoids are then subjected to alkaline conditions that allows for the unwinding of DNA containing strand breaks. The relaxed DNA is electrophoresed and stained with a fluorescent DNA binding dye. The broken DNA fragments migrate more slowly during the electrophoresis than the intact DNA, creating a comet-like appearance, with intact DNA in comet head and broken DNA in the comet tail. The ratio of DNA in tail vs head is analysed to determine the extent of DNA damage. Using the conventional comet assay, only a

limited number of cells could be viewed per slide. A high throughput 96-well version of the comet assay (CometChip Platform) has been developed, allowing for a significantly greater testing capacity than the traditional slide-based protocol while maintaining the same principles as the standard alkaline comet assay (Sykora *et al.*, 2018). Each of the 96 wells contains agarose with 400-500 micropores, enabling hundreds of comets to be analyzed at a time. A data analysis software package is then used to process the large amounts of data generated by the CometChip Platform.

Micronuclei are small nuclear bodies formed when whole chromosomes do not segregate correctly during mitosis (aneuploidy) or when broken chromosome fragments are not incorporated into daughter nuclei (clastogenicity) (Kuo *et al.*, 2022). The micronucleus (MN) assay assesses cytogenetic damage by evaluating the presence of micronuclei in cultured mammalian cells (Collins *et al.*, 2008). The traditional *in vitro* MN test involves treating cells with a test chemical and comparing MN frequencies with control treated cells. MN frequencies are generally measured by manually counting micronuclei in interphase cells using microscopy (Kuo *et al.*, 2022; Sommer *et al.*, 2020). The traditional MN assay is relatively time consuming. The newer microflow assay is a high-throughput version of the traditional MN assay that makes use of automated flow cytometry for the MN detection, which allows for the evaluation of a greater number of cells in a shorter time frame and eliminates scoring bias (Witt *et al.*, 2008; Sommer *et al.*, 2020). A differential staining procedure is used to stain the chromatin of dead/dying cells so that they may be distinguished from healthy cells which minimizes the impact of apoptosis and necrosis on the MN frequency (Bryce *et al.*, 2010). *In vitro*, the microflow assay may also use counting beads to determine the cell density, which is useful as it provides insight into the survival and replication of cells being treated.

In addition to high-throughput approaches, high-content techniques are also very important to the toxicological assessment of chemical substances. High-content techniques provide a vast amount of information about a substance that can be examined in great depth. For example, transcriptomics enables the expression profiling of the entire set of genes in a biological system. Determining characteristic chemical response profiles using transcriptomics provide invaluable insights into the mechanisms and toxic potential of a chemical of interest (Solorio-Rodriguez *et al.*, 2023; Boyadzhiev *et al.*, 2021; Rahman *et al.*, 2017; Halappanavar *et al.*, 2015). Pathway analysis and clustering can be performed using the expression results to examine perturbed biological functions and any similarities/differences between chemicals. Gene expression analysis is commonly performed using microarrays and RNA sequencing, and techniques are constantly being refined and improved. However, given the large amount of data that is produced by global transcriptomic techniques, specific skills and expertise is a must for the bioinformatic processing and correct interpretation of large data (Rao *et al.*, 2018).

Rationale for Thesis

A large amount of research has shown that ENMs including MONPs have the potential to induce numerous forms of toxicity in various experimental models. The limited number of published epidemiological studies have identified ENM exposure in the workplace as a potential health concern (Pietroiusti *et al.*, 2018). While certain physicochemical properties such as size and solubility are believed to contribute to the toxicity and adverse outcomes induced by ENMs, few systematic studies have effectively differentiated between the effects of each characteristic; thus, systematic studies are needed to address these knowledge and data gaps.

I used FE1 lung epithelial cell line in this study to address these gaps. FE1 cells are derived from the lungs of the MutaMouse transgenic rodent system and they are well suited for testing suspected mutagens *in vitro* (White *et al.*, 2003). The cells possess both type I and type II alveolar epithelial cell characteristics and have been used in mechanistic genotoxicity and molecular toxicity studies to test various ENMs (such as carbon black, multiwalled carbon nanotubes, silica NPs, and CuO NPs) (Jacobsen *et al.*, 2011; Poulsen *et al.*, 2013; Decan *et al.*, 2016; Boyadzhiev *et al.*, 2021). This mouse lung epithelial cell line was used as inhalation is a major route of exposure to MONPs and it is the same cell line that was used to generate the in-house data for other MONP that the results of this study will be compared to. My thesis applies high-throughput and high-content techniques to evaluate the toxicity of two MONPs of different chemical composition in their nano, micro, and ionic forms. These two MONPs were chosen because they were part of a group of eight MONPs identified as high priority for assessment by Health Canada.

Hypotheses

1) MONP-induced genotoxicity is influenced by their size, chemical composition, and solubility.

2) MONPs cause perturbations in molecular pathways that are associated with the manifestation of toxicological responses such as inflammation, genotoxicity, and metabolic disturbances.

Objectives

The overarching objective of this project is to investigate and compare the toxicities of different sizes, chemical compositions, and solubilities of two MONPs. Specifically, the effects of the NP and MP forms of MnO_2 and Fe_2O_3 , as well as the respective ion equivalent for Mn, on cytotoxicity, genotoxicity, and gene expression were assessed in a mouse lung epithelial cell line (FE1).

Specific objectives:

- 1) *Examine how MnO_2 and Fe_2O_3 NP size and solubility affect cytotoxicity, genotoxicity, and global transcription.* The toxic effects induced by soluble/insoluble MONPs and MPs were compared to those induced by equivalent concentrations of dissolved Mn ions to provide insight into the importance of size and solubility on the toxicity of each MONP
- 2) *Compare how MnO_2 and Fe_2O_3 NP toxicity differs from that induced by other MONPs.* The features of toxicity (cytotoxicity, genotoxicity, and global transcriptional perturbations) for MnO_2 and Fe_2O_3 NPs were compared to cytotoxicity, genotoxicity, and transcriptional perturbations induced by three forms of CuO (NP, MP, dissolved ion equivalent), which is more soluble than the two MONPs investigated in the present study. Comparing the toxic effects of various soluble and insoluble MONPs may reveal characteristic molecular pathways involved in their toxicity.

Methods/Materials

Materials

MnO_2 NPs with a manufacturer reported average primary particle size of 40 - 60 nm (Product number: 4910DX) and MnO_2 MPs with a manufacturer reported average primary

particle size of ~5000 nm (D50; <10000 nm (D90)) (Product number: 4930DX) were purchased from Skyspring Nanomaterials, Inc (Houston, TX). Fe₂O₃ NPs (Product number: US3160) with a manufacturer reported average primary particle size of 30 nm and Fe₂O₃ MPs (Product number: US1139M) with a manufacturer reported average primary particle size of ~5000 nm were obtained from US Research Nanomaterials Inc. Cell culture grade manganese (II) sulfate monohydrate (MnSO₄ · H₂O) was procured from Sigma-Aldrich. Tables 1 and 2 provide a summary of the particles used in my thesis and their property characteristics as obtained by the manufacturer and as characterised in-house.

In-house Particle Characterization

A JEM-2100F Field Emission transmission electron microscope (TEM) (JEOL, USA) was used to capture several non-overlapping transmission electron micrographs of dry MnO₂ and Fe₂O₃ NPs. A JSM-7500F Field Emission scanning electron microscope (SEM) (JEOL, USA) and a Tescan Vega-II XMU Scanning Electron Microscope (SEM) were used to capture several non-overlapping scanning electron micrographs of dry MnO₂ and Fe₂O₃ MPs to measure particle size. All electron microscopy images were imported into ImageJ for particle size analysis. At least 90 particles were measured for each type. The size distributions were plotted in histogram format using Microsoft Excel, and the mean length and width were reported with 95% confidence interval (CI). Aspect ratio was reported with standard deviation.

Brunauer-Emmett-Teller Specific Surface Area Analysis

The Brunauer-Emmett-Teller (BET) method uses the adsorption of non-reactive gas molecules onto a solid surface to calculate and quantify specific surface area (Nasrollahzadeh, 2019). Samples were run through a built-in leaking test method to ensure that there was no moisture or organic volatiles on the particle surface before the measurement was taken. The cell

containing the sample was weighed prior to degassing. The samples were heated at 20°C/min to 120°C and held for 12 hours. Specific surface area was determined by the multipoint BET method. Performed at McGill University using an Autosorb iQ (Anton Paar Canada Inc, QC, Canada) instrument.

Table 1. Particle information.

Particle	CAS #	Supplier	Product Number	Multi-Point BET (m ² /g)	Manufacturer Average Particle Size (nm)	Measured Length (nm) [CI 95%]	Measured Width (nm) [CI 95%]	Aspect Ratio +/- SD
MnO ₂ NP	1317-34-6	Skyspring Nanomaterials, Inc.	4910DX	42.16	40-60	28.3 [24.9-32.1]	12.0 [11.1-13.0]	2.80 +/- 1.97
Fe ₂ O ₃ NP	1309-37-1	US Research Nanomaterials, Inc.	US3160	44.88	30	28.3 [26.6-30.0]	22.8 [21.5-24.2]	1.26 +/- 0.24
MnO ₂ MP	1313-13-9	Skyspring Nanomaterials, Inc.	4930DX	2.55	~5000 (D50; <10000 (D90))	3510 [3186-3868]	2187 [1988-2407]	1.66 +/- 0.47
Fe ₂ O ₃ MP	1309-37-1	US Research Nanomaterials, Inc.	US1139M	9.52	5000	224.3 [202.0-369.7]	194.7 [177.2-305.0]	1.16 +/- 0.16

Table 2. Manganese (IV) oxide (MnO₂) and Iron (III) oxide (Fe₂O₃) NP/MP dissolution in water and DMEM+2%FBS (data retrieved from Avramescu *et al.*, 2022).

Particle	Initial concentration	% Dissolved 24h Water		% Dissolved 48h Water		% Dissolved 24h DMEM		% Dissolved 48h DMEM	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
MnO ₂ NP	10mg/L	0.17	0.07	0.2	0.08	0.29	0.01	4.79	0.94
	100mg/L	0.035	0.006	0.026	0.001	0.76	0.03	3.87	0.2
Fe ₂ O ₃ NP	10mg/L	nd	nd	nd	nd	nd	nd	nd	nd
	100mg/L	nd	nd	0.0019	0.0006	nd	nd	nd	nd
MnO ₂ MP	100mg/L	0.113	0.018	0.113	0.011	0.269	0.017	1.366	0.084
Fe ₂ O ₃ MP	100mg/L	nd	nd	nd	nd	nd	nd	nd	nd

nd = not detected

Particle Suspension

Both MnO₂ NPs and MPs were prepared at stock concentrations of 5 mg/mL (40 mL total) while the Fe₂O₃ NPs and MPs were prepared at stock concentrations of 1 mg/mL (100 mL total) in ultrapure, cell culture grade H₂O. Sonication was conducted using a Branson Digital SonifierTM 450 (Branson Ultrasonics, Markham, ON, Canada) and a ½” disruptor horn probe with a removable flat tip (MnO₂ NPs and MPs) or with an extension and removable flat tip (Fe₂O₃ NPs and MPs). The MnO₂ NP and MP stocks were sonicated at 40% output (8 seconds on, 2 seconds off) for 1 minute and 30 seconds. The Fe₂O₃ NPs and MPs were sonicated at 55% output (8 seconds on, 2 seconds off) for 10 minutes. The particles were sonicated in dH₂O and then serially diluted in treatment media at the appropriate concentrations. For MnO₂ NPs and MPs, the 5 mg/mL stocks were serially diluted to 1 mg/mL in media then further diluted to 5, 10, 25, 50, and 100 µg/mL exposure dilutions. For Fe₂O₃ NPs and MPs, the 1 mg/mL stocks were serially diluted in media to 5, 10, 25, 50, and 100 µg/mL exposure dilutions. The MnSO₄ was serially diluted down to 100 µg/mL in media from a 10 mg/mL stock, then subsequently diluted

to 3.75, 7.5 and 15 $\mu\text{g/mL}$. The 3.75, 7.5, and 15 $\mu\text{g/mL}$ MnSO_4 treatments release an equivalent amount of Mn ions to what would be released in 40, 80, and 160 $\mu\text{g/mL}$ MnO_2 NP treatments. Dynamic light scattering (DLS) was conducted on particle suspensions using a Zetasizer Nano Zsp (Malvern Panalytical, Westborough, MA, USA). The stocks of MnO_2 NPs suspended in ultrapure H_2O (5 mg/mL for MnO_2) and in cell culture media (1 mg/mL) then diluted to 50 $\mu\text{g/mL}$ in H_2O and in DMEM/F12+2% FBS media. The stocks of Fe_2O_3 NPs were suspended in ultrapure H_2O (1 mg/mL) and then diluted to 50 $\mu\text{g/mL}$ in H_2O and in DMEM/F12+2% FBS media. The mean hydrodynamic diameter (measure of aggregate size of particles in suspension), polydispersion index (measure of the heterogeneity of the particles in the sample based on size), and zeta potential (measure of surface charge of particles) were determined. The measurements were conducted in triplicate and the mean values are reported (Table 3).

Table 3. Manganese (IV) oxide (MnO_2) and Iron (III) oxide (Fe_2O_3) NP DLS results.

Sample Name	Hydrodynamic Diameter (D_h), nm (+/-SD)	PdI (+/-SD)	Zeta Potential, mV (+/-SD)
MnO ₂ NP Sample diluted to 50 $\mu\text{g/ml}$ in H ₂ O	134 $\pm$ 1.53	0.20 $\pm$ 0.03	-15.3 $\pm$ 1.71
MnO ₂ NP Sample diluted to 50 $\mu\text{g/ml}$ in DMEM/F12+2% FBS	151 $\pm$ 2.42	0.19 $\pm$ 0.03	-12.3 $\pm$ 0.87
Fe ₂ O ₃ Sample diluted to 50 $\mu\text{g/ml}$ in H ₂ O	187 $\pm$ 15.5	0.33 $\pm$ 0.04	-38.6 $\pm$ 1.60
Fe ₂ O ₃ Sample diluted to 50 $\mu\text{g/ml}$ in DMEM/F12+2% FBS	193 $\pm$ 12.9	0.28 $\pm$ 0.03	-11.4 $\pm$ 0.86

Cell Culture

The FE1 cell line was maintained in phenol red containing Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12HAM (DMEM F12HAM; Life Technologies, Burlington, ON, Canada) culture media supplemented with 2% fetal bovine serum (FBS; Life Technologies, Burlington, ON, Canada), 1 ng/mL epidermal growth factor (EGF; Life Technologies, Burlington, ON, Canada), 100 U/mL penicillin G, and 100 µg/mL streptomycin (Life Technologies, Burlington, ON, Canada) and maintained at 37 °C with 5% CO₂ (Boyadzhiev *et al.*, 2021). All experiments were performed using media that did not contain phenol red.

A preliminary dose selection experiment was conducted to ensure that appropriate concentrations of each particle suspension were used. The trypan blue assay was used to assess cell viability of FE1 cells exposed to MnO₂ NPs and Fe₂O₃ NPs over several concentrations – 1, 5, 10, 25, 50, and 100 µg/mL. A concentration of 100 µg/mL was the highest concentration tested, as higher concentrations of particles than 100 µg/mL (especially poorly soluble particles) create a thin layer on top of cells in the plate, creating a suffocative environment, which may lead to cytotoxicity that is challenging to discern from actual responses induced by the particles.

Cell Viability

Cytotoxicity was measured using the trypan blue exclusion assay. Trypan blue is a dye that stains dead cells in solution by penetrating their permeable cell membranes and entering their cytoplasm. The intact membranes of living cells prevent the dye from passing through and entering their cytoplasm. The viable (unstained) and non-viable (stained blue) cells in solution are counted using a hemocytometer and the percentage of viable cells is calculated (Strober, 2015). Trypan blue was used because MONPs have been observed to interfere with the reagents used in other high throughput assays (such as LDH and MTT) (Holder *et al.*, 2012). A cell

suspension of 10 μ L was mixed with 10 μ L of trypan blue and incubated for 5-7 minutes at room temperature prior to cell counting in the hemocytometer. The ratio of white cells to total cells was used to determine the percent cell viability for each exposure concentration (0, 10, 25, 50, 100 μ g/mL for the MnO₂ and Fe₂O₃ NPs and MPs; 3.75, 7.5, and 15 μ g/mL for MnSO₄) and timepoint (24h and 48h) tested. Three separate experiments were performed for each condition (using different cell passages), and there were two technical replicates per experiment. Cells were seeded at a density of 130 000 cells/well in six well plates and incubated overnight for 24h. Media were then replaced with exposure media containing MnO₂ NPs, MnO₂ MPs, MnSO₄, Fe₂O₃ NPs, Fe₂O₃ MPs, or fresh media (negative control). Following the exposure, the supernatant was collected and cells were washed with phosphate buffered saline (PBS). Images of the exposed cells were taken under a phase-contrast microscope at 4X, 10X, and 20X to assess cell morphology. The cells were then trypsinized and suspended in fresh media, and an aliquot of cell suspension was used in the trypan blue assay. Samples were then washed with PBS and centrifuged (8000 RPM for 10 minutes at 4°C) so that cells pellets could be collected and stored at -80°C for further analysis.

Comet Assay

Genotoxicity was evaluated using the CometChip Platform (high throughput version of the alkaline comet assay). The CometChip Platform uses a 96 well format that allows for more samples to be tested in less time than the traditional method (Sykora *et al.*, 2018). Each of the 96 wells contain an agarose surface with approximately 500 microwells which can hold single cells (Sykora *et al.*, 2018). This allows up to 96 different conditions to be evaluated at once.

For the comet assay, FE1 cells were trypsinized and suspended in phenol red free media before being diluted to approximately 150 000 cells/mL. The CometChip was washed with PBS prior to being inserted into the macrowell former system, and any excess PBS was aspirated from the wells using a VacuSafe system (Integra LifeSciences, Toronto, ON, Canada). The trypsinized and washed cell suspension was passed through a 70 μ m cell strainer (Fisher Scientific, Whitby, ON, Canada) then 100 μ L of the suspension was loaded into the wells of the chip. The system was subsequently incubated for 15 minutes, removed and rocked north-south and east-west 3-4 times, then put back in the incubator for 5 minutes. Once the cells were loaded, excess media were aspirated from the wells and replaced by 50 μ L of fresh media and the system was put into the incubator until the exposure. The cells were exposed to various concentrations (0, 10, 25, 50, 100 μ g/mL for the MnO₂ and Fe₂O₃ NPs and MPs; 3.75, 7.5, and 15 μ g/mL for MnSO₄) of MnO₂ NPs, MnO₂ MPs, MnSO₄, Fe₂O₃ NPs, Fe₂O₃ MPs for 2h and 4h. Each experiment had a 4h media control and cells exposed to 1h of 100 μ M H₂O₂ as positive control. Three or four separate experiments were performed for each condition using different cell passages (between p17 – p26), with four technical replicates in each experiment. CometChip® Electrophoresis Starter Kits were used to detect DNA strand breaks and Trevigen Comet software was used for analysis. Following exposure, the chip was removed from the macrowell former and rinsed with PBS. It was then slowly overlayed with low melting agarose and allowed to solidify at room temperature for 3 minutes and at 4°C for 12 minutes under light occlusion. The chip was then lysed and subjected to alkaline conditions (200 mM NaOH + 1 mM EDTA; pH $\geq$ 13). Electrophoresis was carried out in the dark under alkaline conditions (200 mM NaOH + 1 mM EDTA + 0.1% Triton-X; pH $\geq$ 13,) at a constant voltage (20 V) and variable current (280 mA) for 50 minutes. Once finished, the chip was neutralized in 400 mM and 20 mM Tris-HCl buffers

(pH 7.4) then incubated in a 0.2X SYBR Gold dilution (10,000× SYBR GOLD in DMSO obtained from Life Technologies Corporation, Burlington, ON, Canada) in the dark for 14h overnight.

The following day, the chip was destained in 20 mM Tris-HCl buffer in the dark for 1h at room temperature then imaged using a Leica DMI8 automated confocal fluorescence microscope (Leica Microsystems, Wetzlar, Germany) at 5× magnification. The images obtained were uploaded into the proprietary Trevigen Comet Software (Bio-Techne, Devens, MA, USA) for analysis. Quality control was performed to remove comets with artifacts and adjust the comets (head and tail) that were not identified correctly by the software. Wells containing fewer than 50 comets were not included in the final analysis.

Microflow Assay

The MN assay is a genotoxicity test that detects chromosome breakage (clastogenicity) and/or whole chromosome loss (aneugenicity) after exposure to a substance of interest. The microflow assay is a high throughput version of the MN assay that makes use of automated flow cytometry for the detection of MN in a 96 well plate format.

FE1 cells were exposed to various concentrations (2.5, 5, 10, and 20 µg/mL for MnO₂ NPs and MPs; 0.9, 1.8, 3.6 and 7.2 µg/mL for MnSO₄; 5, 10, 20, 40 µg/mL for Fe₂O₃ NPs, and MPs) of MnO₂ NPs, MnO₂ MPs, MnSO₄, Fe₂O₃ NPs, and Fe₂O₃ MPs in addition to Methyl methanesulfonate (MMS, purchased from Sigma-Aldrich; 500 µM positive control) and fresh media (negative control) for 40h. Three separate experiments (n=3) were performed for each condition using different cell passages (<p25). A Litron In Vitro *MicroFlow*® 96 Well Plate *Format* Kit was used to conduct the assay. FE1 cells were trypsinized and suspended in phenol

red free media before being diluted to approximately 25 000 cells/mL. 200 μ L of the cell suspension was added to each well of the 96 well plate so that each well contained approximately 5000 cells. Cells were then incubated in the 96 well plate at 37°C, 5% CO₂ for 24h. After the 24h incubation, the supernatant was aspirated using a VacuSafe system (Integra LifeSciences, Toronto, ON, Canada) and replaced with 100 μ L of the media suspension consisting of particles prior to being incubated for 40h. Following the 40h incubation, the 96 well plates were placed on wet ice for 20 minutes then the exposure media were carefully aspirated from each well. 50 μ L of an initial nucleic acid dye solution (composed of Nucleic Acid Dye A - Ethidium monoazide (EMA) and 1X Buffer Solution) was added to each well of the 96 well plate then the uncovered plates were placed on wet ice under visible light for 30 minutes. After the 30 minutes, the light source was turned off and 0.15 mL of a cold 1X buffer solution (composed of 10X buffer, dH₂O, and FBS) was added to each well and exposure to light was limited beginning at this step. Supernatants were then carefully aspirated and 100 μ L of Lysis Solution 1 (composed of Incomplete Lysis solution 1, Nucleic Acid Dye B – SYTOX Green nucleic acid stain, RNase Solution, and Counting Beads) was added to each well and plates were gently mixed on a shaker for 5 seconds prior to being incubated for 1h in the dark at 37°C. Post-incubation, 100 μ L of Lysis Solution 2 (composed of incomplete Lysis Solution 2 and Nucleic Acid Dye B - SYTOX Green nucleic acid stain) was added to each well, gently mixed, and incubated for 30 minutes in the dark at room temperature. Samples were stored at room temperature under light occlusion prior to Flow cytometric analysis. A BD LSRFortessa™ Cell Analyzer (BD Biosciences, San Jose, CA) was used to count the MN, according to the manufacturer's recommendations. The uptake volume was set to 100 μ L and 5000 events were examined.

RNA Extraction

Following the cell viability experiments, samples were washed with PBS, centrifuged (8000 RPM for 10 minutes at 4°C) and cell pellets were collected and stored at -80°C for RNA extraction. For the RNA extraction, TRIzol reagent was used to isolate RNA from the undesired cellular components. The RNA was then purified using a Direct-zol RNA Miniprep Kit (Zymo Research Corp, Irvine, USA) according to the manufacturer's instructions. The RNA concentration and purity were determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, USA). An Agilent TapeStation 4200 (Agilent Technologies, Inc., Santa Clara, USA) was used to determine the RNA integrity and quality. RNA samples had appropriate 260/280 and 260/230 ratios and RNA integrity numbers greater than 8.8.

Microarray

Transcriptional analysis was conducted using Agilent DNA microarrays. Microarrays detect changes in gene expression based on the interaction between immobilized DNA probes on a chip and target cDNA molecules (Govindarajan *et al.*, 2012). RNA is extracted from cells and used to synthesize cDNA that is labelled with fluorescent dyes. A randomized block design was used, where samples were labeled with Cy5 and reference cRNA was labeled with Cy3 (Kerr and Churchill, 2001). The fluorescently labelled cDNA molecules are then hybridized to their complementary probes on the microarray. A series of washes are used to remove non-binding cDNA molecules while the bound molecules fluoresce after being scanned by a laser beam. The intensity of the fluorescent signal at each spot of the array is a measure of gene expression (Bumgarner, 2013).

Using a low Input Quick Amp Labeling Kit (Agilent Technologies Inc., Santa Clara, CA, USA), 200 ng of purified RNA from the exposure samples (MnO₂ NPs, MnO₂ MPs, MnSO₄, Fe₂O₃ NPs, Fe₂O₃ MPs, or fresh media (control)), and a universal mouse RNA reference (Agilent Technologies Inc., Santa Clara, CA, USA) were used to synthesize double stranded cDNA followed by fluorescent cRNA. Fluorescent molecules cyanine 3-cytidine triphosphate (Cy3; reference RNA) or cyanine 5-cytidine triphosphate (Cy5; sample RNA) were incorporated into the cRNA. Three separate experiments were performed for each condition (using different cell passages, ranging from p17 – p26). The fluorescently labelled cRNA was then purified using RNeasy Mini Kits according to the manufacturer's instructions (Qiagen N.V., Venlo, the Netherlands). An equimolar amount of the sample RNA and reference RNA were mixed then subsequently hybridized onto 8x60K Agilent SurePrint G3 Mouse Gene Expression v2 Microarrays (Agilent Technologies, Inc., Santa Clara, USA). Slides were rotated at 10 rpm in a hybridization chamber for 17h at 65°C. Slides were then washed prior to being scanned using an Agilent Scanner G2505B (Agilent Technologies Inc., Santa Clara, CA, USA). Agilent Feature Extraction software version 11.01.1 was used to extract the data from the images.

Data Processing and Statistics

Cell Viability and DNA Damage Assay Statistics

Statistical analysis was performed using version 15.0 of SigmaPlot (Systat Software Inc., San Jose, CA, USA). The cell viability and comet assay data containing 3-4 biological replicates per condition were analyzed using the Kruskal-Wallis test with a Dunnett's *post hoc* to determine the statistically significant differences between the treated samples and their respective controls. Note that the replicates of certain samples were not normally distributed and could not be

normalized via transformation so the Kruskal-Wallis test was used in place of one-way analysis of variance. The statistical significance of the micronuclei percentage data containing three biological replicates was determined using one-way analysis of variance with a Dunnett's *post hoc* while the % relative survival was normalized via log transformation and analyzed with a one-way analysis of variance with a Dunnett's *post hoc*.

Microarray Data Quality Analysis

The microarray data quality analysis was performed as in Webster *et al.* (2014). The background of the arrays were assessed with a negative control 3xSLv1 probe, which does not hybridize well with labelled samples as it forms a hair pin structure. There are 182 technical replicates of the probe distributed across the microarray, and their trimmed mean (trim=0.05) and standard deviation was used to measure the background fluorescence and variability between each array. The trimmed mean was used in order to reduce the influence of outliers that may have been present.

Hierarchical clustering analysis using average linkage and one minus the Pearson correlation as the dissimilarity metric was performed in R to identify microarrays with poor data quality using normalized log ratios for all probes. Signal intensities were adjusted to control for any slide effects (variation between slides that may have occurred as an artifact of experimental conditions as opposed to a true-treatment related effect) by using a linear model to subtract estimated slide effects from the log ratios. Single biological replicates for the 48h 50 µg/mL Fe₂O₃ NP treatment and 24h 50 µg/mL MnO₂ NP treatment did not cluster with the rest of the samples from the previous slides, so they were repeated on another slide.

Microarray Statistical Analysis

The statistical analysis of the microarray data was conducted as in Boyadzhiev *et al.* (2021). Data were normalized using the locally weighted scatterplot smoothing (LOWESS) regression modeling method and statistically significant differences in gene expression were determined using a microarray analysis of variance (MANOVA) performed in R (Wu *et al.*, 2003). Treatment effects were tested for using the F s statistic, and p-values were estimated using the permutation method for each statistical comparison. The false discovery rate (FDR) multiple testing correction was used to adjust p-values for multiple comparisons (Benjamini and Hochberg, 1995). Fold change calculations of differentially expressed genes (DEGs) were based on the least-square means (Searle *et al.*, 1980). Genes were identified as DEGs if they had a fold change $\geq |1.5|$ and an $FDR \leq 0.05$ when compared to media controls.

Pathway Analysis of Microarray Data

Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, Redwood City, CA, USA) was used to conduct canonical pathway analysis on DEGs. Fisher's exact tests were used to measure the significance of the association between the DEGs and the canonical pathways in IPA. Pathways were considered significantly perturbed with a $-\log(p\text{-value}) \geq 1.3$ and a minimum of three DEGs in the pathway. The heatmap and hierarchical clustering of the top pathways was performed using Euclidean distance as the dissimilarity metric and complete linkage using the pheatmap package in R. Figures examining overlap (and Jaccard index) between perturbed pathways of different treatment conditions were generated using the website <https://molbiotools.com/listcompare.php>. The Jaccard index is a metric used to determine the similarity between two groups by dividing the number of shared objects between two groups by

the total number of objects in the groups (Fuxman Bass *et al.*, 2013). JVenn was also used to generate Venn diagrams to compare the overlap between the altered pathways and DEGs between treatments (Bardou *et al.*, 2014).

Results

Cell Viability

Cell viability was conducted using a trypan blue assay following the exposure of FE1 cells to MnO_2 and Fe_2O_3 NPs and MPs (Figure 1A) as well as dissolved Mn ions for 24h and 48h (Figure 1B). The percent cell viability after exposure to MnO_2 NPs was similar to media controls for all concentrations tested at 24h and 48h timepoints. Only the 100 $\mu\text{g/mL}$ concentration at 48h showed a slight but statistically significant reduction in viability (95.7%) relative to the controls (99.3%). MnO_2 MPs did not reduce cell viability for any of the concentrations examined. When examined under a light microscope, there appeared to be a decrease in cell density of cells compared to the control and some of the cells were slightly elongated following MnO_2 NP exposure at the highest concentration for 48h. MnO_2 MP and MnSO_4 exposure did not significantly change the cell morphology.

The Fe_2O_3 NPs induced a small but statistically significant reduction in cell viability compared to the controls (97%) at the 50 $\mu\text{g/mL}$ and 100 $\mu\text{g/mL}$ concentrations after 24h (93.6% and 89%) and at the 100 $\mu\text{g/mL}$ after 48h (87.6%). However, Fe_2O_3 MPs did not reduce cell viability for any of the concentrations tested. The cell morphology was not significantly altered following exposure to Fe_2O_3 NPs and MPs.

Overall, the MnO_2 and Fe_2O_3 NPs induced a greater reduction in cell viability than MPs and the equivalent soluble ion fraction at the highest concentrations.

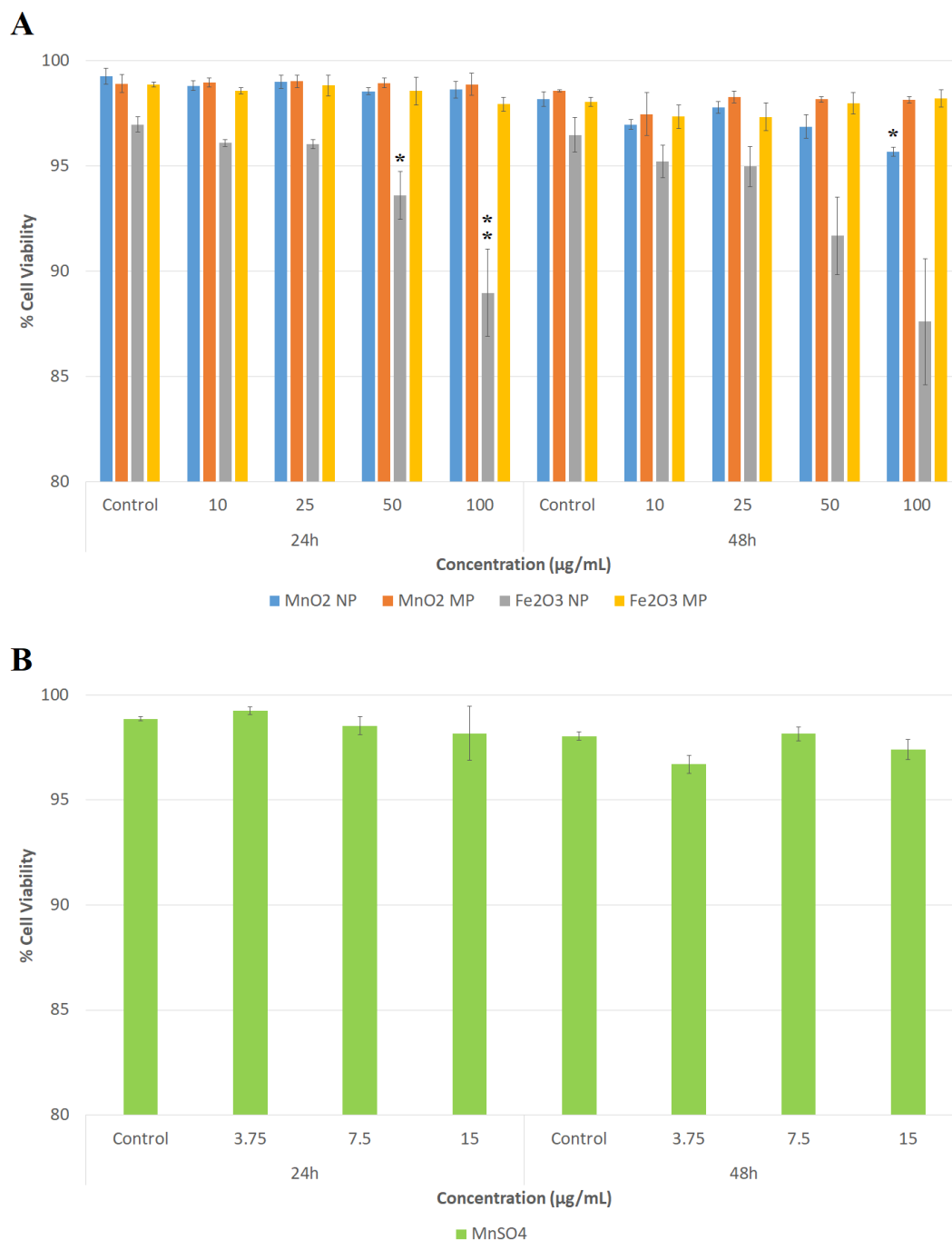


Figure 1. FE1 cell viability after exposure to Manganese (IV) oxide (MnO₂) NPs and MPs and Iron (III) oxide (Fe₂O₃) NPs and MPs (A), and Manganese (II) sulfate (MnSO₄) (B) for 24h and 48h. Statistically significant differences between the treated samples and their respective media controls were determined using the Kruskal-Wallis test with a Dunnett's *post hoc*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Error bars denote $\pm$ standard error of the mean (SEM; $n = 3$). The 3.75, 7.5, and 15 $\mu\text{g/mL}$ MnSO₄ treatments release an equivalent amount of Mn ions to what would be released in 43.3, 99.5, and 239.8 $\mu\text{g/mL}$ MnO₂ treatments.

Comet Assay

DNA strand breaks (measured by the % DNA in the comet tail) were evaluated using the comet assay after exposing FE1 cells to MnO_2 and Fe_2O_3 NPs and MPs (Figure 2A) as well as dissolved Mn ions (Figure 2B) for 2h and 4h. The MnO_2 NPs caused a concentration- and time-dependent increase in DNA strand breaks that was statistically significant at the 100 $\mu\text{g/mL}$ concentration (29.1%) relative to the control (4.9%) at the 4h timepoint. The MnO_2 MPs also showed a concentration- and time-dependent increase in DNA strand breaks the 100 $\mu\text{g/mL}$ concentration (12.4%) and the control (4.9%) at the 4h timepoint. The magnitude of DNA damage was higher in the MnO_2 NPs than the MnO_2 MPs.

Exposure to Fe_2O_3 NPs caused a very slight increase in DNA strand breaks that was statistically significant at the 100 $\mu\text{g/mL}$ concentration at 2h (5.9%) and 4h (5.9%) relative to the control (4.4%). The Fe_2O_3 MPs also appeared to induce a small concentration- and time-dependent increase in DNA stand breaks (8.9% in 100 $\mu\text{g/mL}$ concentration at 4h timepoint) relative to the control (3.7%), although none of the tested concentrations were statistically significant.

The MnO_2 NPs and MPs induced greater amounts of DNA strand breaks than the Fe_2O_3 NPs and MPs. The NPs were more potent than the MPs with respect to DNA stand break induction for MnO_2 , while the DNA strand break induction was low for both Fe_2O_3 particles. The Mn ions released by MnSO_4 did not induce DNA damage, which suggests that the dissolved fraction of the MnO_2 NPs and MPs may not have contributed to the DNA damage they induced.

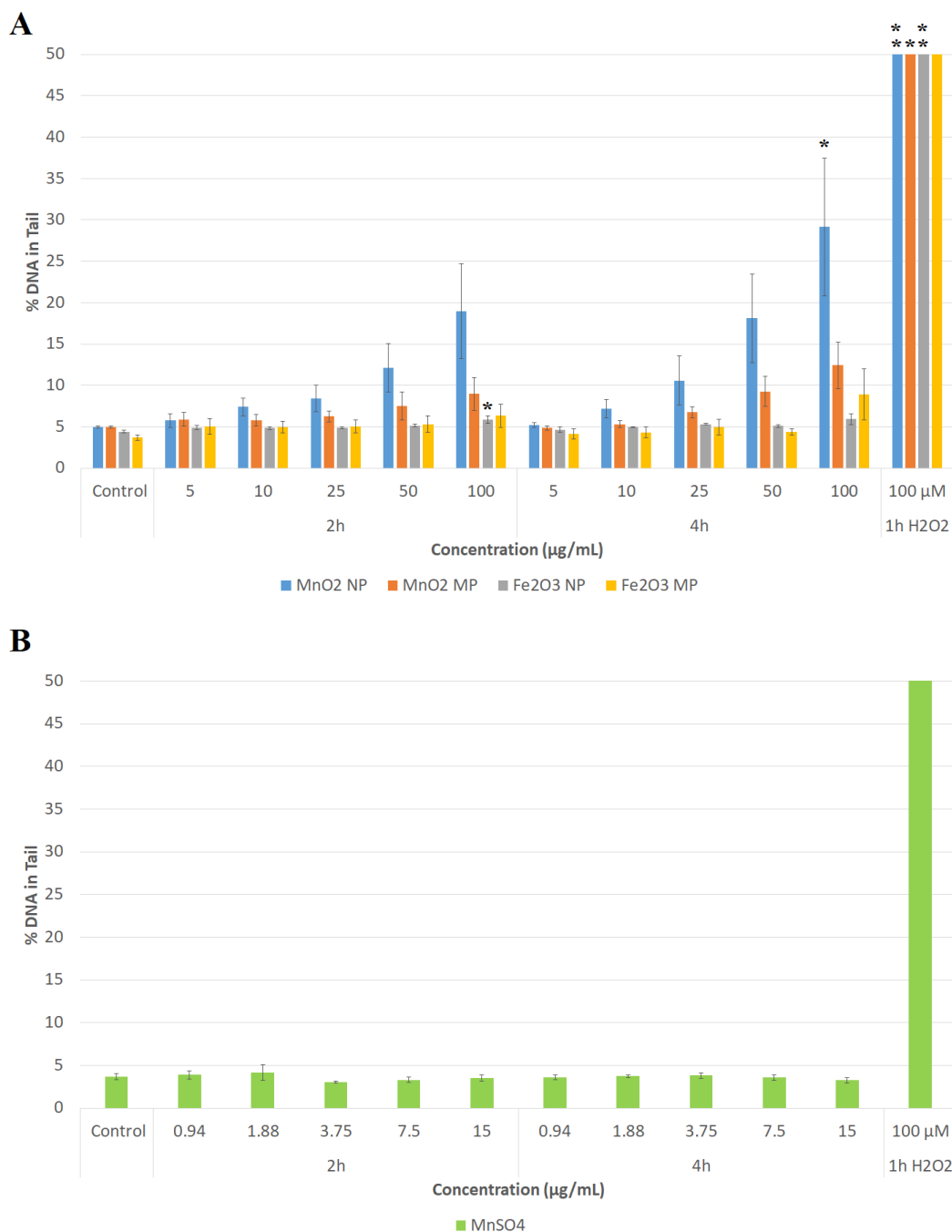


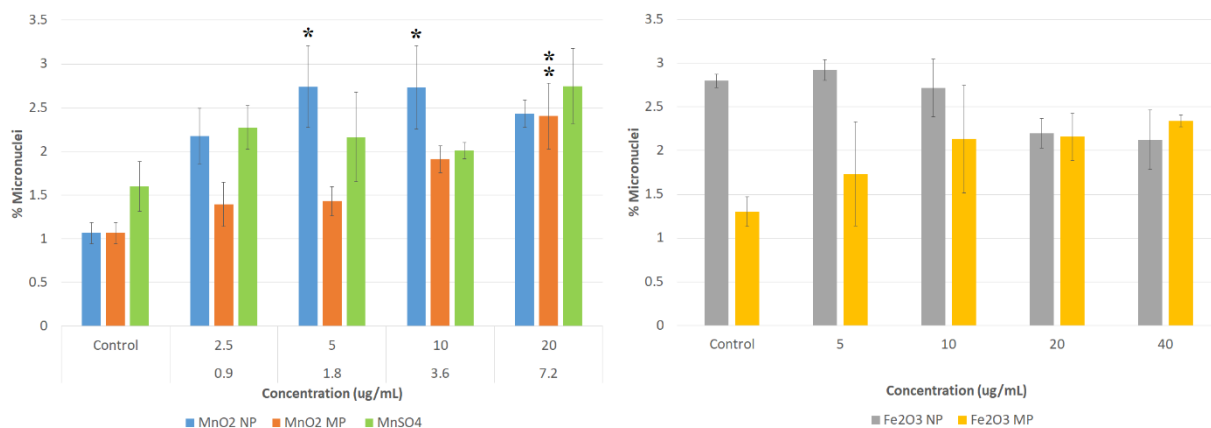
Figure 2. DNA damage, measured by the percentage of DNA in comet tail after exposure to Manganese (IV) oxide (MnO₂) NPs and MPs, Iron (III) oxide (Fe₂O₃) NPs and MPs, and Manganese (II) sulfate (MnSO₄) for 2h and 4h. Statistically significant differences between the treated samples and their respective media control were determined using the Kruskal-Wallis test with a Dunnett's *post hoc*. * $p < 0.05$, ** $p < 0.01$, * $p < 0.001$. Error bars denote $\pm$ standard error of the mean (SEM; $n = 3$ or 4). The 0.94, 1.88, 3.75, 7.5, and 15 $\mu\text{g/mL}$ MnSO₄ treatments release an equivalent amount of Mn ions to what would be released in 10.1, 20.6, 43.3, 99.5, and 239.8 $\mu\text{g/mL}$ MnO₂ treatments.**

Microflow Assay

The microflow assay was used to evaluate chromosome damage in FE1 cells exposed to MnO_2 and Fe_2O_3 NPs and MPs as well as dissolved Mn ions for 40h (Figure 3). The MnO_2 NPs induced a statistically significant increase in the MN frequency at 5 and 10 $\mu\text{g/mL}$ and a concentration-dependent decrease in the % relative survival beginning at the 2.5 $\mu\text{g/mL}$ concentration. There was a slight reduction in the % MN at the highest concentration (20 $\mu\text{g/mL}$) and it was no longer statistically significant. The MnO_2 MPs caused a statistically significant increase in the MN frequency at the highest concentration (20 $\mu\text{g/mL}$) and a concentration-dependent decrease in the % relative survival (starting at 10 $\mu\text{g/mL}$). The overall response to MnO_2 MPs was less than that induced by the MnO_2 NPs. The response to MnSO_4 exposure was not statistically significant.

The Fe_2O_3 NP and Fe_2O_3 MP exposures did not induce significant changes in MN frequency relative to the controls, although they both caused a statistically significant increase in % relative survival at the highest concentration (10 $\mu\text{g/mL}$).

A



B

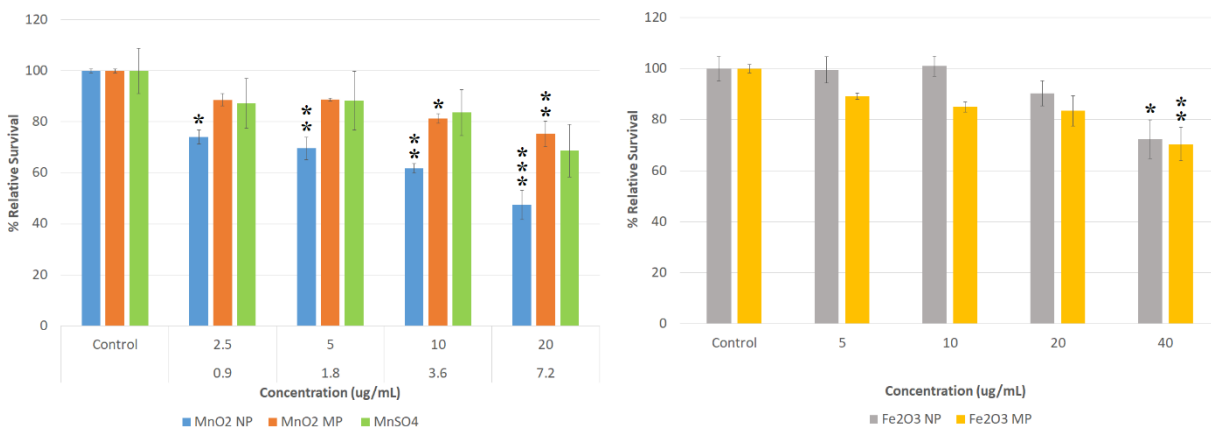


Figure 3. The MN frequency (A) and % relative survival (B) after exposure to Manganese (IV) oxide (MnO₂) NPs and MPs, Iron (III) oxide (Fe₂O₃) NPs and MPs, and Manganese (II) sulfate (MnSO₄) for 40h. Statistically significant differences between the treated samples and their respective media controls was determined using a one-way analysis of variance with a Dunnett's *post hoc*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Error bars denote $\pm$ standard error of the mean (SEM; $n = 3$). Cells were treated with 0.9, 1.8, 3.6, and 7.2 $\mu\text{g/mL}$ of MnSO₄, which release an equivalent amount of Mn ions to 9.7, 19.7, 41.4, and 94.2 $\mu\text{g/mL}$ MnO₂ treatments.

The cell viability and survival (trypan blue assay), DNA damage (comet assay), and micronucleus (microflow assay) data were published by Solorio-Rodriguez *et al.* (2024). The study systematically evaluated the effects of size, solubility, and surface coating on the cytotoxic and genotoxic responses of several MONPs.

No-Observed-Genotoxic-Effect-Level

The no-observed-genotoxic-effect-level (NOGEL) is defined as the highest dose for which there is no statistically significant increase in observed genotoxic effects relative to an appropriate negative control (Gollapudi *et al.*, 2013). In principle, the NOGEL is a point on the concentration-response curve that has an indistinguishable response from the background, meaning the concentrations below should represent low concern. However, because the NOGEL concentration is dependent on the experimental design, it carries a certain degree of uncertainty. Based on the comet assay (Figure 2), the NOGEL of the MnO₂ and Fe₂O₃ NPs was 50 µg/mL while the NOGEL for the of the MnO₂ and Fe₂O₃ MPs was 100 µg/mL. The NOGEL of MnO₂ NPs based on the micronucleus assay (Figure 3) was 5 µg/mL while that of MnO₂ MPs was 10 µg/mL. The Fe₂O₃ NP and MP treatments had a NOGEL of 40 µg/mL.

Gene Expression Analysis

The number of upregulated and downregulated DEGs for all concentrations and particle types is summarized in Figure 4. In general, there were few DEGs in the lower concentrations (25 and 50 µg/mL for particles; 3.75 and 7.5 µg/mL for MnSO₄) compared to the highest concentration (100 µg/mL for particles; 15 µg/mL for MnSO₄) at both timepoints. The number of DEGs increased in a concentration- and time-dependent manner for all materials tested aside from MnSO₄, which did not show a time dependency in response. The NPs induced a greater

number of DEGs than the MPs for both MnO_2 and Fe_2O_3 . MnSO_4 induced more DEGs than the MnO_2 NPs and MPs at the highest concentration (100 $\mu\text{g/mL}$ for particles; 15 $\mu\text{g/mL}$ for MnSO_4) at the 24h timepoint, although MnO_2 NPs induced more DEGs than MnSO_4 after 48h. Between the two particle types tested, MnO_2 NPs and MPs induced a greater number of DEGs than Fe_2O_3 NPs and MPs at both timepoints.

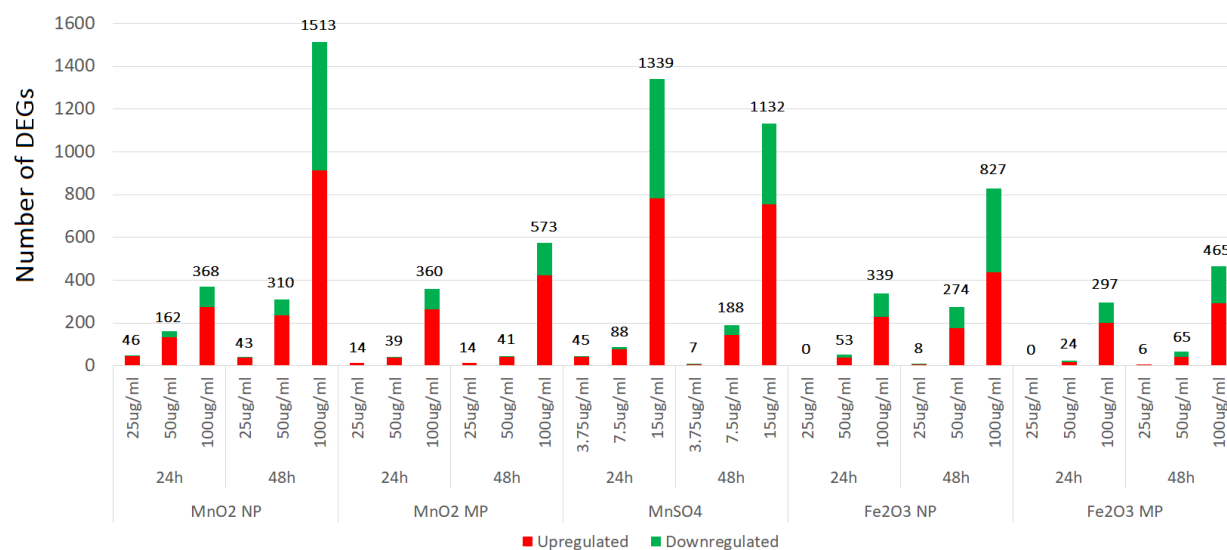


Figure 4. Number of differentially expressed genes (DEGs) following exposure to Manganese (IV) oxide (MnO₂) NPs and MPs, Iron (III) oxide (Fe₂O₃) NPs and MPs, and Manganese (II) sulfate (MnSO₄) equivalent for 24h and 48h. DEGs were defined as genes with a fold change $\pm > |1.5|$ and a false discovery rate (FDR) of ≤ 0.05 ($n = 3$). Red: upregulated DEGs; green: downregulated DEGs. The numbers above the bars indicate the total number of DEGs for each condition.

A heatmap of the DEGs based on fold change is shown in Figure 5. Particles with the same constituent metal showed similar transcriptomic profiles and clustered together (Fe_2O_3 NP and MP; MnO_2 NP, MP, and MnSO_4). The transcriptomic profiles were also similar between treatments with the same timepoint, which can be seen in Figure 5 where particles with the same constituent metal were split into two groups based on whether it was a 24h or 48h exposure. NPs and MPs with the same chemical formula tended to group with particles of the same size, indicating that there may be a size related difference in transcriptional response. With respect to solubility, it appeared that MnSO_4 exhibited a transcriptional response that was closer to that of the MnO_2 NPs rather than MPs, possibly indicating that Mn ions contribute to the response induced by MnO_2 NP.

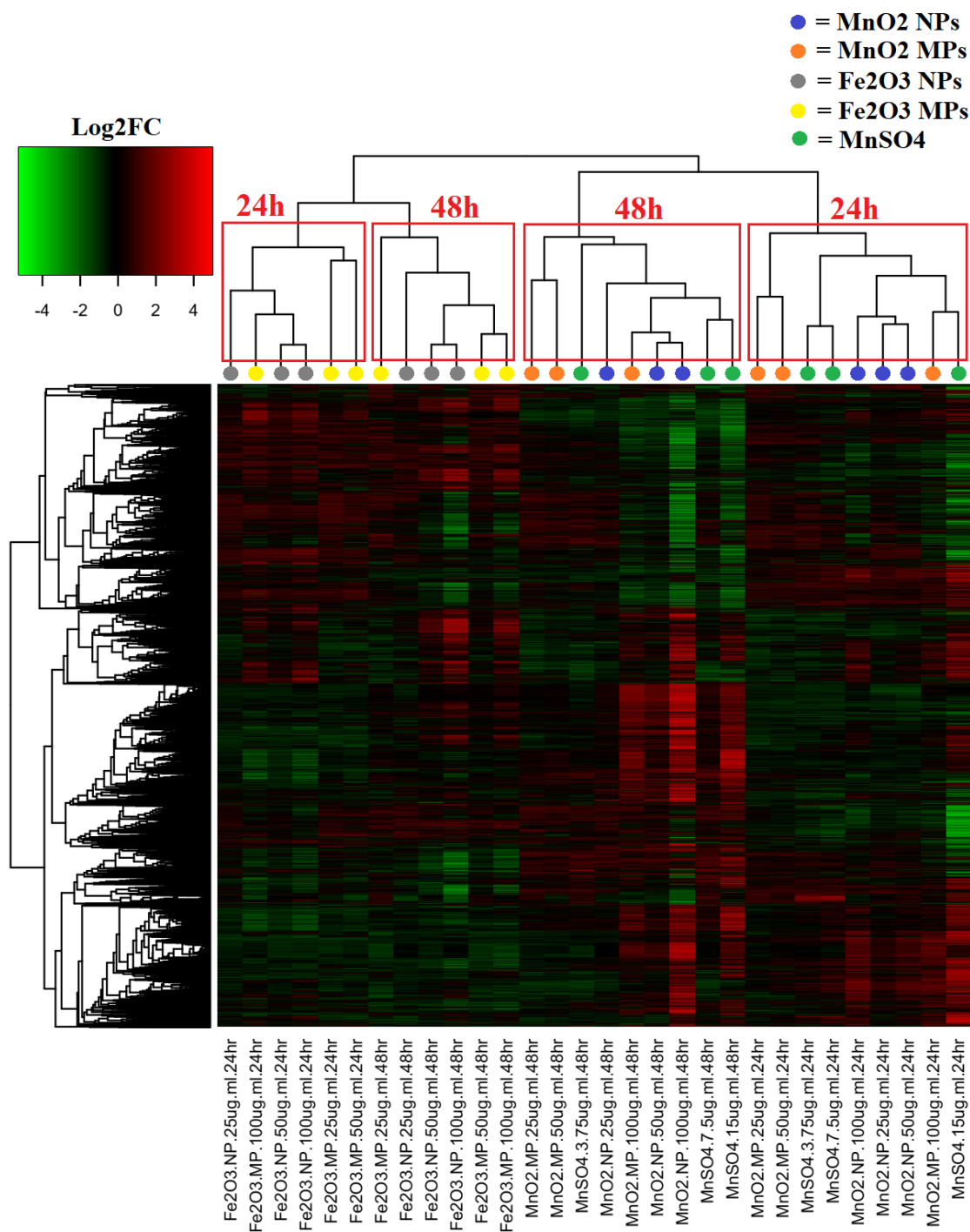


Figure 5. All DEGs (fold change $\geq |1.5|$ and an FDR ≤ 0.05) after 24h and 48h of exposure to Manganese (IV) oxide (MnO₂) NPs and MPs, Iron (III) oxide (Fe₂O₃) NPs and MPs at 100 $\mu\text{g/mL}$, and Manganese (II) sulfate (MnSO₄) at 15 $\mu\text{g/mL}$. The clustering was based on 1- the Spearman correlation as the dissimilarity metric with average linkage. Red: upregulated. Green: downregulated.

Most perturbed genes

To explore the greatest impacts on the transcriptome, I first evaluated the top 10 most altered (based on fold-change) genes within each concentration at both time points for all of the substances. I then considered differences and similarities between the particle size and solubility of these highest responding genes and compare the two metals.

Gene expression changes induced by MnO₂ NPs (most perturbed DEGs)

After 24h, the top 10 DEGs were primarily associated with hypoxia (Table 4). The DEGs that were commonly perturbed across all the concentrations included mitochondrial organization and hypoxia related mitochondria localized glutamic acid rich protein (*MGARP*), cell cycle related BBX high mobility group box domain containing (*BBX*), cellular iron uptake (endocytosis) related transferrin receptor (*TFRC*), apoptosis and hypoxia related BCL2 interacting protein 3 (*BNIP3*), transmembrane protein 74B (*TMEM74B*), and potassium voltage-gated channel subfamily B member 1 (*KCNB1*). Hypoxia related HIG1 hypoxia inducible domain family member 1A (*HIGD1A*) and egl-9 family hypoxia inducible factor 1 (*EGLN1*) were highly altered in both the 25 and 50 µg/mL concentrations. Hypoxia and energy regulation related adenylate kinase 4 (*AK4*), and hypoxia and apoptosis related family with sequence similarity 162 member A (*FAM162A*) were only perturbed in the 25 µg/mL concentration. Actin cytoskeleton related TRIO and F-actin binding protein (*TRIOBP*) and GTP binding related DIRAS family GTPase 2 (*DIRAS2*) were only altered in the 50 µg/mL concentration. The proteases trypsinogen serine protease 3 (*PRSS3*) and matrix metalloproteinase 10 (*MMP10*) were increased along with immune/inflammation related gene C-C motif chemokine ligand 5 (*CCL5*),

UTP6 small subunit processome component (*UTP6*), and cytoskeletal motor related kinesin family member 2C (KIF2C) only in the 100 µg/mL concentration.

After 48h, the top 10 perturbed DEGs were primarily associated with immune response (Table 4). The DEGs that were commonly altered (both increased) across all concentrations were interferon-induced and ISGylation regulation related ubiquitin specific peptidase 18 (*USP18*) and bacterium defense response and autophagy regulation related interferon inducible GTPase 1 (*Iigp1*). The common DEGs that were the most highly increased in the 25 and 50 µg/mL concentrations were: (interferon-induced) innate antiviral response related 2'-5'-oligoadenylate synthetase 2 (*OAS2*), antiviral response related interferon induced protein with tetratricopeptide repeats 1B (*IFIT1B*), interferon-induced antiviral response related 2'-5' oligoadenylate synthetase-like 2 (*Oasl2*), and innate immune response related interferon regulatory factor 7 (*IRF7*). The most highly perturbed DEGs common to the 50 and 100 µg/mL concentrations were (interferon-induced) innate antiviral response related 2'-5'-oligoadenylate synthetase 1 (*OAS1*) and tripartite motif-containing 30A (*Trim30a/Trim30d*). Interferon induced and antiviral related receptor transporter protein 4 (*RTP4*), interferon induced antiviral response related MX dynamin-like GTPase 2 (*Mx2*), antiviral signaling related DExH-box helicase 58 (*DHX58*), and innate immune response related guanylate binding protein 4 (*GBP4*) were increased only in the 25 µg/mL concentration. ISG15 ubiquitin like modifier (*ISG15*) and the transporter activity and cholesterol binding related gene apolipoprotein D (*APOD*) were upregulated only in the 50 µg/mL concentration. Radical S-adenosyl methionine domain containing 2 (*RSAD2*), lymphocyte antigen 6 complex, locus A (*Ly6a*), PHD finger protein 11 (*PHF11*), C-X-C motif chemokine ligand 10 (*CXCL10*), interferon induced protein with tetratricopeptide repeats 1B (*IFIT1B*), and

the expression of serine protease 3 (*PRSS3*) were highly increased in the 100 µg/mL concentration.

Table 4. Top 10 upregulated and downregulated DEGs after 24h and 48h of exposure to Manganese (IV) oxide (MnO₂) NPs.

MnO ₂ NP											
24h						48h					
25 µg/mL		50 µg/mL		100 µg/mL		25 µg/mL		50 µg/mL		100 µg/mL	
Upregulated		Upregulated		Upregulated		Upregulated		Upregulated		Upregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
MGARP	5.2	MGARP	8.3	MGARP	10.2	OAS2	3.3	OAS1	11.3	OAS1	43.1
BBX	4.8	KCNB1	4.9	CCL5	8.2	IFIT1B	3.2	ligp1	8.4	RSAD2	28.4
TFRC	3.9	TMEM74B	4.7	PRSS3	6.2	USP18	3.2	OAS2	8.3	Ly6a (includes others)	27.3
BNIP3	3.0	TFRC	4.1	TMEM74B	5.5	Oasl2	3.1	USP18	7.9	PHF11	24.4
TMEM74B	2.9	BBX	3.9	BBX	5.4	ligp1	3.0	IFIT1B	7.7	Trim30a/Trim30d	22.6
KCNB1	2.9	BNIP3	3.7	MMP10	5.4	RTP4	2.9	ISG15	7.4	CXCL10	22.0
HIGD1A	2.7	TRIOBP	3.0	KCNB1	4.8	Mx2	2.9	Oasl2	7.3	USP18	21.8
EGLN1	2.2	HIGD1A	2.9	BNIP3	4.4	DHX58	2.9	APOD	6.8	ligp1	21.7
AK4	2.1	EGLN1	2.7	C11orf96	4.3	IRF7	2.7	Trim30a/Trim30d	6.6	IFIT1B	20.8
FAM162A	2.1	DIRAS2	2.6	IFIT1B	4.1	GBP4	2.6	IRF7	6.4	PRSS3	20.0
Downregulated		Downregulated		Downregulated		Downregulated		Downregulated		Downregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
FXYD3	-1.6	GAS1	-2.1	UTP6	-5.6	CADM4	-2.0	ID2	-2.7	CABLES1	-5.5
PADI3	-1.5	CYP2S1	-2.0	KIF2C	-5.1	NDRG2	-1.6	ANGPTL7	-2.7	ID2	-5.1
		DHRS3	-1.9	MGLL	-4.7	NXPH3	-1.6	CNN1	-2.4	Fip1l1	-5.0
		CFH	-1.9	MPZL3	-4.5	GSTA3	-1.6	ID1	-2.2	Gm21941	-4.4
		NOL4L	-1.9	PWWP2A	-3.2			Gm34047	-2.2	LUM	-4.2
		F830014O1	-1.8	GAS1	-3.1			NDRG2	-2.2	PATZ1	-4.2
		ID2	-1.8	DHRS3	-2.9			LUM	-2.1	EPHX2	-4.1
		PROKR1	-1.7	HMG20B	-2.7			GAREM2	-2.1	Or52ae7	-4.0
		FAM107A	-1.7	CFH	-2.6			SCRG1	-2.0	CHCHD6	-4.0
		PRR15L	-1.7	MGP	-2.4			CADM4	-2.0	SLC35A1	-3.9

Gene expression changes induced by MnO₂ MP (most perturbed DEGs)

After 24h, the most of the top 10 significantly altered DEGs were associated with hypoxia and oxidative stress (Table 5). The perturbed DEGs common across all the concentrations were: mitochondrial organization and hypoxia related mitochondria localized glutamic acid rich protein (*MGARP*), the apoptosis and hypoxia related BCL2 interacting protein 3 (*BNIP3*), and potassium voltage-gated channel subfamily B member 1 (*KCNB1*). DEGs upregulated in the 25 and 50 µg/mL concentrations were the cellular iron uptake (endocytosis) related transferrin receptor (*TFRC*), hypoxia related HIG1 hypoxia inducible domain family member 1A (*HIGD1A*), phospholipid transport related PRELI domain containing 2 (*PRELID2*), redox regulation related selenoprotein H (*SELENOH*), and transmembrane protein 45A (*TMEM45A*). Transmembrane protein 74B (*TMEM74B*) and growth factor and hormone activity related prolactin family 2 subfamily c member 2 (*Pr12c2*) were increased in the 50 and 100 µg/mL concentrations. Apoptosis and hypoxia related family with sequence similarity 162 member A (*FAM162A*) and the glycolytic enzyme phosphoglycerate kinase 1 (*PGK1*) were increased in the 25 µg/mL. Protease related DEGs including serine protease 3 (*PRSS3*) and matrix metalloproteinase 10 (*MMP10*) were upregulated in the 100 µg/mL concentration along with DEGs associated with immune response including C-C motif chemokine ligand 5 (*CCL5*), receptor transporter protein 4 (*RTP4*) and interferon induced protein with tetratricopeptide repeats 1B (*IFIT1B*).

After 48h, the most significantly altered DEGs were primarily associated with immune response (Table 5). The DEGs that were commonly perturbed across all the concentrations were all increased: immune response related 2'-5' oligoadenylate synthetase-like 2 (*Oasl2*), receptor transporter protein 4 (*RTP4*), 2'-5'-oligoadenylate synthetase 2 (*OAS2*), and ubiquitin specific

peptidase 18 (*USP18*; 2.1, 2.6, 12.2-fold increase). DEGs altered in the 25 and 50 µg/mL concentrations were: immune response related DExH-box helicase 58 (*DHX58*) and redox related glutathione peroxidase 1 (*GPX1*), selenoprotein W (*SELENOW*), and selenoprotein H (*SELENOH*). Serine protease 3 (*PRSS3*) and interferon induced protein with tetratricopeptide repeats 1B (*IFIT1B*) were increased in the 50 and 100 µg/mL concentrations. Immune response related DEGs ISG15 ubiquitin like modifier (*ISG15*; 2.4, 11.6-fold increase) and 2'-5'-oligoadenylate synthetase 1 (*OAS1*; 2.1, 20.0-fold increase) were upregulated in the 25 and 100 µg/mL concentrations. Immune response related interferon inducible GTPase 1 (*Iigp1*), and the transporter activity and cholesterol binding related apolipoprotein D (*APOD*) were increased in the 100 µg/mL concentration.

Table 5. Top 10 upregulated and downregulated DEGs after 24h and 48h of exposure to Manganese (IV) oxide (MnO₂) MPs.

MnO ₂ MP											
24h						48h					
25 µg/mL		50 µg/mL		100 µg/mL		25 µg/mL		50 µg/mL		100 µg/mL	
Upregulated		Upregulated		Upregulated		Upregulated		Upregulated		Upregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
TFRC	2.7	MGARP	4.1	MGARP	7.9	Oasl2	2.6	SELENOW	4.4	OAS1	20.0
MGARP	2.5	TFRC	2.9	PrI2c2 (includes others)	5.4	RTP4	2.6	GPX1	2.9	OAS2	14.7
HIGD1A	1.9	BNIP3	2.3	KCNB1	4.7	GPX1	2.5	SELENOH	2.8	Oasl2	13.0
BNIP3	1.8	TMEM74B	2.3	PRSS3	4.7	DHX58	2.4	PRSS3	2.8	IFIT1B	12.8
PRELID2	1.7	HIGD1A	2.3	TMEM74B	4.6	SELENOW	2.4	Oasl2	2.8	PRSS3	12.3
SELENOH	1.7	KCNB1	2.2	CCL5	4.2	SELENOH	2.4	RTP4	2.8	USP18	12.2
KCNB1	1.7	SELENOH	2.2	MMP10	4.1	OAS2	2.4	USP18	2.6	ISG15	11.6
FAM162A	1.7	PrI2c2 (includes others)	2.1	RTP4	3.9	ISG15	2.4	DHX58	2.6	RTP4	11.3
TMEM45A	1.6	TMEM45A	2.1	BNIP3	3.6	OAS1	2.1	IFIT1B	2.6	ligp1	10.9
PGK1	1.6	PRELID2	2.1	IFIT1B	3.5	USP18	2.1	OAS2	2.6	APOD	10.3
Downregulated		Downregulated		Downregulated		Downregulated		Downregulated		Downregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
N/A	N/A	LAMA1	-1.6	MGP	-2.6	N/A	N/A	SERPINA9	-1.6	SYT17	-3.4
				D7Erttd143e	-2.4					KCNN3	-3.3
				INSC	-2.3					TRDMT1	-3.3
				Sprr2a3	-2.2					LUM	-3.0
				CFH	-2.1					NF1	-3.0
				LAMA1	-2.0					CNN1	-2.9
				PTX3	-2.0					GPR34	-2.8
				AI594671	-2.0					SCRG1	-2.6
				Gm9975	-2.0					ANGPTL7	-2.6
				GAS1	-1.9					RASL11B	-2.5

Gene expression changes induced by MnSO₄ (most perturbed DEGs)

After 24h, the top 10 DEGs were associated with hypoxia, although there was more variety in terms of function at the higher concentrations (Table 6). The DEGs that were commonly increased across all the concentrations were the hypoxia related mitochondria localized glutamic acid rich protein (*MGARP*), BCL2 interacting protein 3 (*BNIP3*), transmembrane protein 74B (*TMEM74B*), and potassium voltage-gated channel subfamily B member 1 (*KCNB1*). HIG1 hypoxia inducible domain family member 1A (*HIGD1A*) and transferrin receptor (*TFRC*) were upregulated in the 3.75 and 7.5 µg/mL concentrations and DIRAS family GTPase 2 (*DIRAS2*) was increased in the 7.5 and 15 µg/mL. Hypoxia related family with sequence similarity 162 member A (*FAM162A*), glucose metabolism/homeostasis related pyruvate dehydrogenase kinase 1 (*PDK1*), collagen synthesis related prolyl 4-hydroxylase subunit alpha 2 (*P4HA2*), and the glycolytic enzyme phosphoglycerate kinase 1 (*PGK1*) were upregulated in the 3.75 µg/mL concentration. Microtubule associated serine/threonine kinase family member 4 (*MAST4*), hypoxia related egl-9 family hypoxia inducible factor 1 (*EGLN1*), and olfactory receptor family 5 subfamily AN member 6 (*Or5an6*) increased in the 7.5 µg/mL concentration. Protease inhibition related gene serpin family B member 5 (*SERPINB5*), prolactin family 2 subfamily c member 2 (*Pr12c2*), neural development related roundabout guidance receptor 3 (*ROBO3*), piercer of microtubule wall 1 (*PIERCE1*), and C-C motif chemokine ligand 5 (*CCL5*) were upregulated in the 15 µg/mL concentration.

After 48h, the top 10 DEGs were largely related to immune response at the higher concentrations (Table 6). The DEGs altered across all concentrations were cell proliferation and differentiation related FosB proto-oncogene, AP-1 transcription factor subunit (*FOSB*), transferrin receptor (*TFRC*), guanine deaminase (*GDA*), H4 histone 16 (*H4C16*), osteoblast

differentiation and growth related glycerophosphodiester phosphodiesterase domain containing 2 (*GDPD2*), extracellular matrix related versican (*VCAN*), and melanosome biogenesis related melan-A (*MLANA*). The DEGs that were commonly increased across the 7.5 and 15 µg/mL concentrations were immune response related DEGs such as receptor transporter protein 4 (*RTP4*), interferon induced protein with tetratricopeptide repeats 1B (*IFIT1B*), 2'-5'-oligoadenylate synthetase 2 (*OAS2*), ubiquitin specific peptidase 18 (*USP18*), 2'-5'-oligoadenylate synthetase-like 2 (*Oasl2*), interferon inducible GTPase 1 (*Iigp1*), guanylate binding protein 4 (*GBP4*), ISG15 ubiquitin like modifier (*ISG15*), 2'-5'-oligoadenylate synthetase 1 (*OAS1*). MX dynamin-like GTPase 2 (*Mx2*) and apolipoprotein D (*APOD*) were also upregulated in the 7.5 and 15 µg/mL concentrations, respectively.

Table 6. Top 10 upregulated and downregulated DEGs after 24h and 48h of exposure to Manganese (II) sulfate (MnSO₄).

MnSO ₄											
24h						48h					
3.75 µg/mL		7.5 µg/mL		15 µg/mL		3.75 µg/mL		7.5 µg/mL		15 µg/mL	
Upregulated		Upregulated		Upregulated		Upregulated		Upregulated		Upregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
MGARP	3.9	MGARP	5.2	MGARP	12.6	FOSB	2.5	RTP4	5.1	OAS2	14.4
TFRC	3.4	MAST4	3.5	SERPINB5	8.5	TFRC	1.9	IFIT1B	5.1	OAS1	11.8
BNIP3	2.6	BNIP3	3.4	PrI2c2 (includes others)	7.0	GDA	1.8	OAS2	4.5	IFIT1B	10.8
TMEM74B	2.4	TMEM74B	3.4	ROBO3	6.9	H4C16	1.6	USP18	4.5	APOD	10.8
HIGD1A	2.3	TFRC	3.0	DIRAS2	6.4	GDPD2	1.6	Oasl2	4.5	RTP4	10.4
FAM162A	2.2	Or5an6	2.9	PIERCE1	6.2	VCAN	1.6	Mx2	4.4	USP18	10.1
KCNB1	2.1	HIGD1A	2.7	KCNB1	6.1			ligp1	4.4	Oasl2	9.9
PDK1	2.1	KCNB1	2.7	TMEM74B	5.9			GBP4	4.1	ISG15	9.5
P4HA2	2.0	DIRAS2	2.5	BNIP3	5.6			ISG15	4.0	ligp1	8.8
PGK1	2.0	EGLN1	2.4	CCL5	5.5			OAS1	3.8	GBP4	8.0
Downregulated		Downregulated		Downregulated		Downregulated		Downregulated		Downregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
Ccl2	-1.7	SDF2L1	-1.7	PTX3	-4.6	MLANA	-1.7	PRMT1	-2.2	SERPINA11	-4.1
SDF2L1	-1.5	EMILIN1	-1.6	MGP	-4.3			GAREM2	-2.2	MLANA	-3.6
FGFBP1	-1.5	MGP	-1.6	Ccl2	-4.0			MLANA	-2.1	GAREM2	-3.5
		UBE2M	-1.6	SDF2L1	-3.2			CADM4	-2.1	FXVD3	-3.5
		MT-ND1	-1.6	GAS1	-3.1			AKT1S1	-2.0	FAM180A	-3.5
		DHCR7	-1.6	MVD	-3.1			GCNT1	-2.0	ANGPTL7	-3.3
		Ccl2	-1.5	PRMT1	-3.0			FAM180A	-2.0	EPYC	-3.2
		PCNX2	-1.5	Spr2a3	-2.9			EPYC	-1.9	ID2	-3.2
		TRPC6	-1.5	SLC2A6	-2.9			SERPINA11	-1.8	RASL11B	-3.1
		CHD5	-1.5	SLC25A43	-2.8			MMRN1	-1.8	FRAS1	-3.1

Gene expression changes induced by Fe₂O₃ NP (most perturbed DEGs)

After 24h, there were no significant DEGs at the 25 µg/mL concentration (Table 7). The top 10 DEGs following exposure to 50 and 100 µg/mL were related to a wide variety of functions. The DEGs commonly altered in the 50 and 100 µg/mL concentrations were atonal bHLH transcription factor 8 (*ATOH8*) (related to endothelial cell proliferation, migration, and differentiation), inhibitor of DNA binding 2 (*ID2*) (regulates a variety of cellular processes – growth, senescence, differentiation, apoptosis, etc.), predicted gene 19990 (*Gm19990*), G-protein coupled receptor 5-hydroxytryptamine receptor 2B (*HTR2B*), acyl-CoA synthetase bubblegum family member 1 (*ACSBG1*), prolactin family 2 subfamily c member 2 (*Pr12c2*), ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 (*ST8SIA1*), ATP dependent transporter ATP binding cassette subfamily C member 3 (*ABCC3*), cannabinoid receptor 1 (*CNR1*), tumor protein p63 regulated 1 (*TPRG1*), cathepsin W (*CTSW*), and the protease ADAM metalloproteinase with thrombospondin type 1 motif 16 (*ADAMTS16*). Serpin family A member 11 (*SERPINA11*), which is related to endopeptidase inhibitor activity, was only increased in the 100 µg/mL concentration.

After 48h, the top 10 DEGs were again related to a wide variety of functions (Table 7). The DEGs commonly perturbed across all the concentrations were ADAM metalloproteinase with thrombospondin type 1 motif 4 (*ADAMTS4*) (protease; degrades aggrecan and brevican), FAM20A golgi associated secretory pathway pseudokinase (*FAM20A*) (related to the biomineralization of teeth), and atonal bHLH transcription factor 8 (*ATOH8*). DEGs that decreased in the 50 and 100 µg/mL concentrations were inhibitor of DNA binding 2 (*ID2*), chemokine (C-X-C motif) ligand 15 (*Cxcl15*), ADAM metalloproteinase with thrombospondin, and calponin 1 (*CNN1*) (related to actin binding activity) while type 1 motif 16 (*ADAMTS16*),

interleukin 6 (*IL6*) (inflammation), and PDZ and LIM domain 3 (*Pdlim3*) (may be involved in cytoskeletal assembly) all increased. Dishevelled binding antagonist of beta catenin 2 (*DACT2*) (cell development related), cytotoxic T lymphocyte-associated protein 2 beta (*Ctla2a/Ctla2b*) (Predicted to be involved in proteolysis), 5'-nucleotidase ecto (*NT5E*), sialic acid binding Ig like lectin 10 (*SIGLEC10*) (immune related), and were all upregulated in the 25 µg/mL concentration while cell migration inducing hyaluronidase 1 (*CEMIP*) (involved in hyaluronan catabolic process; positive regulation of protein phosphorylation; and positive regulation of transport) was downregulated. Lengsin, lens protein with glutamine synthetase domain (*LGSN*), predicted gene 33035 (*Gm33035*) (non-coding RNA), and myomesin 2 (*MYOM2*) (component of the vertebrate myofibrillar M band) were all perturbed in the 50 µg/mL concentration. Periostin (*POSTN*) (extracellular matrix related) was altered in the 100 µg/mL concentration.

Table 7. Top 10 upregulated and downregulated DEGs after 24h and 48h of exposure to Iron (III) oxide (Fe₂O₃) NPs.

Fe2O3 NP											
24h						48h					
25 µg/mL		50 µg/mL		100 µg/mL		25 µg/mL		50 µg/mL		100 µg/mL	
Upregulated		Upregulated		Upregulated		Upregulated		Upregulated		Upregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
N/A	N/A	HTR2B	2.0	HTR2B	3.2	ADAMTS4	1.8	ADAMTS16	3.6	ADAMTS16	8.4
		ACSBG1	1.9	CTSW	2.8	FAM20A	1.8	IL6	3.2	Pdlim3	7.3
		PrI2c2 (includes others)	1.9	ADAMTS16	2.6	DACT2	1.8	FAM20A	3.2	POSTN	6.5
		CNR1	1.8	CNR1	2.5	Ctla2a/Ctla2b	1.8	Pdlim3	3.1	IL6	5.8
		IL6	1.8	SERPINA11	2.5	NT5E	1.6	MYOM2	2.9	NUPR1	5.2
		ADAMTS16	1.8	ACSBG1	2.5	SIGLEC10	1.5	Erv3	2.9	Erv3	5.1
		MYOM2	1.8	MYOM2	2.4			NUPR1	2.9	ADAMTS4	5.1
		TMEM140	1.8	IL6	2.3			ADAMTS4	2.8	NFATC1	5.1
		SLC40A1	1.7	PrI2c2 (includes others)	2.3			ZNF184	2.8	FAM20A	5.0
		CTSW	1.7	SORCS2	2.3			USP18	2.8	PI16	4.8
Downregulated		Downregulated		Downregulated		Downregulated		Downregulated		Downregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
N/A	N/A	ATOH8	-2.2	Gm19990	-3.0	ATOH8	-1.8	LGSN	-6.3	ID2	-10.3
		ID2	-2.1	ATOH8	-2.6	CEMIP	-1.8	Gm33035	-4.7	Cxcl15	-7.2
		Gm19990	-2.1	ID2	-2.6			ID2	-4.4	ATOH8	-5.6
		ST8SIA1	-1.8	ABCC3	-2.6			Cxcl15	-4.1	CNN1	-4.6
		ABCC3	-1.8	ST8SIA1	-2.4			CNN1	-3.2	CD14	-4.0
		TPRG1	-1.8	TPRG1	-2.4			ATOH8	-2.9	ID3	-4.0
		ID3	-1.8	PROKR1	-2.4			BRINP3	-2.9	FXYD3	-4.0
		RASSF6	-1.7	FGF7	-2.3			HPCAL1	-2.8	EPHX2	-3.9
		FGF7	-1.7	PRR15L	-2.2			CELF2	-2.7	MMRN1	-3.8
		TRIB2	-1.7	CD14	-2.2			FIZ1	-2.6	ABCC3	-3.7

Gene expression changes induced by Fe₂O₃ MP (most perturbed DEGs)

After 24h, there were no significant DEGs at the 25 µg/mL concentration (Table 8). The top 10 DEGs following exposure to 50 and 100 µg/mL were related to a wide variety of functions. The increased DEGs common to the 50 and 100 µg/mL concentrations were cathepsin W (*CTSW*) and acyl-CoA synthetase bubblegum family member 1 (*ACSBG1*) while ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 1 (*ST8SIA1*) and matrix Gla protein (*MGP*) (related to organic matrix of bone and cartilage) decreased. Heparan sulfate-glucosamine 3-sulfotransferase 1 (*HS3ST1*) (Important to the biosynthesis of anticoagulant heparan sulfate), predicted gene 19990 (*Gm19990*), UDP-N-acetylglucosamine pyrophosphorylase 1 like 1 (*UAP1L1*), chemokine (C-X-C motif) ligand 15 (*Cxcl15*), transmembrane protein 140 (*TMEM140*), glucoside xylosyltransferase 2 (*GXYLT2*), and neuraminidase 1 (*NEU1*) were altered in the 50 µg/mL concentration. 5-hydroxytryptamine receptor 2B (*HTR2B*), myomesin 2 (*MYOM2*), solute carrier family 40 member 1 (*SLC40A1*) (related to iron homeostasis), neuronal regeneration related protein (*NREP*; 2.6-fold increase) (related to axonal regeneration), 2-cell-stage variable group member 3 (*Tcstv3*), and cannabinoid receptor 1 (*CNRI*) were perturbed in the 100 µg/mL concentration.

After 48h, the most significantly altered DEGs were primarily associated with immune response (Table 8). The top 10 DEGs commonly increased across all the concentrations were immune response related DEGs: receptor transporter protein 4 (*RTP4*) and FAM20A golgi associated secretory pathway pseudokinase (*FAM20A*). The DEGs commonly upregulated in the 25 and 50 µg/mL concentrations included interferon regulatory factor 7 (*IRF7*), and PHD finger protein 11 (*PHF11*). The DEGs commonly downregulated in the 50 and 100 µg/mL concentrations were: (C-X-C motif) ligand 15 (*Cxcl15*) and inhibitor of DNA binding 2 (*ID2*)

while 2'-5' oligoadenylate synthetase-like 2 (*Oasl2*), ubiquitin specific peptidase 18 (*USP18*), myomesin 2 (*MYOM2*), and actin cytoskeleton related PDZ and LIM domain 3 (*Pdlim3*) were upregulated. Guanine deaminase (*GDA*) and dehydrogenase/reductase 3 (*DHRS3*; 1.6-fold increase) were increased in the 25 µg/mL concentration. 2'-5'-oligoadenylate synthetase 1 (*OAS1*) and cytotoxic T lymphocyte-associated protein 2 beta (*Ctla2a/Ctla2b*) were upregulated in the 50 µg/mL concentration. Periostin (*POSTN*) and RIKEN cDNA 0610009K14 gene (*0610009K14Rik*) were increased in the 100 µg/mL concentration.

Table 8. Top 10 upregulated and downregulated DEGs after 24h and 48h of exposure to Iron (III) oxide (Fe₂O₃) MPs.

Fe2O3 MP											
24h						48h					
25 µg/mL		50 µg/mL		100 µg/mL		25 µg/mL		50 µg/mL		100 µg/mL	
Upregulated		Upregulated		Upregulated		Upregulated		Upregulated		Upregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
N/A	N/A	UAP1L1	1.8	HTR2B	3.6	RTP4	3.8	RTP4	2.9	Pdlim3	8.2
		ACSBG1	1.8	MYOM2	3.5	IRF7	2.7	Oasl2	2.8	POSTN	4.9
		TMEM140	1.7	CTSW	3.5	PHF11	2.4	USP18	2.7	FAM20A	4.4
		GXYLT2	1.7	SLC40A1	2.9	GDA	1.7	OAS1	2.5	USP18	4.4
		NEU1	1.7	ACSBG1	2.9	DHRS3	1.6	MYOM2	2.4	MYOM2	4.4
		CTSW	1.6	NREP	2.6	FAM20A	1.5	IRF7	2.4	0610009K14Rik	4.2
		FBXO32	1.6	Tcstv3	2.6			Ctla2a/Ctla2b	2.3	RTP4	4.1
		DPP7	1.6	CNR1	2.5			Pdlim3	2.3	Oasl2	4.1
		FUCA2	1.6	TENM4	2.5			FAM20A	2.2	NUPR1	4.0
		Ctla2a/Ctla2b	1.6	Gm21818	2.5			PHF11	2.1	ISG15	3.9
Downregulated		Downregulated		Downregulated		Downregulated		Downregulated		Downregulated	
DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change	DEG	Fold Change
N/A	N/A	HS3ST1	-1.9	MGP	-3.0	N/A	N/A	Cxcl15	-3.1	Cxcl15	-6.8
		ST8SIA1	-1.8	ST8SIA1	-2.8			ID2	-2.6	ID2	-6.6
		Gm19990	-1.8	ID2	-2.5			LRATD1	-2.1	RASL11A	-3.7
		Cxcl15	-1.7	TPRG1	-2.5			FXVD3	-1.8	ATOH8	-3.1
		PROKR1	-1.6	PROKR1	-2.4			RSPO2	-1.7	CD14	-3.1
		Nrp	-1.6	ABCC3	-2.3			ATOH8	-1.7	H2BC9	-3.1
		MGP	-1.6	TP63	-2.2			ABCC3	-1.7	ID3	-2.9
				CD14	-2.2			AHSG	-1.6	MMRN1	-2.8
				IL33	-2.1			C3	-1.6	C3	-2.8
				FGF7	-1.9			ID1	-1.6	CNN1	-2.8

Influence of particle size on gene expression

Size related effects were analyzed by comparing the gene expression changes induced by MnO₂ and Fe₂O₃ NPs with their associated MPs. Overall, the number of DEGs induced by the NPs was substantially greater than that induced by the MPs among the concentration groups, particularly after 48h at the higher concentrations (Figure 4). Venn analysis of the MnO₂ NPs and MPs after 24h showed 12 overlapping genes in the low concentration (25 µg/mL) group, 30 in the medium concentration (50 µg/mL) group, and 205 in the highest concentration (100 µg/mL) group (Figure 6). The 12 common genes among both MnO₂ particle types and all concentration groups were related to hypoxia (*MGARP*, *BNIP3*, *HIGD1A*, and *FAM162A* (also cell death)), glucose metabolism (*PGMI*, *PGKI*), nucleotide homeostasis (*AK4*), collagen synthesis (*P4HA2*), iron homeostasis (*TFRC*), and membrane/transport (*PRELID2*, *TMEM45A*, and *KCNBI*). Analysis of the MnO₂ NPs and MPs after 48h showed 9 overlapping genes in the low concentration (25 µg/mL), 33 in the medium concentration (50 µg/mL), and 497 in the highest concentration (100 µg/mL) groups. The 9 common genes among both MnO₂ particle types and all concentration groups were related to immune/inflammatory response (*OAS2*, *USP18*, *Oasl2*, *RTP4*, *DHX58*, *PHF11*, *ISG15*, *Apol9a/Apol9b*) and transport (*APOD*). Overall, there were many overlapping DEGs between MnO₂ NPs and MPs, although the NPs had more unique DEGs than the MPs as they induced a greater number of total DEGs. There were more upregulated DEGs induced by both the NPs and MPs (Figure 4) and the DEGs induced by the NPs generally had higher fold changes than those induced by the MPs.

Fe₂O₃ NP and MP comparison of DEGs at the 24h time point showed 11 overlapping genes in the medium concentration (50 µg/mL) group, and 169 overlapping genes in the highest concentration (100 µg/mL) group (there were no DEGs in the 25 µg/mL concentration group).

There were no common genes among the Fe₂O₃ particle types across all concentration groups at 24h. Fe₂O₃ particles after 48h had 1 overlapping DEG in the 25 µg/mL concentration, 56 in the medium concentration (50 µg/mL), and 382 in the highest concentration (100 µg/mL) group. The only common gene was associated with the biomineralization of teeth (*FAM20C*). The most significant DEGs induced by the Fe₂O₃ NPs and MPs had similar fold changes (slightly higher in the NPs), unlike the MnO₂ NPs and MPs that had a dramatic difference in fold change (much higher in the NPs). Additionally, the Fe₂O₃ NP and MP induced DEGs with the highest fold changes were often split between upregulation and downregulation, whereas those induced by the MnO₂ NPs and MPs were only upregulated.

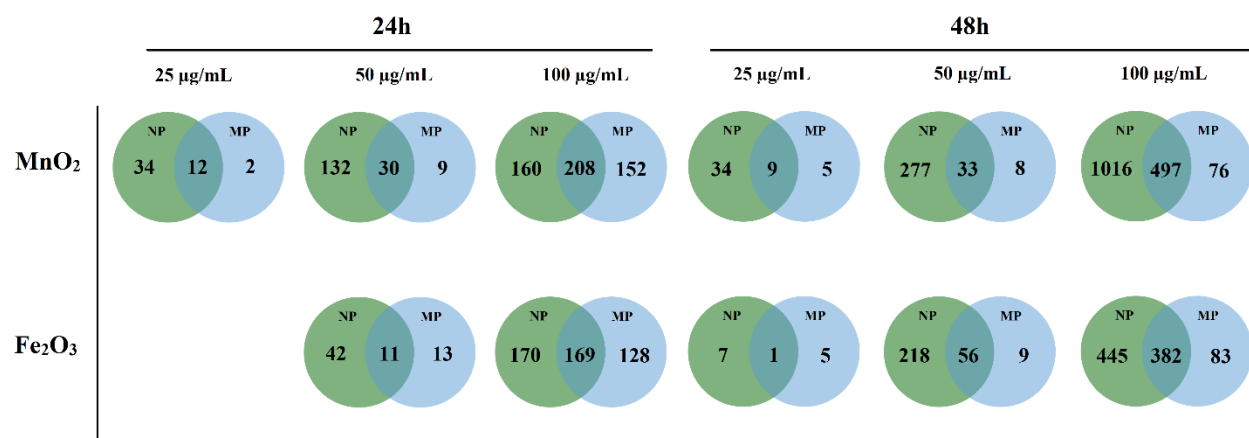


Figure 6. Venn diagrams comparing the number of DEGs based on Manganese (IV) oxide (MnO₂) and Iron (III) oxide (Fe₂O₃) particle size at 25, 50 and 100 µg/mL at 24 and 48h.

Influence of solubility on gene expression

Solubility related effects were analyzed by comparing the expression responses of MnO₂ NPs and MPs with MnSO₄. MnO₂ NPs and MnSO₄ showed 29 overlapping genes at 24h in the low concentration (25 µg/mL/3.75 µg/mL) group, 55 in the medium concentration (50 µg/mL/7.5 µg/mL) group, and 273 in the highest concentration (100 µg/mL/15 µg/mL) group. A total of 29 DEGs were common across the two compounds and concentrations, which were primarily related to hypoxia and glucose/energy metabolism. MnO₂ NPs and MnSO₄ at 48h showed 3 overlapping genes in the low concentration (25 µg/mL/3.75 µg/mL) group, 126 in the medium concentration (50 µg/mL/7.5 µg/mL) group, and 673 in the highest concentration (100 µg/mL/15 µg/mL) group. The extracellular matrix (*VCAN*) and iron homeostasis (*TFRC*) related genes were in common across the compounds and concentrations.

MnO₂ MPs and MnSO₄ had 11 overlapping DEGs at 24h in the low concentration (25 µg/mL/3.75 µg/mL) group, 25 in the medium concentration (50 µg/mL/7.5 µg/mL) group, and 262 in the highest concentration (100 µg/mL/15 µg/mL) group. The 11 common genes among all concentration groups of MnO₂ MPs and MnSO₄ were genes associated with hypoxia (*MGARP*, *HIGD1A*, *BNIP3*, and *FAM162A*), iron homeostasis (*TFRC*), membrane/transport (*PRELID2* and *KCNB1*), glucose metabolism (*PGK1* and *PGM1*), collagen synthesis (*P4HA2*), and nucleotide homeostasis (*AK4*). MnO₂ MPs and MnSO₄ at 48h had no overlapping genes in the low concentration (25 µg/mL/3.75 µg/mL), 31 overlapping genes in the medium concentration (50 µg/mL/7.5 µg/mL), and 468 overlapping genes in the highest concentration (100 µg/mL/15 µg/mL). There were no common DEGs among MnO₂ MPs and MnSO₄ at any concentrations as there was a low number of DEGs induced by MnSO₄ at the low (3.75 µg/mL) concentration. The most significant DEGs induced by the MnO₂ NPs and MPs had higher fold changes than MnSO₄

(much higher in the NPs). Additionally, the top DEGs induced by the MnO₂ NPs, MPs, and MnSO₄ were upregulated rather than down regulated.

Canonical Pathway Analysis

I next explored the enrichment of canonical pathways in my gene set to understand the differential effects of the metal oxides on the biology of the FE1 mouse pulmonary epithelial cells more deeply. There was a concentration- and time-dependent increase in perturbed pathways for all treatment conditions except for the highest and lowest concentration of MnSO₄ (Figure 7). Overall, the NPs enriched more pathways than the MPs, and the MnO₂ NPs and MPs enriched more pathways than the Fe₂O₃ NPs and MPs at all concentrations aside from the top concentration of Fe₂O₃ NPs at 48h. The MnSO₄ treatments perturbed a greater number of pathways than the MnO₂ particles after 24h and a similar amount after 48h.

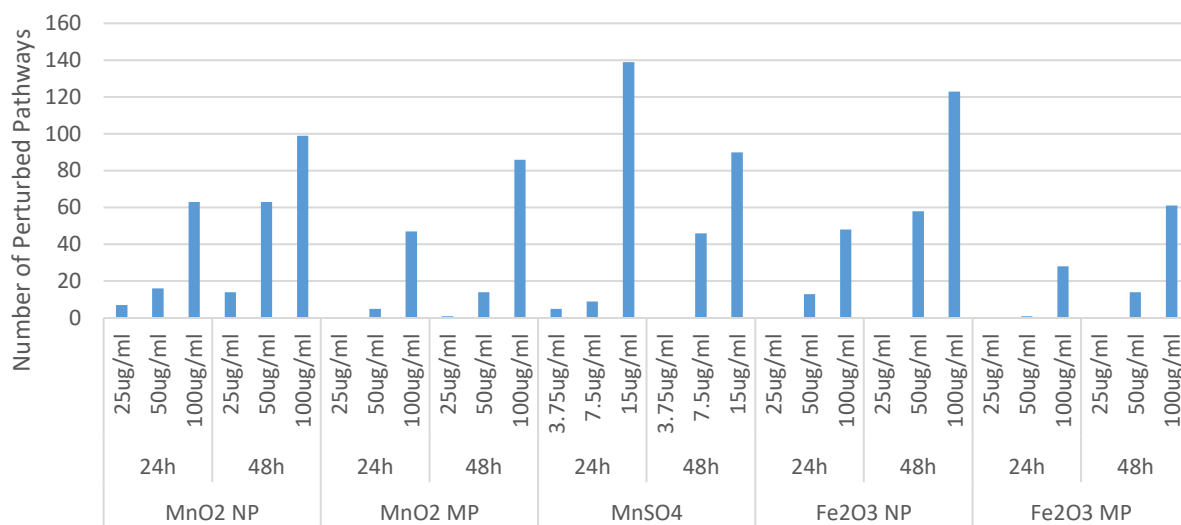
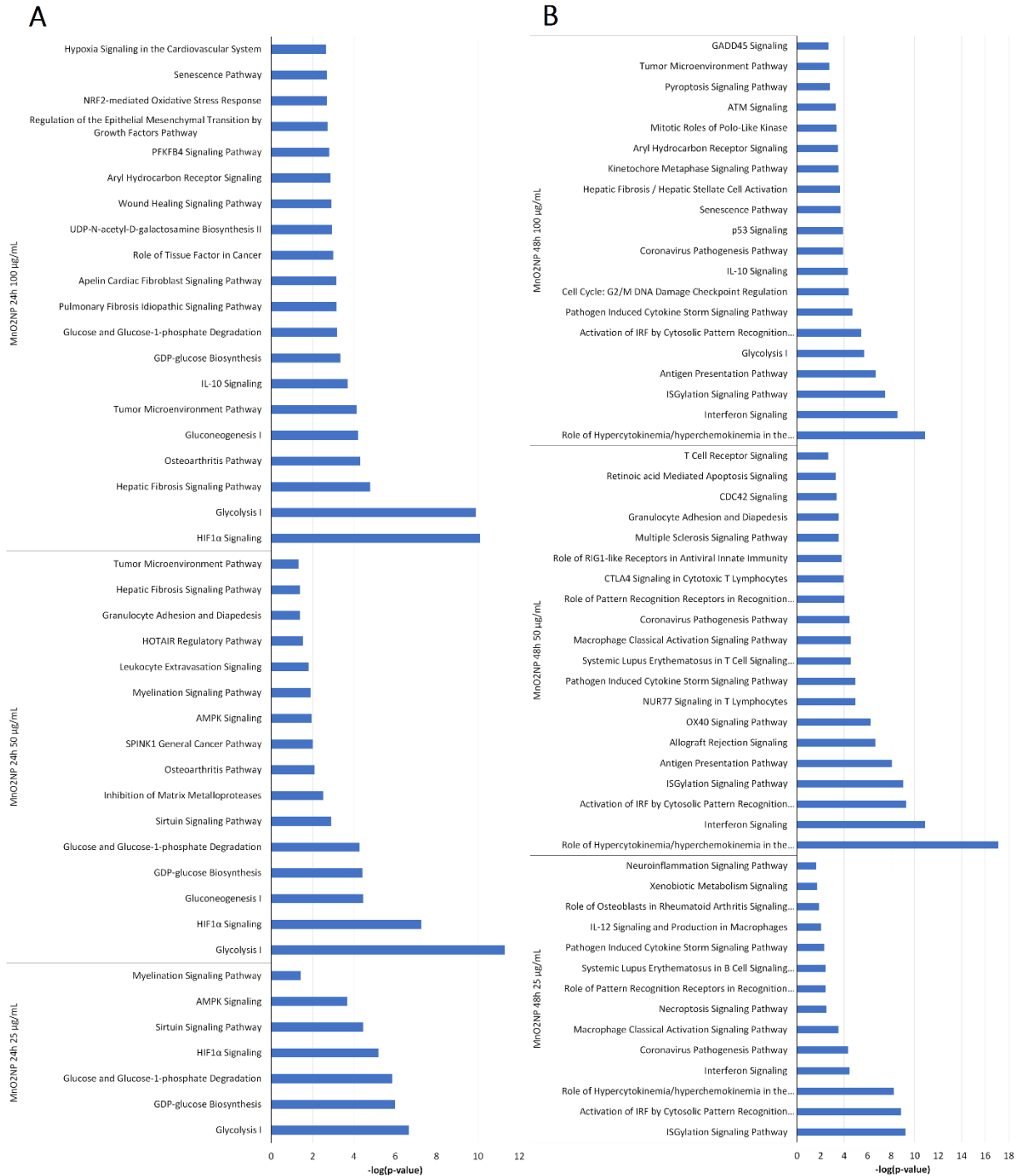


Figure 7. Number of significantly perturbed canonical pathways following exposure to 25, 50, and 100 µg/mL of Manganese (IV) oxide (MnO₂) NPs and MPs, Iron (III) oxide (Fe₂O₃) NPs and MPs, and Manganese (II) sulfate (MnSO₄) equivalent for 24h and 48h. The bars in the graph represent the number of perturbed canonical pathways with a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$.

Pathway analysis of MnO₂ NPs

The 20 most significantly perturbed IPA canonical pathways for each concentration of MnO₂ NPs are shown in Figure 8. Exposure to MnO₂ NPs resulted in the enrichment of hypoxia/oxidative stress, glucose metabolism, and some inflammatory related pathways at the 24h timepoint. Immune/inflammatory and cell cycle/DNA damage related pathways were the most perturbed after 48h. There was a large overlap in affected pathways between the concentrations at 24h, as all seven significant pathways in the 25 µg/mL treatment and 15/16 pathways in the 50 µg/mL pathway were in the list of 63 pathways altered in the 100 µg/mL treatment group. Additionally, 10/14 significant pathways in the 25 µg/mL treatment and 44/63 significant pathways in the 50 µg/mL treatment were also in the list of 99 pathways perturbed in the 100 µg/mL treatment at 48h. The pathways from the 50 µg/mL treatment that were not in the 100 µg/mL group were mostly inflammation/immune and signaling pathways that had -log(p-values) closer to the significance cutoff (-log(p-value) = 1.3). Many of the pathways that were uniquely perturbed in the 100 µg/mL concentration were cell cycle/DNA damage related.



Pathway analysis of MnO₂ MPs

The 20 most significantly perturbed IPA canonical pathways for each concentration of MnO₂ MPs are shown in Figure 9. Similarly to the MnO₂ NPs, the MPs resulted in the enrichment of hypoxia/oxidative stress, glucose metabolism, and some inflammatory related pathways at the 24h timepoint and primarily immune/inflammatory and cell cycle/DNA damage pathways at 48h. There was a large overlap in the pathways between concentrations, with 3/5 significantly perturbed pathways in the 50 µg/mL being among the 47 pathways perturbed in the 100 µg/mL group at 24h. At 48h, the only significant pathway in the 25 µg/mL group and 11/14 significant pathways in the 50 µg/mL group were among the 86 pathways perturbed by the 100 µg/mL exposure.

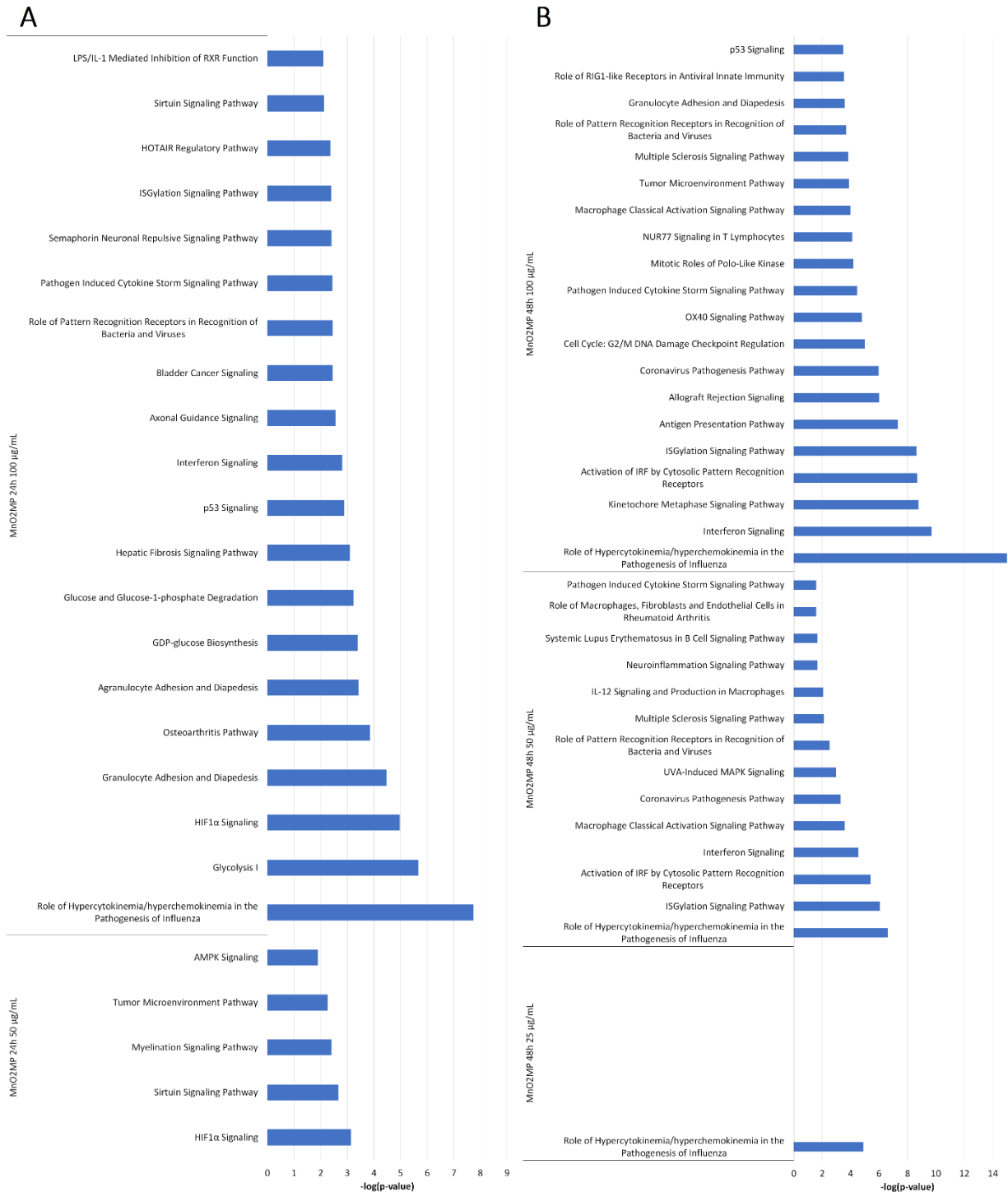


Figure 9. List of the top 20 most significantly perturbed pathways after exposure to 25, 50, and 100 µg/mL Manganese (IV) oxide (MnO₂) MPs for 24h (A) or 48h (B). Each bar in the graph represents a perturbed canonical pathway with a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$. For concentrations with < 20 affected pathways, all affected pathways are shown.

Pathway analysis of MnSO₄

The 20 most significantly perturbed IPA canonical pathways for each concentration of MnSO₄ are summarized in Figure 10. Exposure to MnSO₄ enriched pathways related to metabolism (cholesterol and glucose), hypoxia/oxidative stress, immune/inflammatory response, and cellular/tissue development at 24h. Perturbed pathways at 48h were mostly related to cell cycle/DNA repair and metabolism (particularly cholesterol) along with a few additional immune/inflammatory response pathways. All 5 significant pathways from the 3.75 µg/mL treatment group and all 9 significant pathways in 7.5 µg/mL were in the list of 139 pathways perturbed by the 15 µg/mL exposure at 24h. At 48h, 27/46 significantly perturbed pathways from the 7.5 µg/mL treatment group overlapped with the list of 90 perturbed pathways in the 15 µg/mL exposure group. Most of the common pathways were immune/inflammatory response pathways with a high -log(p-value). The pathways only in the 7.5 µg/mL group were generally immune/inflammatory response pathways with a lower -log(p-value). The pathways unique to the 15 µg/mL group were largely cell cycle/DNA repair and metabolism (particularly cholesterol) related pathways along with a few additional immune/inflammatory response pathways.



Figure 10. List of the top 20 most significantly perturbed pathways after exposure to 3.75, 7.5, and 15 µg/mL Manganese (II) sulfate (MnSO₄) for 24h (A) or 48h (B). Each bar in the graph represents a perturbed canonical pathway with a minimum of 3 DEGs and a -log(p-value) ≥ 1.3. For concentrations with < 20 affected pathways, all affected pathways are shown.

Pathway analysis of Fe₂O₃ NPs

The 20 most significantly perturbed IPA canonical pathways for each concentration of Fe₂O₃ NPs are shown in Figure 11. Fe₂O₃ NP exposure resulted in the perturbation of pathways related to hypoxia/oxidative stress, metabolism (glucose, cholesterol, and xenobiotic), immune/inflammatory response, and cellular/tissue development at 24h. At 48h primarily immune/inflammatory response pathways and cellular/tissue development pathways were altered. Many pathways overlapped between concentrations, as 9/13 significant pathways in the 50 µg/mL treatment group were within the list of 48 perturbed pathways in the 100 µg/mL group at 24h. At 48h, 52/58 pathways from the 50 µg/mL treatment group were among the 123 pathways perturbed by the 100 µg/mL group. The 71 pathways unique to the 100 µg/mL treatment at 48h were related to a wide variety of functions including immune/inflammatory response, metabolism, and cellular/tissue development.

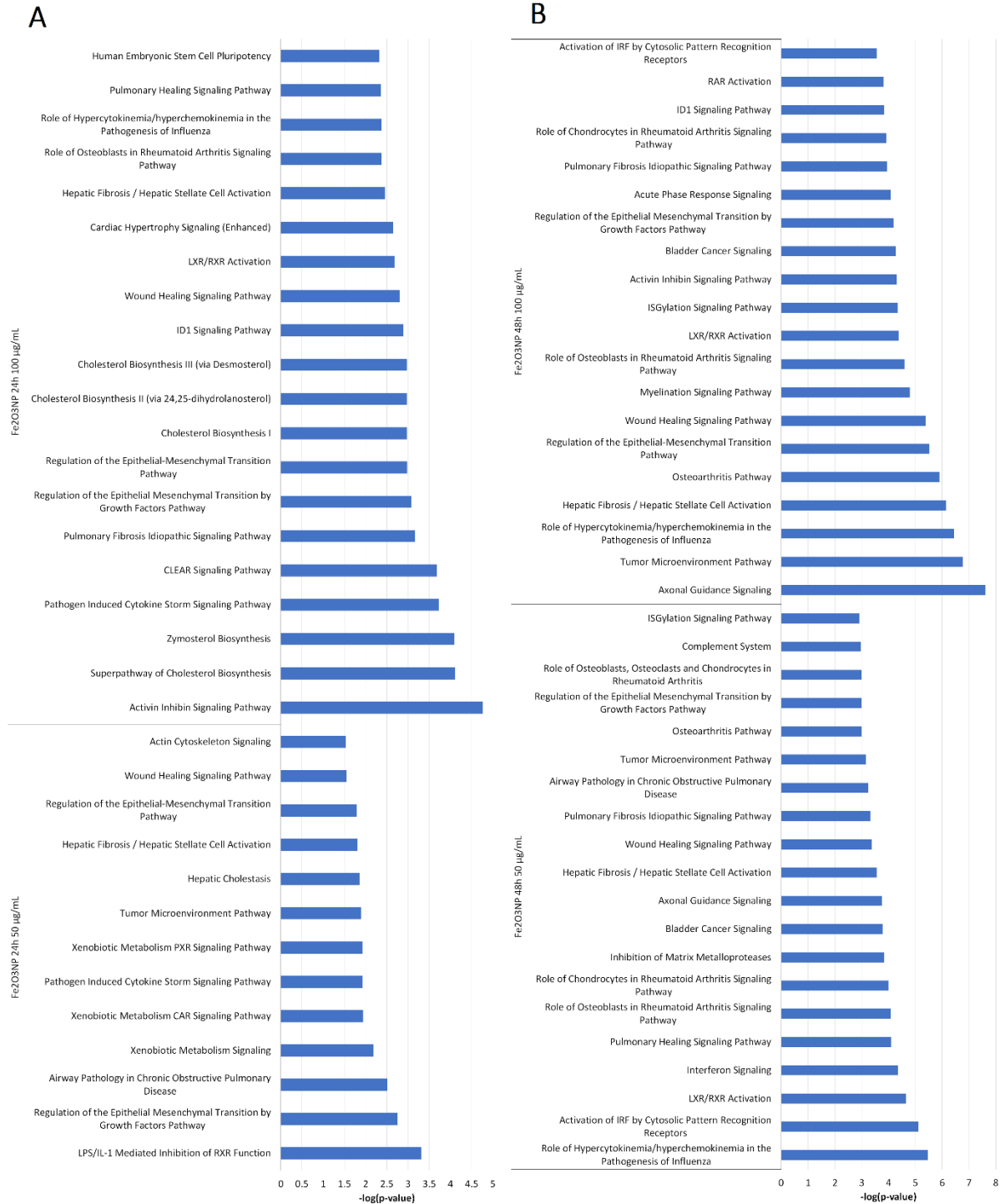


Figure 11. List of the top 20 most significantly perturbed pathways after exposure to 50 µg/mL and 100 µg/mL Iron (III) oxide (Fe₂O₃) NPs for 24h (A) or 48h (B). Each bar in the graph represents a perturbed canonical pathway with a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$. For concentrations with < 20 affected pathways, all affected pathways are shown.

Pathway analysis of Fe₂O₃ MPs

The 20 most significantly perturbed IPA canonical pathways for each concentration of Fe₂O₃ MPs are summarized in Figure 12. Perturbed pathways following exposure to Fe₂O₃ MPs were mostly related to metabolism, phagosome maturation/lysosomes, and cellular/tissue development at 24h. At 48h, the enriched pathways were immune/inflammatory response and cellular/tissue development related, which was similar to the effects of the Fe₂O₃ NPs. The only pathway affected in the 50 µg/mL treatment group was one of the 28 perturbed pathways in the 100 µg/mL Fe₂O₃ MP exposure group. At 48h, there was also a large overlap in affected pathways between concentrations; the only significant pathway in the 25 µg/mL group and 11/14 significant pathways in the 50 µg/mL group were among the 61 pathways perturbed by the 100 µg/mL group. The majority of the pathways unique to the 100 µg/mL group were additional inflammatory and cell/tissue development pathways.



Figure 12. List of the top 20 most significantly perturbed pathways after exposure to 50 µg/mL and 100 µg/mL of Iron (III) oxide (Fe₂O₃) MPs for 24h (A) or 48h (B). Each bar in the graph represents a perturbed canonical pathway with a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$. For concentrations with < 20 affected pathways, all affected pathways are shown. Summary of Top Significantly Perturbed Pathways

At 24h, the significantly perturbed pathways were most similar among particles with the same chemical composition; the groups [MnO₂ NP and MnO₂ MP] and [Fe₂O₃ NP and Fe₂O₃ MP] tended to alter similar pathways while the soluble MnSO₄ perturbed a more unique set of pathways (Figure 13). The MnO₂ particles and MnSO₄ highly enriched pathways related to energy/metabolism and hypoxia at 24h (MnO₂ NPs were the most potent in that respect). Some inflammatory and developmental related pathways were also perturbed (MnO₂ MPs were the most potent in that respect). MnSO₄ induced lipid related and biosynthesis pathways (cholesterol/zymosterol/geranylgeranyldiphosphate biosynthesis and LXR/RXR activation) that were not affected by the MnO₂ particles. For the Fe₂O₃ particles, there was more variety with respect to the significant pathways induced by each substance at 24h. While they both perturbed some of the same inflammatory/developmental and lysosome (CLEAR signaling) related pathways, Fe₂O₃ NPs altered lipid related pathways (cholesterol/zymosterol biosynthesis and LXR/RXR activation) and some inflammatory/developmental related pathways that were not significantly enriched by Fe₂O₃ MPs. Fe₂O₃ MPs also induced phagosome, microautophagy, and xenobiotic metabolism related pathways that were not significantly altered by Fe₂O₃ MPs.

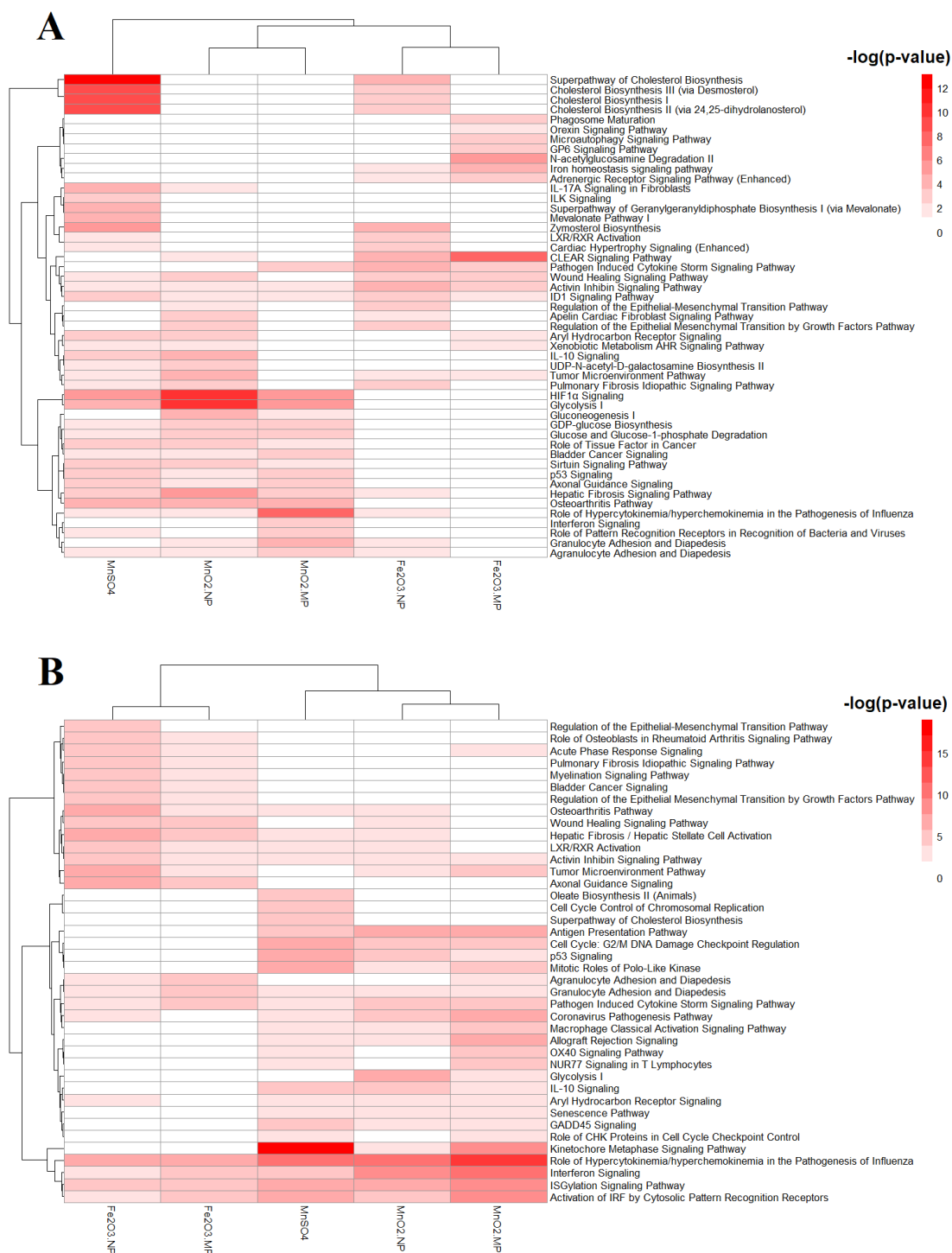


Figure 13. Top 15 perturbed canonical pathways after 24h (A) and 48h (B) of exposure to Manganese (IV) oxide (MnO₂) NPs/MPs and Iron (III) oxide (Fe₂O₃) NPs/MPs) at 100 µg/mL, and Manganese (II) sulfate (MnSO₄) at 15 µg/mL. The top 15 perturbed canonical pathways were considered significantly perturbed if they had a minimum of 3 DEGs and a -log(p-value) ≥ 1.3. The dendrograms on the columns and rows represents the Euclidean distance via complete linkage. Canonical pathways below the cutoff were coloured white.

At 48h, the significantly perturbed pathways were most similar among particles with the same chemical composition and constituent metal; the groups [MnO₂ NP, MnO₂ MP, and MnSO₄] and [Fe₂O₃ NP and Fe₂O₃ MP] tended to enrich similar pathways (Figure 13). MnSO₄ altered similar pathways to the MnO₂ particles and resembled them more closely than it did at 24h. The MnO₂ particles and MnSO₄ enriched pathways related to inflammatory and cell cycle/DNA damage processes (MnO₂ MPs and MnSO₄ were often slightly more potent than the MnO₂ NPs in perturbing common pathways). Pathways related to cell cycle/DNA damage were more perturbed after exposure to MnSO₄ than MnO₂ NP and MnO₂ MP. MnSO₄ also enriched certain lipid related pathways (cholesterol/oleate biosynthesis) that the MnO₂ particles did not. The Fe₂O₃ NPs and MPs had a distinct pathway perturbation profile that was fairly consistent between them - many of the same pathways associated with immune/inflammatory response and cellular development were affected by both of them. However, the Fe₂O₃ NPs altered more development-related pathways than the Fe₂O₃ MPs, while the Fe₂O₃ NPs altered more inflammatory-related pathways (each by a small amount).

The most common immune/inflammatory response pathways among all particles at the 100 µg/mL concentration after 48h were: 'Role of Hypercytokinemia/hyperchemokineemia in the Pathogenesis of Influenza', 'ISGylation Signaling Pathway', 'Interferon Signaling', 'Activation of IRF by Cytosolic Pattern Recognition Receptors', and 'Pathogen Induced Cytokine Storm Signaling Pathway'. There was a significant amount of overlap among DEGs associated with each immune/inflammatory response pathway across all particles at the highest concentration at 48h.

Cell cycle/DNA damage related pathways were also significantly perturbed at the 100 µg/mL concentration after 48h, such as 'Kinetochore Metaphase Signaling Pathway', 'Cell

Cycle: G2/M DNA Damage Checkpoint Regulation', 'Mitotic Roles of Polo-Like Kinase', 'p53 Signaling', 'Senescence Pathway', and 'Cell Cycle Control of Chromosomal Replication'. Only the MnO₂ NPs, MnO₂ MPs, and MnSO₄ treatment groups induced perturbations in these cell cycle/DNA damage related pathways. There was a significant amount of overlap in the DEGs found in these pathways among the MnO₂ NPs, MnO₂ MPs, and MnSO₄ treatments.

Gene Expression and Pathway Analysis of CuO NPs, MPs, and CuCl₂

Transcriptomic data for CuO NPs, CuO MPs, and CuCl₂ was originally published by Boyadzhiev *et al.* (2021). The number of upregulated and downregulated genes for CuO NPs and MPs along with CuCl₂ are summarized in Figure 14. There were substantially more DEGs induced by CuO NPs than CuO MPs at all the concentrations tested. CuCl₂ induced fewer DEGs than the concentration induced by CuO NPs, but more than that induced by CuO MPs. The CuO NPs and CuCl₂ at 48h induced a comparable number of upregulated and downregulated DEGs, while the DEGs induced by the CuO MPs and CuCl₂ at 24h were slightly more downregulated. The CuO NPs produced a concentration-dependent response at both timepoints. After 24h, 1, 147, 1759, and 4044 DEGs were induced from the 1 µg/mL concentration to the 25 µg/mL concentration. After 48h, 13, 3731, and 7212 DEGs were induced from the 1 µg/mL concentration to the 10 µg/mL concentration. There were no DEGs following exposure to the 25 µg/mL concentration at 48h because it was too cytotoxic for the transcriptional response to be assessed. The CuO MPs induced few DEGs: 1, 2, and 1 DEGs from the 5 µg/mL concentration to 25 µg/mL concentration after 24h, and 1, 1, 4, 3 from the 1 µg/mL to the 25 µg/mL concentration after 48h. The 7 µg/mL concentration of CuCl₂ induced 5 DEGs at 24h and 714 DEGs at 48h.

The CuO NPs induced many more DEGs than MnO₂ and Fe₂O₃ NPs at lower concentrations. However, the CuO MPs induced fewer DEGs than the MnO₂ and Fe₂O₃ MPs over the concentrations tested. Overall, there was a much larger difference between the number of DEGs induced by the NP and MP forms for CuO than for MnO₂ and Fe₂O₃. The concentration of CuCl₂, which represented 2.64x the predicted extracellular Cu expected to be released from 10 µg/mL of CuO NPs, did not induce a response comparable to that of the CuO NPs (it was much smaller). This indicates that despite being highly soluble, the toxicity induced by CuO NPs cannot be explained by their solubility alone. Conversely, the largest MnSO₄ treatment, which represented the equivalent amount of Mn ions to what would be released in 239.8 µg/mL MnO₂ NP treatments, induced a similar number of DEGs to the 100 µg/mL concentration of MnO₂ NPs after 48h.

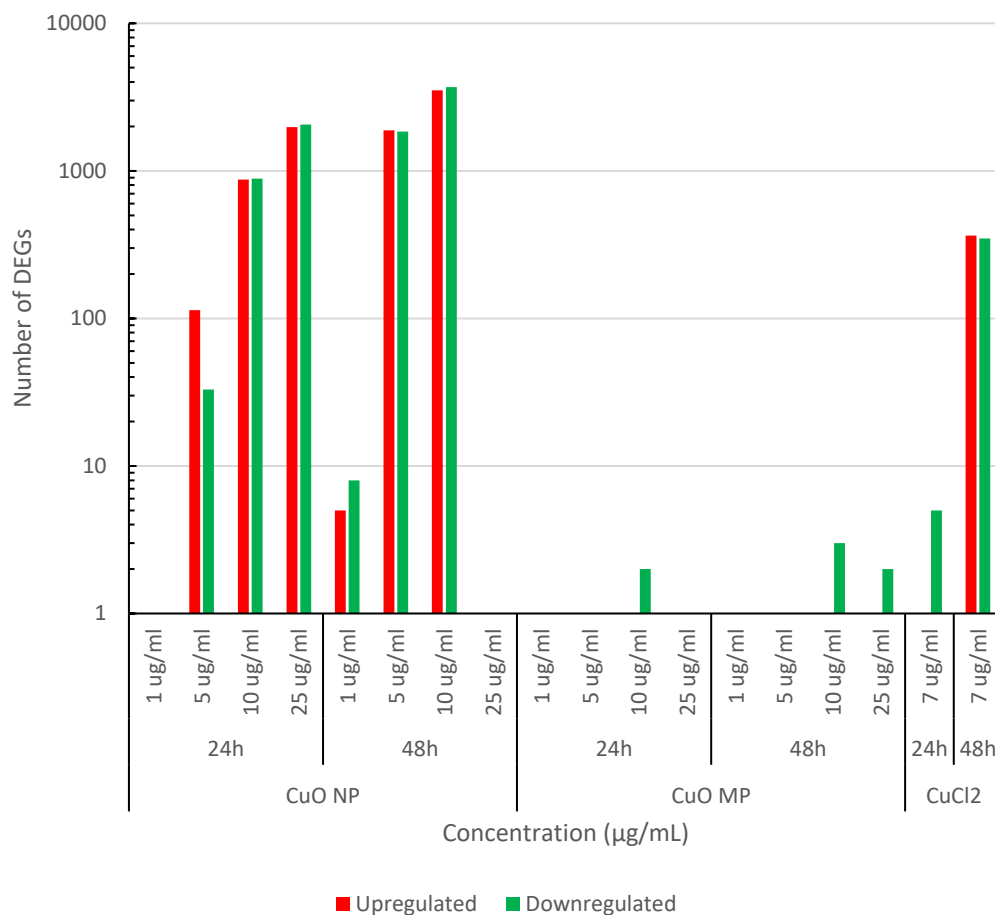


Figure 14. Number of differentially expressed genes (DEGs) following exposure to 1, 5, 10, and 25 µg/mL of Copper (II) oxide (CuO) NPs and MPs, and 7 µg/mL of Copper (II) chloride (CuCl₂) for 24h and 48h. The 7 µg/mL CuCl₂ concentration represents an amount of Cu ions that is 2.64 times the predicted extracellular Cu expected to be released from 10 µg/mL of CuO NPs based on 48h dissolution experiments. DEGs were defined as genes with a fold change $\pm > 1.5$ and a false discovery rate (FDR) of ≤ 0.05 ($n = 3$). Red shows upregulated DEGs while green shows downregulated DEGs.

Canonical Pathway Analysis

The number of significantly perturbed canonical pathways associated with DEGs following treatment with the CuO NPs and CuCl₂ is shown in figure 15. There were no perturbed pathways following exposure to CuO MPs. In general, the CuO NPs perturbed pathways related to hypoxia/oxidative stress, cell cycle/DNA damage, and cellular/tissue development at both 24h and 48h. Exposure to CuCl₂ primarily enriched pathways related to hypoxia/oxidative stress, metabolism, cell cycle/DNA damage. This differs from the response observed following exposure to the MnO₂ and Fe₂O₃ particles and MnSO₄, where many immune/inflammatory pathways were perturbed, particularly at 48h.

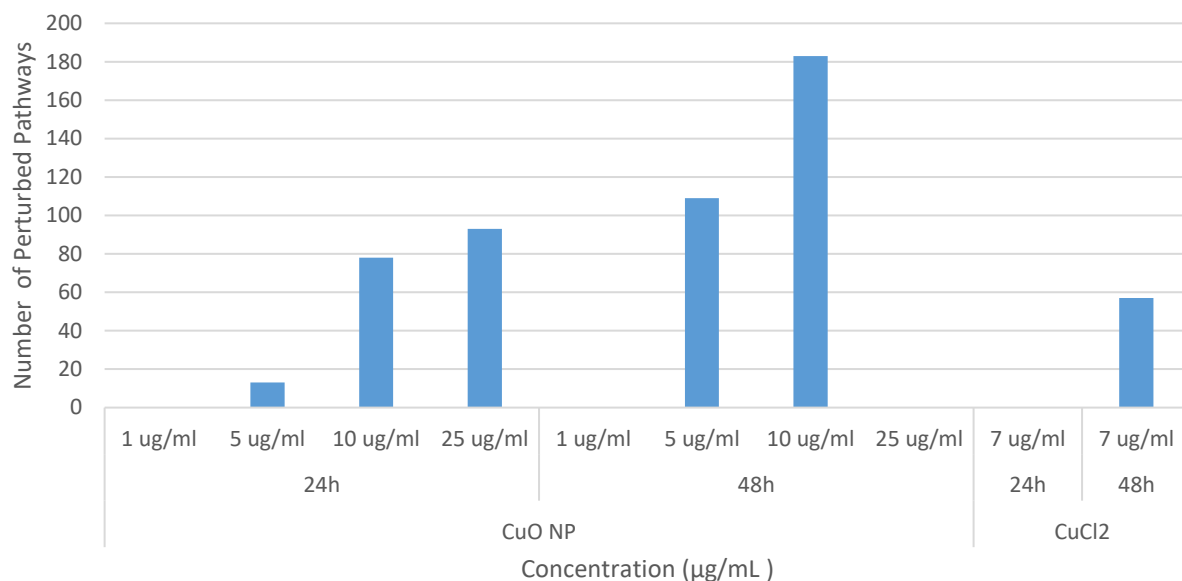


Figure 15. Number of significantly perturbed canonical pathways following exposure to 1, 5, 10, and 25 µg/mL of Copper (II) oxide (CuO) NPs and 7 µg/mL of Copper (II) chloride (CuCl₂) for 24h and 48h. The 7 µg/mL of CuCl₂ is equivalent to. The bars in the graph represent the number of perturbed canonical pathways with a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$.

Comparing the overlapping perturbed pathways and their respective Jaccard indexes provides insight into which particles may behave similarly (Figure 16). The MnO₂ NPs, MnO₂ MPs, and MnSO₄ had the greatest numbers of overlapping pathways and the highest Jaccard indexes at both timepoints, indicating that their responses are the most similar. Many of the shared pathways were involved in the immune/inflammatory response and cell cycle/DNA damage. The Fe₂O₃ NPs and Fe₂O₃ MPs showed a lot of overlap with each other and had a relatively high Jaccard index at both timepoints (more dramatically at 48h). Interestingly, CuO NPs, which are highly soluble, did not have as much overlap in pathway perturbations (based on the intersection size or Jaccard index) as the MnO₂ NPs, MnO₂ MPs, and MnSO₄ or the Fe₂O₃ NPs and Fe₂O₃ MPs, respectively.

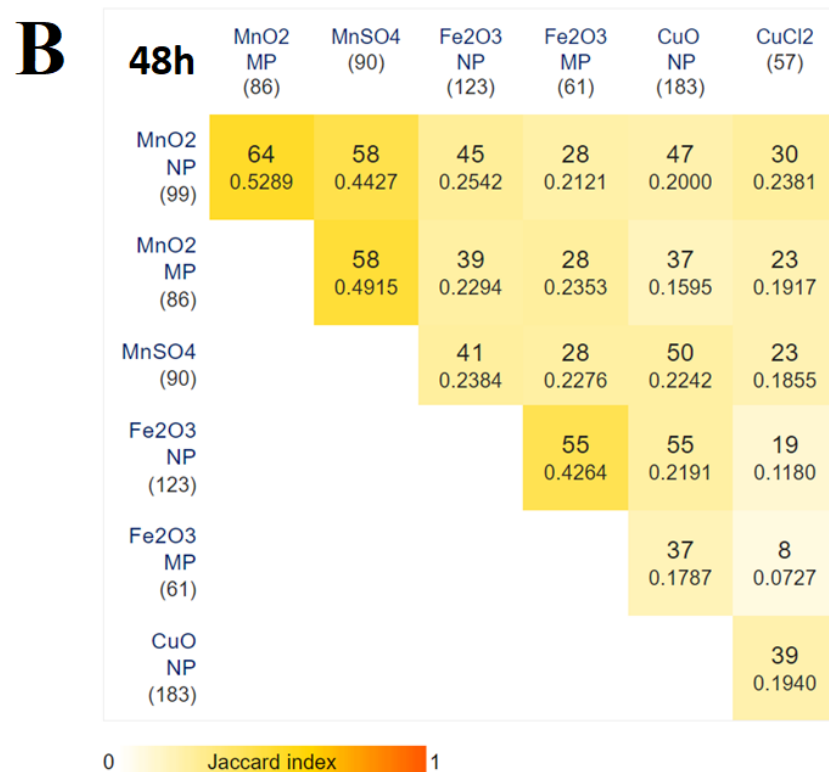
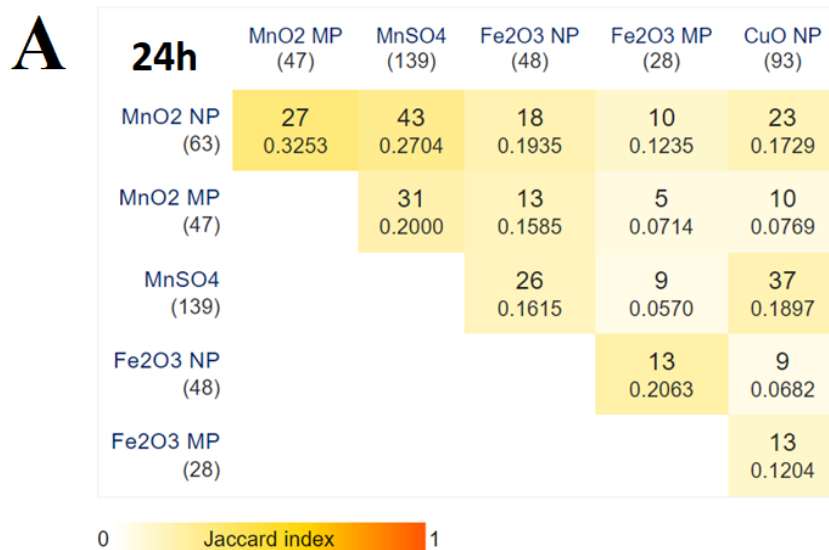


Figure 16. Diagram showing the number of overlapping perturbed pathways in each condition after 24h (A) and 48h (B) of exposure to Manganese (IV) oxide (MnO₂) NPs/MPs and Iron (III) oxide (Fe₂O₃) NPs/MPs at 100 µg/mL, Manganese (II) sulfate (MnSO₄) at 15 µg/mL, Copper (II) Oxide (CuO) NPs at 10 µg/mL, and Copper (II) Chloride (CuCl₂) at 7 µg/mL. The Jaccard index is shown below the number of overlapping perturbed pathways.

The CuO NPs did not group with the other particles at 24h using hierarchical clustering (there were no significantly perturbed pathways induced by CuO MPs or CuCl₂ after 24h) (Figure 17). The pathways enriched by the CuO NPs were primarily related to cell cycle/DNA damage, and to a lesser extent oxidative stress/autophagy/lysosomal regulation/phagosome at 24h. Similarly at 48h, the CuO NPs and CuCl₂ formed a cluster apart from the other particles (CuO MPs did not significantly alter any pathways at 48h) (Figure 17). The CuO NPs primarily enriched pathways related to cell cycle/DNA damage, xenobiotic metabolism, and oxidative stress while CuCl₂ primarily perturbed cell cycle/DNA damage and some autophagy related pathways at 48h. In general, the CuO particles/ions differed from the MnO₂ and Fe₂O₃ particles/ions in that many fewer immune response/inflammation related and development related pathways were perturbed.

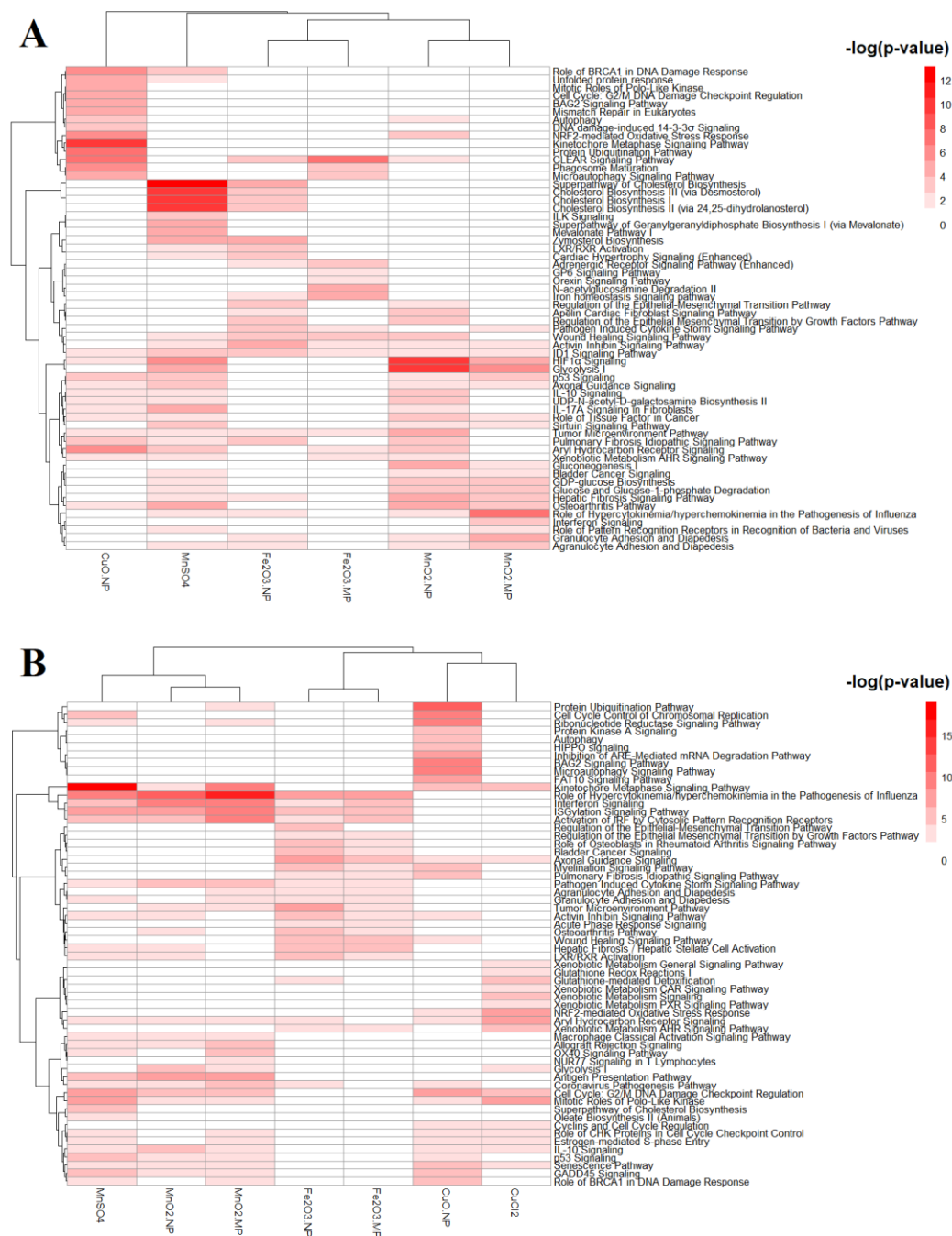


Figure 17. Top 15 perturbed canonical pathways after 24h (A) and 48h (B) of exposure to Manganese (IV) oxide (MnO₂) NPs/MPs and Iron (III) oxide (Fe₂O₃) NPs/MPs at 100 μ g/mL, Manganese (II) sulfate (MnSO₄) at 15 μ g/mL, Copper (II) Oxide (CuO) NPs at 10 μ g/mL, and Copper (II) Chloride (CuCl₂) at 7 μ g/mL. The top 15 perturbed canonical pathways were considered significantly perturbed if they had a minimum of 3 DEGs and a $-\log(p\text{-value}) \geq 1.3$. The hierarchical clustering of the columns and rows was generated using the Euclidean distance via complete linkage. Canonical pathways below the cutoff are shown in white.

Discussion

I evaluated the toxicity of two MONPs (MnO_2 NPs and Fe_2O_3 NPs), their respective MP counterparts (MnO_2 MPs and Fe_2O_3 MPs), and a dissolved fraction of Mn ions (MnSO_4) in mouse lung epithelial cells to characterize their response and examine the influence of size and solubility on the toxicity. Cell viability experiments were conducted to examine the cytotoxicity of the MONPs and determine treatment concentrations that did not cause overt cell death. I assessed DNA damage (DNA strand breaks and micronucleus formation) to determine the genotoxic potential of the MONPs and to investigate the role of size and solubility in this outcome. To identify underlying mechanisms involved in the toxicity induced by the MONPs, I characterized transcriptional responses and compared profiles based on chemical composition, size, and solubility.

Cytotoxic effects of the MONPs

The toxicity of MnO_2 and Fe_2O_3 NPs and MPs is believed to result from particle interactions with the cellular membrane and internal cellular components as well as the dissolved fraction of ions (which can be modulated by physicochemical properties). Inflammation and oxidative stress are thought to be the main contributors to their toxicity (Valdiglesias *et al.*, 2015; Frick *et al.*, 2011). However, the influence of size and relative contributions of the particle and ion fractions to the toxicity induced by MnO_2 and Fe_2O_3 NPs have not been particularly well studied and is not fully understood.

I found that the overall levels of cytotoxicity were low using the trypan blue assay at the concentrations tested. I used this assay because many MONPs interfere with common enzymatic techniques such as the LDH and MTT (Han *et al.*, 2011; Andraos *et al.*, 2020). My results

showed that all cells treated with particles had viabilities greater than 87.6% from 24-48 hours post-exposure. Only the highest concentration (100 $\mu\text{g/mL}$) of MnO_2 NPs (at 48h) caused a statistically significant reduction in cell viability (and only to 95.7%). Only the two highest concentrations of Fe_2O_3 NPs (50 and 100 $\mu\text{g/mL}$) at 24h significantly reduced cell viability (to 93.6% and 89%, respectively). Treatment with MnO_2 MPs, Fe_2O_3 MPs, and MnSO_4 did not reduce cell viability.

In contrast, the % relative survival from the microflow assay for MnO_2 NPs showed significant decrease at all concentrations tested. I also found a concentration dependent decrease in % relative survival following exposure to MnO_2 MPs (although to a lesser degree than the MnO_2 NPs), with the top two concentrations (10 and 20 $\mu\text{g/mL}$) being statistically significant. Although the % relative survival decreased following MnSO_4 exposure, it was not statistically significant. Exposure to Fe_2O_3 NPs and Fe_2O_3 MPs significantly decreased the % relative survival at the highest concentrations (40 $\mu\text{g/mL}$). It is worth noting differences in the concentration used for two particles and their individual forms. For example, the concentrations of Fe_2O_3 particles (5, 10, 20, 40 $\mu\text{g/mL}$) used for the microflow assay were double those of the MnO_2 particles (2.5, 5, 10, 20 $\mu\text{g/mL}$) and MnSO_4 dissolved ion equivalent (0.9, 1.8, 3.6, and 7.2 $\mu\text{g/mL}$). The concentrations for all particle treatments used were higher (10, 25, 50, 100 $\mu\text{g/mL}$) in the trypan blue assay. Additionally, the microflow assay was performed only at 40h post-exposure, while the trypan blue assay was performed at 24h and 48h.

The overall trends in the microflow assay for the particles were fairly consistent with the trypan blue assay results, albeit with higher sensitivity. The MnO_2 NPs caused reductions in cell viability/% relative survival in both the trypan blue and microflow assays (although to a greater extent in the microflow assay). MnSO_4 was not cytotoxic in either experiment, while MnO_2 MPs

were cytotoxic in the microflow assay but not the trypan blue assay. Fe₂O₃ NPs induced cytotoxicity at the highest concentration in both assays, while Fe₂O₃ MPs did not reduce cell viability in the trypan blue assay but did significantly decrease the % relative survival in the microflow assay. Given that each experiment measured a different metric (membrane integrity for the trypan blue assay and a ratio of nuclei to counting beads in the microflow assay) and were performed using different concentrations, it is unsurprising that there are some differences in the results between the two techniques.

The literature shows that MnO₂ NPs are generally more cytotoxic than Fe₂O₃ NPs, which is consistent with the results observed in my experiment (Karlsson *et al.*, 2009; Karlsson *et al.*, 2008; Oliveira *et al.*, 2019; Valdiglesias *et al.*, 2015). However, the literature is not consistent as positive and negative results have been produced for both MnO₂ NPs and Fe₂O₃ NPs with respect to cytotoxicity. A few studies have systematically compared the toxicity induced by MnO₂ NPs and Fe₂O₃ NPs while taking into account size and solubility. My trypan blue and microflow assay results show that NPs generally induce more cytotoxicity than their MP counterparts, which supports the idea that smaller sized particles usually induce greater levels of toxicity than larger particles. Only the Fe₂O₃ NPs in the microflow assay didn't show greater cytotoxicity than its respective MP (Fe₂O₃ NP and MP are much closer in size than the MnO₂ NP and MP). The lower cytotoxicity observed following treatment with the MPs may be due to less efficient uptake and internalization. Lung epithelial cells efficiently internalize particles up to 3 µm *in vitro*, which is smaller than the MnO₂ MPs used in this study (average in the 4 µm range) (Foster *et al.*, 2001). Thus, my results suggest that size does play a role in the response induced in lung epithelial cells; however, it isn't the sole property responsible for the observed effects.

My results also suggest that the solubility of MnO₂ NPs may not contribute to their cytotoxic response, as the MnSO₄ treatments (which release Mn²⁺ ions) did not induce any cytotoxicity. However, given that the uptake mechanisms of particles and ions differ, perhaps the ions were unable to enter the cells as quickly/efficiently as the particles. That could explain the lack of response. It is possible that a longer exposure time with MnSO₄ would result in a cytotoxic response more similar to the MnO₂ NPs. Polar molecules such as ions are unable to cross the plasma membrane on their own and must rely on specialized membrane-transport protein channels, whereas NPs can be internalized through endocytosis upon contact with the cell membrane and deposited in endolysosomes (Verma & Stellacci, 2010). Frick *et al.* (2011) observed that the uptake of Mn₃O₄ NPs was greater than MnSO₄ (at corresponding equivalent concentrations to the Mn₃O₄ NPs) over the same periods of time (4h and 24h) in rat alveolar epithelial cells (resulting in low levels of intracellular Mn that were difficult to detect following MnSO₄ exposure). While MnO₂ NPs are poorly soluble (only a small fraction of Mn ions released) and Fe₂O₃ NPs are insoluble in water and biological media, they are efficiently internalized by the cell and are able to dissolve in environments with low pH such as lysosomes. The dissolution into toxic ions may destabilize/damage the lysosomal membrane, which can result in the release of its contents into the cytoplasm. Known as the Trojan horse-type mechanism, the toxicity is driven by overwhelming the antioxidant defenses of the cell and inducing oxidative stress (Stark, 2011; Nordberg and Costa, 2022). A mixture of Mn₃O₄, Mn₂O₃, and MnO NPs (40 µg/mL in artificial lysosomal fluid resulted in 60% of particles dissolving after 24h) and Fe₂O₃ NPs (100 µg/mL in simulated pulmonary fluid pH= 3.0 reached a dissolution degree of 2.33% after 24h) dissolved to a greater extent in acidic biological fluid simulated solutions than to those with a neutral pH (Stebounova *et al.*, 2018; Godymchuk *et al.*,

2022). Thus, these results suggest that given the poorly soluble or insoluble nature of MnO NPs and Fe₂O₃ NPs, solubility may not be the factor influencing the responses observed in my study.

CuO NPs, which are much more soluble than MnO₂ and Fe₂O₃ NPs, are far more cytotoxic at lower concentrations. CuO NP concentrations (45.9 nm average length and 64.8 nm average width) of 10 and 25 µg/mL significantly decreased cell viability to 71.4% and 13.1% at 48h, while equivalent concentrations of CuO MPs did not result in any viability reduction using FE1 cells and the trypan blue assay (Boyadzhiev *et al.*, 2021). A 7 µg/mL concentration of CuCl₂, which represents 2.64x the predicted extracellular Cu expected to be released from 10 mg/mL CuO NPs based on 48 h particle dissolution experiments, did not reduce cell viability. Karlsson *et al.* (2008) treated A549 cells with a variety of MONPs and CuO NP (20-40 nm). Concentrations of 40 and 80 µg/mL were the most cytotoxic (>90% non-viable cells) using the trypan blue assay at 18h. An 80 µg/mL Fe₂O₃ NP (30-60 nm) treatment at the same timepoint resulted in low cytotoxicity (6% non-viable cells) and other insoluble MONPs (Fe₃O₄ and TiO₂ NPs) were no different than the control. The influence of particulate CuO and Cu ions on cytotoxicity was also compared over a concentration range of 6-50 µg/mL for CuO NPs and 11-85 µg/mL of CuCl₂ (which represents equivalent concentrations to the CuO NPs). The CuO NPs produced a much steeper linear dose response curve than CuCl₂; at the top concentration, CuO NP exposure resulted in ~90% non-viable cells, while CuCl₂ resulted in ~12.5% non-viable cells. In a separate study, CuO MPs caused cytotoxicity (31% non-viable cells) to a much smaller extent than CuO NPs (96% non-viable cells) (Karlsson *et al.*, 2009). The NP form appears to be particularly important in inducing cytotoxicity following treatment with CuO. The difference in cytotoxicity among the NP, MP, and ion for MnO₂ and Fe₂O₃ was much less pronounced. These results suggest that in addition to size and solubility, the chemical composition of metal oxides

and the mechanisms by which they are internalised by the cells are the other factors that contribute to the cellular responses to exposures.

Genotoxic Effects of the MONPs

Statistically significant increases in DNA damage based on the comet assay only occurred at the highest concentration (100 µg/mL) of MnO₂ NPs at 48h (29% DNA in the tail) and the highest concentration (100 µg/mL) of Fe₂O₃ NPs at 24h (5.9% DNA in the tail). The MnO₂ MPs displayed a dose response at both timepoints but were not significant. The Fe₂O₃ MPs and MnSO₄ did not significantly differ from the control at any concentration or timepoint. Overall, the Fe₂O₃ particles and MnSO₄ had a much smaller percentage of DNA in the comet tails than the MnO₂ particles.

The microflow and comet assay results were generally concordant, with increased sensitivity using the microflow assay. The frequency of micronuclei significantly increased after treatment with 5 and 10 µg/mL of MnO₂ NPs and the highest concentration (20 µg/mL) of MnO₂ MPs. Fe₂O₃ NPs, MPs, and MnSO₄ exposure did not induce micronuclei formation. Based on both assays, MnO₂ NPs were the most potent particle at inducing DNA damage/micronuclei, followed by MnO₂ MPs. The Fe₂O₃ MPs and MnSO₄ did not cause genotoxicity in either assay, while the Fe₂O₃ NPs were negative in the comet assay but caused an increase in micronucleus frequency. The concentrations of Fe₂O₃ particles (5, 10, 20, 40 µg/mL) used for the microflow assay were double those of the MnO₂ particles (2.5, 5, 10, 20 µg/mL) and MnSO₄ ion equivalent (0.9, 1.8, 3.6, and 7.2 µg/mL), although all concentrations used in the microflow assay were less than those used in the comet assay (5, 10, 25, 50, 100 µg/mL). It has been established that the micronucleus assay is more sensitive than the comet assay as it consistently detects genotoxicity at lower concentrations for the same chemicals (Kawaguchi *et al.*, 2010; Le Hégarat *et al.*, 2014).

DNA strand breaks may be repaired, resulting in a lower amount of damage observed in the comet at these early timepoints (Kawaguchi *et al.*, 2010). However, my research shows that there are sufficient DNA strand breaks to be converted into micronuclei, which are permanent, irreversible changes in chromosome structure. If the level of double strand breaks (DSBs) exceeds the DNA repair capacity of the dividing cell within a specified time frame, chromosome/chromatid fragments may arise and lead to the formation of micronuclei (Fenech *et al.*, 2011; Luzhna *et al.*, 2013). The misrepair of DSBs is primarily due to defects in the homologous recombination DNA repair pathway and/or defective enzymes involved in the non-homologous end joining pathway. Simultaneous excision repair of damaged and/or inappropriately incorporated bases on complementary strands in close proximity may also give rise to DSBs and micronucleus formation, particularly if the gap-filling step is not completed (Fenech *et al.*, 2011). Micronucleus formation could also be the result of disrupted actin cytoskeletal transport system *in vitro*. Whole chromosomes can be malsegregated at anaphase due to defects in kinetochore and microtubule interaction dynamics, resulting in MN formation (Fenech *et al.*, 2011; Luzhna *et al.*, 2013).

Based on the literature, MnO₂ NPs are generally more potent than Fe₂O₃ NPs with respect to genotoxicity. While the literature for MnO₂ NPs consistently demonstrates DNA damage (Alarifi *et al.*, 2017; Hain *et al.* 2012; Singh *et al.*, 2013), Fe₂O₃ NPs have both produced relatively low positive (Karlsson *et al.*, 2009; Könczöl *et al.* 2011; Ahmed *et al.*, 2013) and negative (Karlsson *et al.* 2008) results. Unfortunately, there are few studies comparing the influence of size and solubility on the genotoxicity of MnO₂ and Fe₂O₃. With respect to size, Singh *et al.* (2013) observed that MnO₂ NPs induce genotoxicity (comet and micronucleus assays) in Wistar rats following oral exposure to 300 and 1000 mg/Kg bw per day over 28 days

while MPs only induced genotoxicity at 1000 mg/Kg bw per day. Karlsson *et al.* (2009) found that Fe₂O₃ and Fe₃O₄ NPs and MPs cause toxicity in A549 cells (comet assays) but with no clear difference in toxicity between particle sizes (in contrast to CuO, where the NPs are much more potent than the MPs). Other studies confirm that CuO NPs are much more potent than CuO MPs and their ionic Cu equivalent (Boyadzhiev *et al.*, 2022; Siivola *et al.*, 2020). In alignment with the literature, my results support that MnO₂ is more potent than Fe₂O₃ in terms of genotoxicity. With respect to size, the NPs are more potent than the MPs for both MnO₂ and Fe₂O₃. Reasons for the difference could be higher uptake and internalization of NPs or greater ability to dissolve once taken up by the cell and trafficked to the lysosome. Additionally, I found that the MnSO₄ did not induce any genotoxicity, indicating that the solubility of MnO₂ NPs is not a large factor in its genotoxic response. However, there may be differing uptake mechanisms between ions and particles – perhaps an exposure time longer than 4h for the comet assay and longer than 40h for the microflow would result in DNA damage levels closer to those of MnO₂ NPs and MPs.

The genotoxicity induced by NPs and their toxic ions are thought to arise through several mechanisms, including direct interaction with DNA and/or DNA repair/maintenance machinery after crossing the nuclear membrane, during mitosis when the nuclear membrane dissolves, and indirect damage from ROS generated by activated inflammatory cells or from the surface of NPs (Magdolenova *et al.*, 2014). The previously mentioned Trojan horse-type mechanism, which involves the uptake and dissolution of NPs in the lysosome followed by membrane damage and the release of the contents into the cytoplasm, is a potential mechanism for MnO₂ and Fe₂O₃ induced genotoxicity (Stark, 2011; Nordberg and Costa, 2022). Many studies have reported increased inflammatory markers and inflammation *in vivo* following exposure to Fe₂O₃ NPs and MnO₂ NPs, indicating that ROS induced by inflammation may be a large contributor to the

observed genotoxicity (Pettibone *et al.*, 2008; Hadrup *et al.*, 2020; Sárközi *et al.* 2009; Singh *et al.*, 2013). Certain NPs including Fe₂O₃ have high binding affinities for DNA (determined using modeling [Derjaguin–Landau–Verwey–Overbeek theory] and confirmed using atomic force microscopy) and been demonstrated to interfere with DNA replication (using a PCR based DNA replication assay) (Li *et al.*, 2013). However, given that the size of the MnO₂ and Fe₂O₃ NPs was ~30nm, it is unlikely that they crossed the nuclear membrane and interacted with the DNA directly (Barua and Mitragotri, 2014). It is more likely that the genotoxicity was caused by ROS induction via inflammation, interaction with DNA during mitosis when the nuclear membrane was dissolved, or interfered with DNA maintenance machinery.

Transcriptomics and comparison to CuO NPs, MPs, and CuCl₂

I used genome wide transcriptional profiling to gain a better understanding of the relative influence of size and solubility on cellular toxicity. My results support that NPs have a greater effect on gene expression than MPs of the same chemical composition. Specifically, the MnO₂ NPs exhibited the largest number of DEGs at both timepoints and all concentrations, the MnO₂ MPs and Fe₂O₃ NPs altered the expression of a comparable number of DEGs, and the Fe₂O₃ MPs had the fewest DEGs. This size-dependent transcriptional response observed is consistent with previously published studies that have suggested that particle size is a major contributor to their biological response following exposure to Fe and Mn oxide NPs (Singh *et al.*, 2013; Máté *et al.*, 2016; Wu *et al.*, 2022). There was significant overlap between the DEGs induced by the NPs and MPs for MnO₂ and Fe₂O₃ at both timepoints, although the NPs had a much larger number of unique DEGs than the MPs particularly after 48h (largely due to having induced a greater number of DEGs than the MPs). The DEGs induced by the particles clustered based on constituent metal and timepoint (MnO₂ NP, MP and MnSO₄ formed a group; Fe₂O₃ NP and MP

formed a separate group), indicating that chemical composition was an important factor in the transcriptional response. The Fe₂O₃ NP and MP induced DEGs with the highest fold changes were often split between upregulation and downregulation, whereas those induced by the MnO₂ NPs and MPs were only upregulated. Few studies have examined the transcriptomic changes induced by MnO₂ NPs and Fe₂O₃ NPs. A study that investigated the transcriptional response of MnO₂ NPs (up to 400 mg/kg bodyweight) in the choroid plexus of rats via intratracheal injection also saw a general upregulation of DEGs (Memg *et al.*, 2023). However, another study that examined the transcriptional response to MnCl₂ (up to 100 µM) in human neuroblastoma cells found that DEGs were primarily downregulated following exposure (Fernandes *et al.*, 2019). Zhang and colleagues (2015) exposed THP-1 and Hep2G cells to Fe₃O₄ NPs and observed a general upregulation of DEGs in THP1 cells while there was a fairly even split between upregulation and downregulation in Hep2G cells. The differences in expression highlight the cell/tissue type dependent responses of MONPs.

Exposure to MnO₂ NPs, MPs, and dissolved Mn ions induced similar downstream pathways. The pathways commonly perturbed by MnO₂ NPs, MPs, and dissolved Mn ions at 24h were primarily related to energy/metabolism and hypoxia, although some immune/inflammatory response, developmental, and p53 signaling pathways were also altered. The treatments also shared many of the same upregulated DEGs related to hypoxia such as ‘MGARP’, ‘BNIP3’, ‘*HIGD1A*’. The Mn ions also enriched additional pathways related to lipid metabolism (‘cholesterol/zymosterol/geranylgeranyldiphosphate biosynthesis’ and ‘LXR/RXR activation’) that the particles did not. At 48h, the transcriptomic response following exposure to MnO₂ NPs, MPs, and dissolved Mn ions were more similar. Common altered pathways included immune/inflammatory response and cell cycle/DNA damage pathways. Many of the same

immune/inflammatory related DEGs, such as 'OAS1', 'OAS2', 'OAS12', 'IFIT1B', and 'USP18' were also upregulated by each of the treatments. The overall fold change of the DEGs was largest after treatment with the MnO₂ NPs, followed by the MnO₂ MPs, and MnSO₄. This aligns with the results of the cytotoxicity and genotoxicity assays where MnO₂ NPs were the most toxic and MnSO₄ was the least toxic. Very few studies have examined the transcriptomic response to MnO₂ NPs. In a study by Memg and colleagues (2023), the expression of transporters, ion channel proteins, and ribosomal proteins in the choroid plexus of rats via intratracheal injection were significantly affected by MnO₂ NPs (up to 400 mg/kg bodyweight; treated once a week for 3 months). There was also some evidence of inflammation as the Th17 cell differentiation pathway was identified following KEGG pathway enrichment. While the MnO₂ NPs primarily induced inflammation in FE1 cells, they also affected a variety of transporters and ions channels such as (*APOD*, *KCNBI*, *PCLO*, *TAPI*, *TFRC*, and *SLC25A12*). The different dosage, cell/tissue type, and *in vivo* nature of the Memg study contributes to the differences to the results of my work.

The Fe₂O₃ NPs and MPs also produced similar transcriptional profiles to one another. At 24h, Fe₂O₃ NPs and MPs both primarily altered immune/inflammatory response and developmental pathways. The Fe₂O₃ NPs enriched lipid related pathways (cholesterol/zymosterol biosynthesis and LXR/RXR activation) and some inflammatory/developmental related pathways that were not enriched by Fe₂O₃ MPs. Fe₂O₃ MPs perturbed some vesicular trafficking (phagosome and microautophagy) and xenobiotic metabolism related pathways that Fe₂O₃ NP did not. Similarly to the Mn particles, the Fe₂O₃ NP and MP primarily induced immune/inflammatory response pathways at 48h along with cellular development pathways. The overall fold changes of the DEGs had slightly larger magnitudes after treatment with the Fe₂O₃

NPs as compared to the Fe₂O₃ MPs. This is consistent with the results from the cytotoxicity and genotoxicity assays as their results were fairly similar. The Fe₂O₃ NPs and MPs being much closer in size than the MnO₂ NPs and MPs may account for their more similar responses. In a study examining the transcriptomic effects of DMSA-coated Fe₃O₄ NPs (50 and 100 µg/mL at 24h), other immune response related genes were most significantly induced in THP-1 cells (*Cxcl13*, *Aamdec1*, *Ebi3*, *Ifi44l*, *Clec7a* and *Ly96*) and HepG2 cells (*Ifi27*, *Ifi6l*, and *Tagln*) (Zhang *et al.*, 2015). While not all these genes were induced after exposing in the FE1 cells to Fe₂O₃ NPs, many of the most significantly induced genes were immune response related. Similarly to our results, GO analysis in the study by Zhang and colleagues (2015) revealed that inflammatory, defense, and immune response related pathways were among the most perturbed in THP-1 cells while HepG2 cells enriched a wider variety of response related biological processes. The transcription of genes related to iron homeostasis (*Tfrc*, *Trf*, *Slc40a1* and *Lcn2*) are also affected by DMSA-coated Fe₃O₄ NPs in RAW264.7 cells (up to 100 µg/mL at 4h, 24h, and 48h) (Liu and Wang, 2013). The NPs likely disturb iron homeostasis due to the release of iron ions after being taken up by the cell and trafficked to the acidic environment of the lysosome (Liu and Wang, 2013). In my study, the *Slc40a1* gene, which is responsible for transferring intracellular iron out of the cell, was upregulated by a similar level at the highest doses of Fe₂O₃ NPs and MPs (and the 50 µg/mL treatment of Fe₂O₃ NPs to a slightly lesser extent). The *LCN12* gene, which is a paralog of *LCN2* (transfers intracellular iron out of the cell), was upregulated at the highest concentration of Fe₂O₃ NPs at 48h. The *TFRC* gene, which is responsible for transferring iron into cells, was downregulated at the highest concentration of Fe₂O₃ MPs at 24h.

Transcriptomic analysis showed that several cell cycle/DNA damage pathways were perturbed following exposure to MnO₂ NPs, MPs, and MnSO₄ but not Fe₂O₃ NPs and MPs after 48h. Pathways such as ‘Kinetochore Metaphase Signaling Pathway’, ‘Cell Cycle: G2/M DNA Damage Checkpoint Regulation’, ‘Mitotic Roles of Polo-Like Kinase’, ‘p53 Signaling’, ‘Senescence Pathway’, and ‘Cell Cycle Control of Chromosomal Replication’ were among the most perturbed. This aligns with the comet and microflow assay results, which showed that the MnO₂ particles are more genotoxic than the Fe₂O₃ particles. Interestingly, MnSO₄, which enriched many of the same cell cycle/DNA damage pathways as MnO₂ NPs and MPs, did not induce any DNA damage in the comet assay or the micronucleus assay. It can perhaps be explained by the scale of the transcriptomic changes – the fold changes of DEGs in the upregulated cell cycle/DNA damage pathways were higher following exposure to MnO₂ NP and MP than after exposure to MnSO₄. MnO₂ NP and MnO₂ MP were potentially able to exceed the DNA repair capabilities of the cell as demonstrated by the comet and micronucleus assays. A difference in uptake and internalization between the particulate and ionic forms could contribute to the lower transcriptomic response observed following MnSO₄ exposure. A longer exposure could potentially result in greater changes in gene expression and a larger response in the genotoxicity tests similar to that of the MnO₂ particles.

CuO NPs, which are more soluble in biological media (12.6% at 10 µg/mL after 48h) than MnO₂ NPs (3.87% at 100 µg/mL after 48h) and Fe₂O₃ NPs (not detected), were very potent with respect to the number of DEGs induced and the magnitude of the fold change (at lower concentrations (1, 5, 10, 25 µg/mL) than the MnO₂ and Fe₂O₃ particles (25, 50, 100 µg/mL)) (Boyadzhiev *et al.*, 2019; Avramescu *et al.*, 2022). Interestingly, CuCl₂ induced an intermediate number of DEGs while CuO MPs induced very few DEGs (perhaps because of inefficient uptake

due to their large 5 μm size). This shows that the perturbations at the transcriptional level caused by CuO NPs are size-dependent, which aligns with the size-dependent toxic endpoints observed in many studies (Boyadzhiev *et al.*, 2022; Siivola *et al.*, 2020; Karlsson *et al.*, 2009). Given that the Cu ions induced a moderate transcriptional response, they are likely a component of the toxicity induced by CuO NPs. Based on previous research, CuO NPs have a higher solubility in lysosomal fluid than MnO_2 NPs, and Fe_2O_3 NPs are only slightly soluble in lysosomes (Avramescu *et al.*, 2022; Zhong *et al.*, 2017). It is possible that the higher solubility of CuO NPs in lysosomes (via the Trojan horse-type mechanism) is responsible for its greater cytotoxicity and genotoxicity due to the release of a greater amount of toxic ions than MnO_2 and Fe_2O_3 NPs. Fe_2O_3 NPs, which are the least soluble of the particles, were the least potent based on the literature and in terms of the toxic endpoints I examined. Based on these particles of differing solubility, it can be generally concluded that greater solubility in biological media and lysosomal fluid results in greater cytotoxicity, genotoxicity (based on DNA strand breaks and micronucleus formation), and transcriptional alterations.

The CuO NPs produced a unique transcriptional profile that was distinct from the MnO_2 and Fe_2O_3 particles. In general, the CuO NPs perturbed pathways related to hypoxia/oxidative stress, cell cycle/DNA damage, autophagy, and cellular/tissue development at both 24h and 48h. This differs from the MnO_2 and Fe_2O_3 particles which both highly enriched immune/inflammatory pathways after 48h. However, the MnO_2 and CuO particles share many perturbed cell cycle/DNA damage pathways (such as ‘Cell Cycle: G2/M DNA Damage Checkpoint Regulation’, ‘Kinetochore Metaphase Signaling Pathway’, ‘Role of BRCA1 in DNA Damage Response’, ‘Senescence Pathway’, and ‘p53 Signaling’) and both induced a greater amount of genotoxicity than the Fe_2O_3 particles. CuCl_2 only produced a meaningful

transcriptional response at 48h and perturbed many similar oxidative stress and cell cycle/DNA damage pathways to the CuO NPs. The weaker responses of the CuCl₂ treatments compared to the CuO NPs at 24h and 48h could be due to the differences in uptake efficiency (particle endocytosis vs specialized membrane transport channels for ions). It is possible that longer exposure times to CuCl₂ could result in a transcriptional response that is more similar to the CuO NPs. The transcriptional response to MONPs appears to be influenced by chemical composition/solubility (based on the varied responses seen in particles with different solubility and the impact of ion equivalent treatments) and size. Similar pathways are perturbed at the transcriptional level by CuO and MnO₂ NPs and their respective dissolved ions (CuCl₂ and MnSO₄), although the more soluble CuO NPs were more potent in inducing transcriptional changes than the less soluble MnO₂ NPs and insoluble Fe₂O₃ NPs. With respect to size, the NPs consistently induced more transcriptional perturbations with higher fold changes than that induced by the MPs regardless of chemical composition.

Conclusion

Summary

In conclusion, the effects of two MONPs (MnO₂ and Fe₂O₃ NPs), their bulk counterparts (MnO₂ and Fe₂O₃ MPs), and a dissolved ion equivalent (MnSO₄) were characterized in mouse lung epithelial cells to investigate the influence of size and solubility on their toxicity. The overall levels of cytotoxicity were low following the trypan blue and microflow assays. NPs induced greater levels of cytotoxicity and MPs at high concentrations and dissolved Mn ions did not appear to contribute to the cytotoxicity induced by the MnO₂ particles. MnO₂ NPs showed moderate levels of DNA damage (comet assay) and micronuclei formation (micronucleus assay).

NPs generally induced more genotoxicity than their MP counterparts and dissolved Mn ions did not appear to contribute to the genotoxicity induced by the MnO₂ particles. The MnO₂ NPs had the largest impact on gene expression in terms of number of DEGs followed by MnSO₄, Fe₂O₃ NPs, MnO₂ MPs, and Fe₂O₃ MPs. The NPs perturbed a greater number of DEGs and produced larger fold changes than the MPs. While the MnSO₄ exposure perturbed a large number of DEGs, fold changes were lower than those of the MnO₂ NPs and MnO₂ MPs. Particles with the same constituent metal perturbed similar pathways: MnO₂ NPs, MPs, and MnSO₄ primarily perturbed immune/inflammatory response and cell cycle/DNA damage pathways while Fe₂O₃ NPs and MPs generally enriched immune/inflammatory response and developmental pathways. The toxicity of the slightly soluble MnO₂ (NP, MP, and ionic forms) and insoluble Fe₂O₃ (NP and MP forms) were compared to that of the more soluble CuO (NP, MP, and ionic forms). The toxicity of the particles coincided with their solubility: CuO was most toxic, MnO₂ was less toxic, and Fe₂O₃ was the least toxic in terms of cytotoxicity, genotoxicity, and transcriptional perturbations. The transcriptomic analysis showed that the MONPs caused perturbations in molecular pathways associated with toxicological endpoints such as inflammation and genotoxicity. Overall, smaller particle size and increased solubility generally resulted in greater levels of toxicity for all endpoints tested.

Fulfillment of Thesis Objectives

Objective 1: Examine how MnO₂ and Fe₂O₃ NP size and solubility affect cytotoxicity, genotoxicity, and global transcription.

Outcome: The toxic effects induced by MnO₂ NPs, MPs, and equivalent concentrations of dissolved Mn ions (MnSO₄) as well as Fe₂O₃ NPs and MPs were compared to provide insight into the importance of size and solubility on the toxicity of each MONP. MONP size appeared to

be a large factor contributing to toxicity, as NPs generally induced a greater amount of cytotoxicity, genotoxicity, and transcriptional perturbations than the MPs for both MnO₂ and Fe₂O₃. Solubility did not appear to play a large role in Mn toxicity, because treatment with Mn ions (from MnSO₄) at concentrations close to what would be released by MnO₂ NP treatments in media did not result in significant levels of cytotoxicity or genotoxicity but did induce transcriptional alterations (although to a lesser extent than the MnO₂ NPs and MPs). However, MnO₂ NPs, which were more soluble than Fe₂O₃ NPs, did induce greater amounts of toxicity.

Objective 2: Compare how MnO₂ and Fe₂O₃ NP toxicity differs from that induced by other MONPs.

Outcome: The features of toxicity (cytotoxicity, genotoxicity, and global transcriptional perturbations) for MnO₂ and Fe₂O₃ NPs were compared to cytotoxicity, genotoxicity, and transcriptional perturbations induced by three forms of CuO (NP, MP, dissolved ion equivalent), which was more soluble than the two MONPs investigated in the present study. MnO₂ and Fe₂O₃ NPs induced slight reductions in cell viability at the highest concentrations after 48h while the MPs and MnSO₄ did not reduce cell viability. In contrast, CuO NPs induced a large reduction in cell viability at lower concentrations than MnO₂ and Fe₂O₃ NPs while CuCl₂ (similar concentration of ions released by CuO NPs) and CuO MPs do not. MnO₂ NPs and MPs were more potent than the Fe₂O₃ particles and MnSO₄ in the induction of DNA strand breaks and micronuclei formation (toxicity not explained by Mn ions released in cell medium). CuO NPs were much more potent than MnO₂ and Fe₂O₃ NPs and its MP form with respect to DNA damage induction and micronuclei formation, and CuCl₂ did not result in significant DNA damage either (toxicity not explained by Cu ions released in cell medium). CuO NPs induced the greatest number of DEGs, followed by MnO₂ NPs and Fe₂O₃ NPs. The MnO₂ NPs, MPs, and

MnSO₄ primarily perturbed pathways related to energy/metabolism/hypoxia, immune/inflammatory response, cellular/tissue development, and cell cycle/DNA damage while the Fe₂O₃ NPs and MPs enriched pathways related to lipid metabolism, immune/inflammatory response, and cellular/tissue development pathways. The CuO NPs and CuCl₂ generally altered pathways related to hypoxia/oxidative stress, cell cycle/DNA damage, autophagy, and cellular/tissue development.

Contribution to scientific knowledge

Health Canada is responsible for the assessment and management of risks associated with engineered nanomaterials. Regulators must evaluate MONPs currently in commerce in Canada to reduce the negative impacts of environmental exposures on the health of Canadians. Several MONPs, including MnO₂ and Fe₂O₃ NPs, have been identified as high priority for assessment. Information regarding the pulmonary toxicity of many MONPs is limited, and the impact of size and dissolution on MONP toxicity has not been well studied. This project characterized the *in vitro* cytotoxic and genotoxic potential of MnO₂ and Fe₂O₃ NPs/MPs and differentiated the biological effects induced by negligibly soluble/insoluble NPs from those induced by dissolved ions. Additionally, transcriptional analysis identified molecular pathway perturbations induced by both types of MONP and their dissolved ions. The results provided useful information to regulators about two data-poor MONPs and gave insight into the relative influence of size and solubility on MONP toxicity. The data generated in this project can be used for future nanomaterial risk assessments.

Future Directions

Given the lack of toxicological information and data pertaining to MnO₂ and Fe₂O₃ NPs, there are additional experiments that could be performed that would provide useful knowledge

about their toxicity. With respect to the cytotoxicity and genotoxicity assays, longer timepoints could be used as neither MnO₂ nor Fe₂O₃ NPs were especially toxic. If longer timepoints had been used, it is possible that the particles and MnSO₄ treatments would have resulted in greater levels of toxicity. Intracellular levels of metal after exposure to NPs/MPs/ions could be examined using inductively coupled plasma mass spectrometry to quantify levels of metal uptake. TEM could also be used to confirm the presence of particles and their localization inside cells, which would help with the understanding of how the particles interact with cellular systems and potentially cause adverse effects. Additionally, a larger variety of particles from different manufacturers possessing different physicochemical properties (size, shape, coatings, charge, etc.) could be examined to characterize how each property contributes to MnO₂ and Fe₂O₃ NP induced toxicity. The results of experiments involving MnO₂ and Fe₂O₃ NPs can be compared to those of other MONPs to identify trends in their toxic responses while taking into account any differences in physicochemical properties. Developments in artificial intelligence and machine learning have led to the emergence of computer based nanotoxicology evaluation models such as quantitative structure activity relationships (QSAR), which is designed to establish statistical relationships between independent variables (Ex: NP physicochemical properties) and dependent variables (toxic effects) (Xuan *et al.*, 2023; Li *et al.*, 2022). The data generated in this study could potentially be included in QSAR modeling databases in order to predict toxic effects of NPs and fill toxicity data gaps for which no experimental data is available.

Concluding Remarks

This work generated toxicological data for two data poor MONPs and showed that MONP-induced cytotoxicity, genotoxicity, and transcriptional perturbations are influenced by

their chemical composition, size, and solubility. While both size and solubility contributed to the toxic responses observed in the study, neither property alone can be attributed as the cause of toxicity. This information about the influence physicochemical properties may be of significance with respect to the potential application of read-across strategies used by regulators in future nanomaterial (MONP) risk assessments.

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