“Investigating the hazards posed by pristine and modified Copper Nanomaterials using in vitro screens combined with in vivo models”

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Abstract

Nanomaterial use has increased as the properties of materials at the nanoscale differ substantially from bulk materials. However, there is still a lack of information concerning their impact on human health and environment.

Part of this study investigated the hazard of copper oxide nanoparticles (CuO NPs), multi-wall carbon nanotubes, silicon dioxide, pigment Red 254 and cobalt coated tungsten carbide using an in vitro hepatocyte model (HepG2/C3A cell lines). Most of the work was then conducted on CuO NPs together with four Safer by Design modifications of the same NPs (either citrate, ascorbate, polyethylenimine (PEI) or polyvinylpyrrolidone coating) and on both copper amine and micronized copper formulations, investigating their potency as inhibitors of fungal growth and analysing the gene expression in vivo on inhalation and ingestion models (Wistar rats).

Copper materials consistently exerted cytotoxicity to HepG2/C3A cells and induced cytokine production 24 hours post exposure. Micronized copper and ascorbate coated CuO were the most and the least toxic respectively.

Copper amine was most effective at reducing the fungal growth of Coniophora puteana, Trametes versicolor and Gloeophyllum trabeum while ascorbate coating enhanced the antifungal effect of CuO NPs.

In vivo, short-term inhalation studies (STIS) were performed with pristine, ascorbate and PEI coatings. All the materials upregulated tumour necrosis factor alpha (TNFα) in lung tissue; in the short-term oral study (STOS) with CuO NPs and micronized copper, only the latter downregulated chemokine C-X-C motif ligand 2 (CXCL2) in the liver and metallothionein 1A in the ileum.

In conclusion, CuO NPs were relatively cytotoxic in vitro and induced pro-inflammatory responses both in vitro and in vivo. However, these NPs were ineffective as antifungal treatment while the ascorbate coating enhanced the antifungal effect together with a significant decrease of cytotoxicity in vitro. The other materials did not induce any significant cytotoxicity nor cytokine production in C3A cells.
This work is dedicated to my wife Roberta who supported me the most through the entire length of the project and who is too modest to admit it.

To my son Ian who tried every strategy that a few month-old baby can use to prevent me to focus on this thesis to be cuddled instead.

To everyone who doubted me, *may you live in interesting times* (Terry Pratchett, 1994).
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"O frati," dissi, "che per cento milia
perigli siete giunti a l'occidente,
a questa tanto picciola vigilia
d'i nostri sensi ch'è del rimanente
non vogliate negar l'esperienza,
di retro al sol, del mondo sanza gente.

Considerate la vostra semenza:
fatti non foste a viver come bruti,
ma per seguir virtute e canoscenza".

Dante Alighieri (1265-1321)
La Divina Commedia
Inferno: C. XXVI, vv. 112-120

‘O brothers!’ I began, ‘who to the west
through perils without number now have reach’d;
to this the short remaining watch, that yet

our senses have to wake,
refuse not proof of the unpeopled world,
following the track of Phoebus.

Call to mind from whence ye sprang:
ye were not form’d to live the life of brutes,
but virtue to pursue and knowledge high’.

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Chapter 1: Introduction
1.1 Short History of Toxicology

Toxicology is defined as the science of poisons or poisoning (Goodman et al. 2011). However, to develop a stringent definition for a poison is rather problematic as, quoting the famous Paracelsus (Figure 1.1) considered the Father of Toxicology (Grandjean 2016), ‘Solely the dose determines that a thing is not a poison’ (Deichmann et al. 1986). This paradigm introduced not only the concept of the dose-dependent toxicity of a substance but paved the way to the novel concept of threshold and led future toxicologists to define the no-adverse-effect level (NOAEL) as the highest dose of a tested substance, at which no-adverse effect is observed (Grandjean 2016). With Mattieu Joseph Bonaventura Orfila (1787-1853), attending physician to Louis XVIII of France and chairman at the University of Paris, modern toxicology developed. Orfila (Figure 1.2) was the first to attempt a systematic correlation between the dose of chemicals and the biologic effects of known poisons (Radenkova-Saeva 2008). But it was only in the second half of the 19th century, due to the chemical and industrial Revolution with the release in the environment of thousands of chemicals, and the subsequent need to protect humans from their toxic effects, toxicology eventually evolved in its modern-day branches: general toxicology, pharmacology, occupational toxicology, clinical toxicology, environmental toxicology (Radenkova-Saeva 2008).
1.2 Nanoparticles and Nanomaterials: an overview

Nanomaterials (NMs) are defined as natural, incidental or manufactured particles, either in an unbound state, as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm (2011/696/EU). Nanoparticles (NPs) are defined as having all three dimensions in the nanoscale (1-100nm) (Buzea, Pacheco and Robbie 2007). In fact, the prefix nano is derived from the Greek νάνος [-νο, νο] (nanos), signifying dwarf. In recent years, an increasing number of nanomaterial containing products have entered the market (Kroll et al. 2009) and even more NMs are being developed because of the unusual and interesting properties that materials exhibit at the nanoscale compared to larger materials (Buzea, Pacheco and Robbie 2007; Oberdörster, Stone and Donaldson 2007; Papp et al. 2008).

These new properties have led to NM incorporation into over 1300 consumer products, and this is predicted to increase in the future (Vance et al. 2015) especially considering they also show an extraordinary opportunity for applications in pharmacology and medicine (Kroll et al. 2009). Despite this continuous expansion in use and the vast number of studies performed on this topic, there are still many uncertainties surrounding the potential risks posed by NMs to human health and the environment (Johnston et al. 2013; Kermanizadeh et al. 2016; de Jong et al. 2017). Moreover, the potential adverse impacts of NMs, due both to occupational, consumer and environmental exposures are of concern and pose a potential threat to the continued industrial use and integration of nanomaterials into commercial products (Graham et al. 2017).

Although nanotechnology is considered to be a relatively new discipline (Stone et al. 2016), naturally occurring, and anthropogenic NMs were already widely distributed throughout the environment (Kermanizadeh 2012), living organisms and even, within proteins and viruses (Yang et al. 2013). As a consequence, humans have been exposed to these materials during their whole evolution; nevertheless, in recent years this exposure increased exponentially due to the release of anthropogenic nanomaterials (Oberdörster et al. 2005).

NMs are widely used in several industrial sectors such as textiles (Ramos and Almeida 2017), cosmetics (Peña 2017), transport (Niaounakis 2015), construction (Abdullaeva 2017; Bossa et
al. 2017), agriculture, food and food contact materials (de Jong *et al.* 2017), and medicine (Caruthers, Wickline and Lanza 2007; Hofmann-Amtenbrink *et al.* 2014). In recent years, several NMs have been used in the art sector such as in restoration and conservation of stony materials (Lazzeri *et al.* 2016; Sierra-Fernandez *et al.* 2017) and painting (Kolman *et al.* 2017; Salama, Ali and El-Sheikh 2017).

However, even if the usage of NMs is continuously increasing, successful exploitation will require a more detailed and organised risk evaluation and an improved understanding of the potential implications of exposure to human health and the environment (Oberdörster *et al.* 2005; Papp *et al.* 2008; Kermanizadeh 2012; Stone *et al.* 2016; Pallardy, Turbica and Biola-Vidamment 2017).
1.3 Physicochemical characteristics

Several variables influence the properties and toxicity of NMs such as the size, surface area, shape, composition, agglomeration and charge (Buzea, Pacheco and Robbie 2007; Kermanizadeh 2012).

1.3.1 Size

NPs possess a large surface area and high particle number per unit mass if compared to their bulk form (Kermanizadeh 2012); for example, the ratio of surface area to mass of a 60 nm particle is 1000 times higher than a particle with a 60 μm diameter (Buzea, Pacheco and Robbie 2007). In some cases, as a consequence of the enhanced surface area ratio, the chemical reactivity is enhanced (Roduner 2006). Another consequence of this decrease in size is linked to the fact that the atoms situated on the surface of the NMs have fewer interactions between them than the bulk atoms resulting in a lower binding energy with extreme consequences such as a decrease in their melting point if compared with the bulk material (Roduner 2006). These changes in physicochemical characteristics could lead to the production of new materials behaving like new chemical substances (Kermanizadeh 2012) even if there is an increase of risks related to human health exposure as together with the decrease in size there is often an increase of toxicity (Johnston et al. 2010). One of the most relevant aspects of dose metrics for toxicity of NPs is their surface area (Schmid and Stoeger 2016).

1.3.2 Shape (Morphology)

The morphology of NMs performs an important role not only in their classification, but also in their properties and toxic effect (Kermanizadeh 2012). Previously, NMs were defined as having at least one dimension below the 100 nm; nevertheless, a further classification can be done in regard of the number of dimensions within this threshold. NMs with only one dimension are usually monolayer forming films such as computer chips or surface coating like in LED system (Huang et al. 2009) while a classical example of two-dimensional NMs are carbon nanotubes
(CNTs) (Buza, Pacheco and Robbie 2007). Materials with all three dimensions in nanoscale include colloids and free NPs with various morphologies (Kermanizadeh 2012). The morphology of NMs can vary enormously and can include either high or low-aspect ratio materials such as nanotubes or nanospherical particles respectively (Buza, Pacheco and Robbie 2007; Kermanizadeh 2012) and a wide variety of shapes (Figure 1.3). NM shape might play an important role in deposition and absorption, especially in the respiratory tract, and also affect the cellular uptake of both NPs and NMs and their clearance from the body (Oberdörster et al. 2005). An example of that is the frustrated phagocytosis of fibre-like NMs, as explained in Section 1.9.

![Figure 1.3 Different nanoparticles shape and morphology.](image)

### 1.3.3 Composition

NMs can be composed of one or more different materials (Kermanizadeh 2012), and they can be categorised as carbon-based, organic or inorganic materials and between the latter as metal or metal oxides compounds or composites which include coated or encapsulated structures
Their biological effects and toxicity of NMs also depend on the surface chemistry, eventual impurities or reactive groups on their surface (Oberdörster et al. 2005).

1.3.4 Uniformity and Agglomeration

NMs can be found in different states such as dispersed in an aerosol, suspended in aqueous media or in colloids depending on their physicochemical properties (Handy, Owen and Valsami-Jones 2008). Nevertheless, they will head to a phenomenon consisting in their gradual agglomeration – defined as a reversible state when disperse particles are held together by weak physical interaction (Mcnaught A.D and Wilson.A 1997) – out of the aqueous phase over time (Buzea, Pacheco and Robbie 2007). This phenomenon will have strong implications for their behaviour and toxicity (Oberdörster et al. 2005; Kermanizadeh 2012).

1.3.5 Charge

The charge of a NM can be negative, positive or neutral and it is determined by the chemistry of its surface – i.e. composition, functionalised groups and capping agents – and plays a key role in their agglomeration state (Jiang, Oberdörster and Biswas 2009; El Badawy et al. 2010; Kermanizadeh 2012; Nur, Lead and Baalousha 2015). The weaker the electrostatic repulsive forces are, the faster is the shift from a well-dispersed state into agglomeration (Lakshminarasimhan, Kim and Choi 2008). This aspect is one of the main determinant of their colloidal behaviour and it can easily influence the organism exposure to NPs by the change in their shape and agglomeration state (Akiyoshi Hoshino et al. 2004).

The charge as for the other physicochemical characteristics is directly linked to NM toxicity (El Badawy et al. 2010) for it is able to affect not only the intracellular fate of the NPs but also their interaction with the blood-brain barrier and the transmembrane permeability (Georgieva et al. 2011). Furthermore, a positive surface charge was directly related to both an increase of cellular uptake (Gatoo et al. 2014) and an enhanced systemic toxicity due the ability to induce haemolysis and platelet aggregation (Goodman et al. 2004). Finally, the surface charge of NPs, together with their size, can also influence the formation and the composition of the protein corona (Hühn et al. 2013).
1.3.6 Corona

The term ‘corona’ appears for the first time in 2007 and it was defined as a spontaneous self-assembling layer of proteins onto the surface of NPs (Cedervall et al. 2007). The concept derived from the work of Bangham (Bangham, Pethica and Seaman 1958) and Vroman (Vroman 1962) describing the protein adsorption and the important role played by it in overall interactions and responses to pristine materials (Hadjidemetriou and Kostarelos 2017).

Protein can interact directly with the NPs or NMIs creating a dynamic multilayer called ‘hard’ corona to which a further multilayer of weakly bonded proteins called ‘soft’ corona can be attached (Winzen et al. 2015; Lundqvist et al. 2017). These protein multilayers can undergo a series of changes as the protein do not form stable and irreversible bounds with the NPs but it can interact with the other proteins present in the different systems and organs of the human body over the time (Lundqvist et al. 2011; Chen et al. 2017). These dynamic interactions might play an important role in term of the toxicity and fate of the NPs within the human body (Lynch et al. 2007) but it is still very difficult to determine the level of their final impact in vivo (Hadjidemetriou and Kostarelos 2017).
1.4 Nanotoxicology

Nanotoxicology is a relatively modern branch of science that started to take place in the second half of the twentieth century. The discipline of nanotoxicology emerged after decades of studies on the toxicity of fibre materials (especially asbestos) and on the characterization of combustion exhaust (Whitby et al. 1975) leading to the subsequent studies on particulate air pollution (Oberdörster, Stone and Donaldson 2007; Kermanizadeh 2012; Santamaria 2012) and respirable crystalline silica (quartz) (Donaldson and Borm 1998) also presents in tobacco and cigarette smoke (Pappas 2011). With these studies scientists all over the world realised the importance of ultrafine particles (nanoscale particulate) exposure as possible cause of adverse health effect and, soon after, that these particles could not only be deposited on the lower respiratory tract in humans but they can also translocate to various organs of the human body such as liver, kidneys and brain (Berry et al. 1977; Heyder et al. 1986; Oberdörster, Stone and Donaldson 2007).

In 1990, the group of Ferin and Oberdörster (Ferin et al. 1990; Oberdörster et al. 1990) described for the first time a significant increase of the levels of pulmonary inflammation and interstitial translocation associated with titanium dioxide (TiO$_2$) NPs and, in 1992, the same group was able to conclude that the toxicity of ultrafine particles (equivalent to NPs in terms of size) was linked to both their ability to translocate, and that toxicity was best correlated when particle dose was expressed as surface area (Ferin, Oberdörster and Penney 1992). Finally, the work of Donaldson (Donaldson et al. 1998; MacNee and Donaldson 2003) identified the importance of particle physicochemical properties, linking characteristics such as size and surface area to the particle toxicity. With these fundamental studies and the scientific implications that they generated in the scientific community, nanotoxicology, term used for the first time in Donaldson et al. (2004) to define ‘a new subcategory of toxicology (…) to address gaps in knowledge and to specifically address the special problems likely to be caused by nanoparticles’ (Donaldson 2004) was finally accepted as a proper and independent branch of science and toxicology (Oberdörster, Stone and Donaldson 2007; Kermanizadeh 2012).
1.5 Human Health and Principal Exposure Routes

Historically the principal sites of human exposure to both NPs and NMs were the lungs, the gastrointestinal tract (GIT) and the skin (Figure 1.4). These organs are in constant contact with the external environment, so it is not surprising to find these systems being primary exposure sites for NPs (Chen et al. 1999; Sadauskas et al. 2009).

![Nanoparticles Life Cycle vs Main exposure routes](image)

*Figure 1.4* Nanoparticles exposure routes. The four main routes under which NPs can enter the human body and both occupational and customer exposure in relation to the NPs life cycle.

**Respiratory System**

The respiratory system is potentially exposed to a myriad of substances including ambient particulate matter (PM), NPs and NMs (Card et al. 2008). Whilst particles larger than 5 µm can be trapped by both mucus and cilia on the surface of epithelium of the upper airways (Fröhlich and Salar-Behzadi 2014), fine particles of less than 2.5 µm aerodynamic diameter (PM$_{2.5}$) were identified as responsible for the adverse health effect in human (Stone et al. 2016). NPs, due to their small size can reach the deep, alveolar region of the lung (Fröhlich and Salar-Behzadi 2014). The literature regarding the effects of NPs in an occupational setting is increasing and suggests that occupational exposure may have a detrimental effect on human health (Stone et al. 2016). In rodents, instillation of fine and ultrafine TiO$_2$ particles showed that, at the same equivalent dose of exposure, NPs could cause inflammation to lung epithelium and cross the alveolar barrier (Sager, Kommineni and Castranova 2008). Evidence of NPs crossing the air-blood barrier were also reported in studies performed on rats exposed to elemental silver NPs (Takenaka et al. 2001) and
hamsters exposed to albumin nanocolloid particles (Nemmar et al. 2001). The toxic effects of NPs on the respiratory epithelium are determined not only by their size but also by other properties, e.g. dose, surface modification and so forth. For example, silver NPs, which showed a high pro-inflammatory activity both in vitro on A549 cells (Che et al. 2017) and in vivo on rats (Wen et al. 2017), can also exert immunomodulatory effects on mouse when functionalised with polyvinylpyrrolidone (PVP) or citrate (Alessandrini et al. 2017). Finally, there is also evidence that NPs such as silica, can also be absorbed via the nasal cavity and reach the brain through the olfactory bulb (Fröhlich and Salar-Bezhadi 2014).

Gastrointestinal Tract

Not considering the accidental exposure to NMs due to involuntary ingestion (further explained in Section 5.1), the dietary consumption of these materials is estimated to be more than 10^{12} NPs/day in developed countries (Martirosyan and Schneider 2014), consisting predominantly in silicates and TiO_{2} (Buzea, Pacheco and Robbie 2007). Both NPs and NMs are widely used as food additives, in supplements and food packaging (Sanguansri and Augustin 2006; Chaudhry et al. 2008), and their potential application by the food industry is still increasing (de Jong et al. 2017). Nevertheless, even if this technology is considered beneficial for the economic growth of the food sector, NMs have the potential to lead to complications for the health of consumers, and that brings to the necessity of performing a further investigation to unravel any possible biological outcome following ingestion (Martirosyan and Schneider 2014).

The gastrointestinal tract (GIT) is a selective barrier and due to its large surface area (approx. 200 m² in the average adult human) plays a crucial role in the interaction with ingested NMs (Bergin and Witzmann 2013). The gut barrier is protected by several layers of cellular epithelium and secreted mucus (Johansson, Sjövall and Hansson 2013) enhanced by secretory IgA and antimicrobial peptides and proteins (Wells et al. 2017); the interaction with these luminal factors might influence NPs characteristics such as stability, aggregation and surface properties (Bellmann et al. 2015). Furthermore, also factors such the decrease of pH in the stomach may influence NM toxicity (Bellmann et al. 2015), e.g. the dissolution of Ag NPs is enhanced at acidic pH (Mwilu et al. 2013). The absorption of NMs by GIT is an energy-dependent endocytosis process (Doherty and McMahon 2009). For example the adsorption of phospholipid-based PEGylated micellar nanoparticles linked to biotin was observed in vitro (CaCo-2 cells) and in vivo model (everted rat
intestinal sac) (Simovic et al. 2015) and also copper oxide NPs proved to be able to translocate across the CaCo-2 monolayer in vitro (Ude et al. 2017).

The ingestion of NMs not only might affect GIT cells but also the composition of the bacterial population of the GIT. For example studies on Escherichia coli as representative model of intestinal microbiota, showed that silver NPs were able to kill the bacteria at lower concentrations than the ones required to damage enterocytes (derived from the Japanese quail) (Coturnix coturnix japonica) (Sawosz et al. 2007) indicating that these NPs might cause adverse to effects selectively affecting the composition of the gastrointestinal microbiota (Fröhlich and Fröhlich 2016). Furthermore, studies on Wistar rats exposed to zinc oxide (ZnO) NPs by oral gavage showed that these NPs not only were toxic to the GIT and induced liver damage but also behavioural changes (Slama 2015). Since several studies already linked the interconnection between microbiota composition of the gut and its alteration may cause change of behavioural patterns in rodents (Hsiao et al. 2013; Stilling, Dinan and Cryan 2014; Wang et al. 2015), it was hypothesised that ZnO NPs might affect the behaviour indirectly by altering the bacterial composition of the gut (Fröhlich and Fröhlich 2016). Finally, once absorbed by the gastrointestinal epithelium, NPs can also elicit their toxic action to other organs via blood and lymph circulation (de Jong et al. 2017). An in vitro study demonstrated that carboxylated polystyrene nanoparticles were able to bypass a CaCo-2 and HT29-MTX (mucin-producing cells) co-culture eliciting their toxic effect also on HepG2/C3A hepatocytes within one microfluidic device (Esch et al. 2014).

**Skin absorption and injection**

In addition to inhalation and ingestion, it should be considered that NPs are often incorporated into a variety of personal care products, e.g. sunscreens, cosmetics, and soaps (Kermanizadeh 2012). As such, exposure of the skin to NMs is likely to occur in occupational (e.g. exploitation of NMs by cosmetic, ink and pigment industries) and consumer (e.g. application of sunscreens to the skin) settings (Lines 2008; Peña 2017). More recently, there has also been interest in the exploitation of nanomaterials as dermal and transdermal drug delivery vehicles (Elsaesser and Howard 2012; Santamaria 2012). Exposure of healthy or damaged skin to NMs may occur, and influence the biological response observed (Nasir, Friedman and Wang 2013; Crosera et al. 2015). This is exemplified by the use of silver NPs in wound dressings to prevent infection (Wilkinson, White and Chipman 2011), and the incorporation of metal oxide NPs in sunscreens which may be applied to sun damaged skin (Mortensen et al. 2008). Finally, with the increase of NPs usage in medicine,
e.g. as drug delivery system, another route of access is through injection by actively damaging the skin barrier (El-Ansary and Al-Daihan 2009; Yah, Iyuke and Simate 2012; Shi et al. 2013; Ellenbecker and Tsai 2015).
1.6 Secondary Organ of Exposure: Liver

Even if the primary site of exposure to NMs is the lungs or the gastrointestinal tract (Nel 2006; Bakand, Hayes and Dechsakulthorn 2012; Kermanizadeh et al. 2012a), skin absorption or injection (Mortensen et al. 2008; El-Ansary and Al-Daihan 2009; Yah, Iyuke and Simate 2012), both NPs and NMs might eventually reach secondary tissues. The liver plays a primary role in accumulating NMs, regardless of the route of exposure (Sadauskas et al. 2009; Diesen and Kuo 2010; Kermanizadeh et al. 2012a, 2012c, 2014b; Stone et al. 2016).

As a secondary exposure site, the liver is extremely important, as it has been shown to accumulate NPs at much higher quantities compared to other organs following both inhalation and ingestion (Semmler-Behnke et al. 2008; Sadauskas et al. 2009). The liver is the metabolic centre of the body (Campbell 2006; Sadauskas et al. 2009; Kermanizadeh et al. 2012a; Reinke and Asher 2016) and has a crucial role in metabolic homeostasis (van den Berghe 1991) as it is responsible for the storage, synthesis, metabolism and redistribution of carbohydrates, fats and vitamins (Grant 1991; Kmiec 2001a, 2001b; Diesen and Kuo 2010; Bak et al. 2011; Kermanizadeh et al. 2012a). Moreover, it produces a large number of serum proteins, an array of enzymes and acute phase proteins (Tavill 1972; Gauldie et al. 1987). It is also the principal detoxification centre of the body, removing xenobiotics and waste products by metabolism or biliary excretion (Kmiec 2001b; Kermanizadeh 2012) and can be responsible for the clearance of both NPs and NMs alongside the kidneys (Tran et al. 2000; Semmler-Behnke et al. 2008; Geiser and Kreyling 2010; Kermanizadeh et al. 2012a). Considering the detoxifying role that the liver plays in the human body, and considering that to play that role the hepatocytes (the main liver cell population) during the Phase 1 of detoxification use as intermediary either reactive oxygen species (ROS) or free radicals (FR), it follows that the equilibrium of ROS and antioxidants is fundamental to prevent oxidative stress that might damage this organ (Betteridge 2000; Kermanizadeh et al. 2012a).

An antioxidant is defined as a substance that is able to significantly delay or prevent oxidation of a substrate (Halliwell 1990) and can be naturally found in hepatocytes – e.g. glutathione (GSH), glutathione peroxidase, superoxide dismutase (SOD) and hemeoxygenase (HO) (Glantzounis et al. 2005b) – or be introduced as part of the diet (Casas-Grajales and Muriel 2015) as for curcumin (Saidi et al. 2017), the melanoidins in coffee (Borrelli et al. 2002) and
the several flavonoids present in tea (Rietveld and Wiseman 2003). Amongst all the antioxidants present in the hepatic tissue, GSH not only plays a key role in most of detoxifying reactions, in regulating the thiol-disulphide status of the cell and in the storage and transfer of cysteine (Kaplowitz 1981; DeLeve and Kaplowitz 1991) but it is the most abundant antioxidant in this tissue (Wu et al. 2004). GSH defends the cell components by reacting with ROS and other free radicals generating glutathione disulphide (GSSH) and then, can be reduced again to its previous form with consumption of NADPH (Figure 1.5) (Nimse and Pal 2015a).

![Figure 1. 5 Antioxidative mechanism of Glutathione. GSH can react with intracellular ROS creating glutathione disulphide (GSSH), the latter can be reduced with consumption of NAPDH by the enzyme Glutathione Reductase.](image)

The chances of surviving for cells are strictly linked with their ability to cope with the damage caused by oxidative stress, restoring the original state (Kermanizadeh et al. 2012a); nevertheless, when the extent of the damage is too severe or long lasting, the oxidative stress can cause cell death (Martindale and Holbrook 2002), and it can enhance the transcription of pro-inflammatory genes (Brown et al. 2004), genotoxicity (Gonzalez, Lison and Kirsch-Volders 2008) and even cancer initiation (Saha et al. 2017).

**Human hepatoma (C3A) cell line**

The C3A cell line (ATCC® CRL-10741™; Kelly, 1992) is clonal derivative of the Hep G2 cell line which was isolated from a 15 years adolescent Caucasian male, with a strong contact inhibition of growth, high albumin production, high production of α-fetoprotein (AFP) and
ability to grow in glucose deficient medium (Figure 1.6). Even if this immortalised cell line has lost some of its key liver functions such as the cytochrome P450 activity (Mingoia et al. 2007; Kim et al. 2011a), and for this reason it is not entirely representative of hepatocytes in vivo, it still has some advantages when compared to primary cells (Kermanizadeh et al. 2012c). For example, primary cells (either from human or rodent), even though they better reflect the metabolic pathways and signalling of the liver, are phenotypically unstable (Kermanizadeh et al. 2012c), expensive and extremely variable depending on the human donors (Liguori, Blomme and Waring 2008; Sahi, Grepper and Smith 2010). Both HepG2 and C3A cells on the other hand, not only are cheaper and easier to maintain, but are also a good model for the acute response to toxicant and allow to easily perform study about the uptake, cytotoxicity, pro-inflammatory cytokine release and albumin production due to NPs exposure (Kermanizadeh et al. 2012c; Gaiser et al. 2013a).

Figure 1.6 C3A hepatoma cell line. High-density cell culture after 48 hours of incubation at 37°C and 5% CO₂ in culture flask; 40x magnification.
1.7 Toxic Mechanisms of Nanomaterials

Not all NMs are equally toxic, and their physico-chemical characteristics are the main reasons that underlie their possible interaction with the human body and explaining the different mechanisms of toxicity (Oberdörster et al. 2005; Buzea, Pacheco and Robbie 2007). While the different toxic mechanisms explained in the following paragraph has been demonstrated for many NPs and NMs, that does not mean that all NMs are toxic, there are exceptions reported in literature such as fullerenes (Bakry et al. 2007) and cerium dioxide, the latter for example, not only is not toxic but suppressed ROS production and induced cellular resistance to an exogenous source of oxidative stress on RAW 264.7 in vitro (Xia et al. 2008).

Cells need oxygen to survive, O₂ is defined as a molecule with elevated chemical ‘aggressiveness’ (Augusto and Miyamoto 2012) as its use in cellular metabolism could bear the risk of oxidative modifications to all biomolecules at the expenses of the cellular integrity due to ROS production, and, as result of this risk, all aerobic species have developed several antioxidant strategies during their evolution (Egea et al. 2017). Determining the sources of ROS and their interactions is still a challenging task as are described different possible sources as major trigger for several diseases, even if the oxygen metabolite superoxide (O₂⁻) or H₂O₂ is the principal by-product of key enzymatic reactions involved in almost all of them (Egea et al. 2017). ROS can either elicit damage to the different cellular compartments or play the role of a signalling molecule in a concentration-dependent manner (Egea et al. 2017).

Nel et al. (2006) proposed a hierarchical model of oxidative stress to NMs consisting of three tiers. At low levels of oxidative stress (Tier 1) antioxidant defences are triggered by the activation of Nrf-2 (nuclear factor E2-related factor 2), which is constantly degraded in non-stressed cells and it represents the major mechanism in the cellular defence against either oxidative or electrophilic stress inducing the transcription of the phase II antioxidant enzymes (Nguyen, Nioi and Pickett 2009). If the situation is not restored back to normal, intermediate levels of oxidative stress (Tier 2) leads to the activation of the MAPK (mitogen-activated protein kinase) and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) cascades, inducing a pro-inflammatory response with production of cytokines and chemokines. The maximum amount of oxidative stress (Tier 3) leads to the opening of the mitochondrial permeability transition pore with subsequent disruption of electron transfer; these alterations
will inevitably lead to either apoptosis or necrosis (Nel 2006). Experiments performed on both *in vitro* (A549 and alveolar macrophages from Sprague-Dawley rats) and *in vivo* (Fischer rats) using metal copper NPs, silver NPs and several sizes of TiO$_2$ NPs supported the role of oxidative stress in the toxicity of NP as they demonstrated that NPs could induce ROS production *in vitro* and elicit pulmonary inflammation *in vivo*, assessed through cytokine production and neutrophil accumulation respectively (Brown *et al.* 2004; Rushton *et al.* 2010), and it is now widely accepted that the main toxic mechanism under which many NPs and NMs can exert their toxic effect is through generation of reactive oxygen species (ROS) and via the stimulation of inflammation (Manke, Wang and Rojanasakul 2013; Fard, Jafari and Eghbal 2015).

In 2011, Burello and Worth developed a theoretical model able to predict the potential oxidative stress of metal oxide nanoparticles comparing the redox potentials of relevant intracellular reactions with the NPs’ energy structure. In other words, when both NPs and biological molecules show a similar energetic state, the reaction involving electron transfer led to oxidative stress either through the increase of ROS production or decrease of antioxidant defences (Burello and Worth 2011). Zhang *et al.* (2012) tested this model using 24 metal oxide NPs demonstrating that the overlap of conduction band energy (Ec) levels with the cellular redox potential (-4.12 to -4.84 eV) was strongly correlated to the ability of Co$_3$O$_4$, Cr$_2$O$_3$, Ni$_2$O$_3$, Mn$_2$O$_3$, and CoO NPs to induce oxygen radicals, oxidative stress, and inflammation *in vitro*, on BEAS-2B (human bronchial epithelium), RAW 264.7 (murine macrophages), and *in vivo*, on C57 BL/6 mice (Zhang *et al.* 2012).

In general, NPs can enter the cell either via endocytosis or pinocytosis depending on their size, physicochemical characteristics and proteins absorbed onto their surface (Shang, Nienhaus and Nienhaus 2014). Once uptaken, they can reach different cellular compartments (lysosome, endoplasmic reticulum, nucleus, *etc.*) and cause damage to a wide variety of cellular components (Figure 1.7) such as lipid peroxidation (Premanathan *et al.* 2011; Winter *et al.* 2016), membrane damage in both bacteria (Sondi and Salopek-Sondi 2004) and mammalian cell (Hussain *et al.* 2005), mitochondrial damage (Sun *et al.* 2011; Mallick *et al.* 2016), lysosomal damage and ion release (Qin *et al.* 2003; Hsin *et al.* 2012; Cronholm *et al.* 2013), protein misfolding and oxidation (Fei and Perrett 2009; Parveen, Shamsi and Fatima 2017) as well as DNA damage (Fairbairn *et al.* 1996; Atha *et al.* 2012; Collins 2014; Karlsson *et al.* 2014).
Another toxic mechanism, namely frustrated phagocytosis (Figure 1.8), is specifically related to the fibre-shaped NMs. This mechanism was firstly identified in asbestos and then extended to other fibres such as carbon nanotubes (Poland et al. 2008; Donaldson et al. 2010; Boyles et al. 2015), consists of the attempt of a macrophage to clear a fibre that is too long to be completely phagocytosed. This inability to fully engulf the whole fibre causes a partial phagocytosis with the release of cytotoxic compound all around the extracellular environment; as a consequence, the macrophage accidentally damages the surrounding cells and recruits new immune cells triggering the inflammatory response. The new immune cells can undergo the same process of frustrated phagocytosis starting a process of chronic inflammation.

To conclude this overview about the toxic mechanisms of NPs, it must be highlighted that another important factor that influences the fate of NPs inside the organism is the protein corona first described in 2007 (Cedervall et al. 2007). This term defines the spontaneous self-assembling and subsequent layering of proteins onto the NM surface, this superficial biotransformation modulates the toxicological profile of both NMs and NPs, and it is widely studied for its implication in nanomedicine (Hadjidemetriou and Kostarelos 2017).
Figure 1.7 Cellular toxicity of NPs. Mechanisms of NPs toxicity exerted to different cellular compartments.

Figure 1.8 Frustrated phagocytosis. Diagram showing the different fate of fibre nanomaterials in function of their size; fibre of small size would be taken by macrophage and undergo clearance while fibre longer in size would cause frustrated phagocytosis and subsequent inflammation.
1.8 Copper and Copper Oxide NPs

Toxicity against microorganisms

Copper is the most widely used fungicide for treating wood in contact with the soil, with no satisfactory alternative available since copper is the only biocide that shows significant effects against soft rot fungi and other soil-borne fungi (Hughes 2004). Copper is also preferred as an antimicrobial wood preservative because it is considered a toxicant with relatively low effect on mammals, including humans (Lebow 1996), even if it shows a relatively high toxicity against aquatic communities (Roales and Perlmutter 1980; Ronald 1990).

Among a wide range of available nanomaterials, copper nanoparticles have shown interesting properties both against a large spectrum of microorganisms (Cioffi et al. 2005), again with its toxicity considered to be low in humans compared to microorganisms (Bosetti et al. 2002). For these reasons, in the last years, many industries have begun to develop formulations using micronized copper, containing a considerable amount of metallic copper nanoparticles (Mcintyre 2010) able to penetrate into wood (Matsunaga, Kiguchi and Evans 2007; Geers et al. 2014). The use of various copper nanoparticles instead of bulk copper is reported to improve the durability of wood against fungal decomposition (Kartal, Green and Clausen 2009; McIntyre and Freeman 2009; Cookson et al. 2010; Akhtari, Taghiyari and Kokandeh 2013). However, there is still little evidence that the different copper nanoparticles can be more efficient than copper salts against soil-borne fungi or Cu-tolerant wood-destroying fungi (Kartal, Green and Clausen 2009; Cookson et al. 2010; Tang et al. 2013).

Copper seems to exert its toxic effect on bacterial cells primarily through the generation of reactive hydroxyl radicals, known to cause protein oxidation, DNA and RNA cleavage (Stohs and Bagchi 1995; Kim et al. 2000) and membrane damage due to lipid peroxidation (Halliwell and Gutteridge 1984; Peña, Koch and Thiele 1998). While the toxic mechanism of copper ions is known, there is still not sufficient information about the exact toxic mechanism of metal copper nanoparticles (Civardi et al. 2015). It seems reasonable to assume that copper-based nanomaterials can elicit their toxic action through both direct particle interaction and ion release (Cioffi et al. 2005; Ivask et al. 2010; Wu et al. 2010; Cioffi and Rai 2012). Copper oxide nanoparticles in particular are highly reactive due to their large surface area (Oberdörster

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2000; Chen et al. 2006) which causes the production of ROS leading to oxidative stress in *Pseudomonas aeruginosa* (Saliba et al. 2006), *Vibrio fischeri*, crustaceans *Daphnia magna* and *Thamnocephalus platyurus* (Heinlaan et al. 2008), and in chronic myeloid leukemia (CML) K562 and A549 cells (Shafagh, Rahmani and Delirez 2015) and DNA damage. In addition, CuO and copper NPs may release copper ions able to elicit conformational changes in protein structure or in the active sites of enzymes, causing inhibition or neutralization of their biological activity (Borkow and Gabbay 2009); Cu$^{2+}$, for example, can induce structural alteration in the two copper chaperone proteins CopZ (*Bacillus subtilis*) and Atox1 (*Homo sapiens*) (Hussain and Wittung-Stafshede 2007) or can inactivate the H-1 related protein tyrosine phosphatase activity of the vaccinia virus *in vitro* (Kim et al. 2000). Moreover, copper can also influence the production of lignocellulose-degrading enzymes (Shah et al. 2010), damage to mitochondria (Chang et al. 2012), cell wall (Heinlaan et al. 2008; Shah et al. 2010) and impaired homeostatic processes (Chang et al. 2012).

Not all fungi are sensitive to copper, in fact, several brown rot fungi can grow at Cu$^{++}$ concentrations of up to 1.6 mM (Hughes 2004) or 100 mg/kg (Gadd 2007). This peculiar category of Fungi causes high economic loss since they are the main cause for the early failure of in-ground timber structures in the US and Europe (Lebow et al. 2003; Bollmus et al. 2012); in Germany, copper tolerant fungi cause failures of 1% of wood poles each year. The total annual expense for replacing wood poles are estimated to be EUR 36,000,000 (Civardi, Schwarze and Wick 2015). The high resistance shown by these fungi is one of the key factors driving the continuous development of copper-based formulations and their alternatives.

*Toxicity against mammalian cells*

Copper is found in almost all cells and tissues with the highest concentration in the brain and in the liver of mammals (Turnlund et al. 1998). Together with other metals, it is one of the essential nutrients for mammalian cells, and its homeostasis is finely regulated by the human body (Arredondo and Núñez 2005) through intestinal absorption, biliary excretion and hepatic storage (Turnlund et al. 1998). In particular, the liver plays an important role being the organ majorly involved in storage, distribution and excretion of this metal in mammals (Gaetke, Chow-Johnson and Chow 2014). At the cellular level, it is a chaperone transport system that allows not only its compartmentalization (Prohaska 2008; Boal and Rosenzweig 2009) but also
the ability to minimise possible participation in redox reactions (Burkitt 2001). Examples of these chaperons include CTR1, a plasma membrane protein present on the surface of microvilli that uptakes copper from intestinal lumen; CCS that donates copper to superoxide dismutase; COX17 that participates to the synthesis of cytochrome c oxidase in the mitochondria; Atox1, delivers copper to the secretory pathway by docking with 2 P-type ATPases or the intracellular hepatic copper-binding proteins COMMD1 (copper metabolism MURR1 domain) and XIAP (X-linked inhibitor of apoptosis protein) (Prohaska 2008; Boal and Rosenzweig 2009).

Copper systemic deficiency can impact on human health such as causing a reduction of intellectual capacity, fatigue, reduced growth and diminished immune response (Arredondo and Núñez 2005). Free Cu(II) ions were historically reported to be teratogenic (Agarwal, Sharma and Talukder 1989) due to their ability to bind DNA (Prasal 1986; Galindo-Murillo et al. 2015). This toxic effect is linked to some of the major neurodegenerative diseases such as Alzheimer and Parkinson’s Disease (Jomova et al. 2010; Gangania et al. 2017). Moreover, the presence of copper is essential for the functioning of more than 30 proteins – e.g. superoxide dismutase, cytochrome c oxidase, ceruloplasmin, lysyl oxidase, tyrosinase, and dopamine β-hydroxylase (Arredondo and Núñez 2005; Ogra 2014). These characteristics depend on the ability of copper, as for other metals such as iron, to participate to reactions involving the exchange of one electron (redox cycling); the same property though, can also generate the production of free radicals with dangerous effects to the cell (Arredondo and Núñez 2005).

As already explained, the main mechanism under which Cu can elicit its toxic effects is the free radical-induced oxidative damage (Gaetke, Chow-Johnson and Chow 2014) as copper can be reduced from Cu^{2+} to Cu^{+} in the presence of superoxide or other reducing agents and the latter can participate in the formation of free radicals via Fenton reaction during the Haber-Weiss reaction (Figure 1.9) (Bremner 1998; Crouch 2012; Gaetke, Chow-Johnson and Chow 2014). Even if the damage driven by ROS production is considered to be the main route under which Cu is able to elicit its toxic effect, there are other possible mechanisms of toxic action (Gaetke, Chow-Johnson and Chow 2014) including:

- altering lipid metabolism, as shown in some studies on Wilson’s disease on murine models (Huster and Lutsenko 2007; Burkhead, Gray and Lutsenko 2011),
- altering expression of hepatic genes involved in cholesterol biosynthesis in a fish model (Gasterosteus aculeatus) (Santos et al. 2010),
• aggregating and leading the formation of inclusions of α-synuclein which lead to degenerative disease such as Parkinson’s and Dementia (Study on transfected SH-SY5Y cells) (Wang et al. 2010),
• trigging apoptosis through the activation of sphingomyelinase and release of ceramide in rat hepatocytes (Brewer 2007; Lang, Ullrich and Gulbins 2011),
• altering the hepatic distribution of Cu in rat models in other studies on Wilson’s disease (Fuentealba et al. 2000; Medici et al. 2006; Ralle et al. 2010),
• altering the interaction between protein and metals in the rat (Rivera-Mancía et al. 2010).

Figure 1. 9 Copper-catalysed Haber-Weiss reaction. Freely adapted from Hopkins (Hopkins 2016). The sub-reaction leading to the production of the reactive species OH· is called Fenton reaction (right part of the diagram).

In addition to the toxicity of copper ions, nanoparticles seem to exert a toxic effect in mammalian cell lines that is not likely to be explained by only their ion effect (Karlsson et al. 2008). Cu and CuO NPs seem to be able to elicit not only a strong cytotoxic response when compared to other materials such as zinc oxide (ZnO), titanium dioxide (TiO₂) or Ag NPs (Karlsson et al. 2008; Cronholm et al. 2013) but also to cause significant DNA damage (Karlsson, Holgersson and Möller 2008; Karlsson et al. 2009, 2014).

One of the possible mechanisms behind this enhanced toxicity could be a Trojan horse effect as recently highlighted by Karlsson and Cronholm (Cronholm et al. 2012, 2013; Karlsson et al. 2014). In a similar way, the toxic effect of CuO NPs seems to be mediated by intracellular
uptake and subsequent release of copper ions (Cronholm et al. 2013) allowing the NPs to avoid *de facto* the cell membrane barrier as described for other metal oxide NPs (Sabella et al. 2014).
1.9 European Project’s Aims

The SUN project was based on the idea that the current knowledge on environmental and health risks of nanomaterials, while limited, can nevertheless guide nano-manufacturing to avoid liabilities and that an integrated approach can be applied to the complete lifecycle of the product. This project (http://www.sun-fp7.eu/) was the first one of its kind that aimed to address the entire lifecycle of nanotechnologies to ensure holistic nanosafety evaluation with the scope of incorporating results into tools and guidelines for sustainable manufacturing, easily accessible by industries, regulators and other stakeholders.

Within the project, the following studies take place specifically within Work Package 6 (WP6: Human Health and Toxicology) that aimed to investigate the hazards of pristine, released and aged nanomaterials using in vitro screens and in vivo models for important routes of exposure.
1.10 PhD Aims and Hypotheses

The general aim was to evaluate the toxicity of a panel of nanomaterials (NMs), at different stages of the life cycle, to the liver, lung and GIT using in vitro (hepatocytes at Heriot-Watt University and macrophages at Karolinska Institutet - Sweden) and in vivo (Male Wistar rats; inhalation and ingestion studies) models, in collaboration with The Netherlands National Institute for Public Health and the Environment (RIVM – The Netherlands).

This study also aimed to assess the ability of surface modifications to CuO NMs to reduce their toxicity by applying a safer by design (SbyD) approach.

Finally, considering the major pathways by which NPs are known to elicit their toxicity, the following endpoints were selected for the in vitro screening: cytotoxicity, inflammatory response and genotoxicity. For the in vivo testing, expression of several genes involved in inflammation and metal homeostasis (i.e. the chemokine (C-X-C motif) ligand 2, Interleukin-1β, Tumour Necrosis Factor α, the Cluster of Differentiation 46, Interleukin-10, Glutathione Peroxidase, Fas-ligand, the Intercellular Adhesion Molecule 1 and Metallothioneins 1A, 3 and 4) from the principal organs of exposure such as lung, liver and different portions of the digestive system were investigated.

The main hypothesis of this PhD was that NPs in general, and CuO NPs in particular, will show changes in their toxic properties related to the specific stage of their life cycle (e.g. when embedded in different matrices).

The second hypothesis was that a SbyD approach could be used to decrease the toxic effect of pristine CuO NPs both in vitro and in vivo maintaining their antifungal property.

Furthermore, it is also hypothesised that the findings from in vitro studies will be predictive of in vivo testing providing a useful screening model able to decrease the number of animals used in future studies.
1.11 Thesis Outline

This thesis is structured in six chapters and includes an appendix which contains data obtained from different SUN project partners. The reason for including this data is due to the complexity of the European project itself as a coordinated work that included experiments and expertise from different partners which are useful when interpreting the data obtained during this PhD.

Chapter 1, states the project’s aims, and hypotheses, and provides general background information which provides a rationale for the research conducted.

Chapter 2 presents the in vitro results that were obtained using the HepG2/C3A (hepatocarcinoma) cell line. Cells were exposed to nano and nano-free copper-based formulations such as copper(II)oxide (CuO) NPs, copper chloride and copper sulphate salts (CuCl₂ and CuSO₄), the copper-amine nano-free formulation (Cu-amine) used as a European benchmark for wood protection, and finally a suspension of micronized copper carbonate (CuCO₃·Cu(OH)₂). Moreover, chapter 2 will also introduce the S₈₉D concept and present data from four capping modifications of the pristine CuO NPs: ascorbate (Asc), polyvinylpyrrolidone (PVP), citrate (Cit) and polyethylenimine (PEI). The endpoints of this study were cytotoxicity and cytokine production which were investigated using the Alamar Blue (AB) assay and the Multiplex analysis respectively. Finally, pristine CuO NPs were further analysed for glutathione depletion as an index of oxidative stress and for genotoxicity using the Comet assay.

In Chapter 3, the emphasis moves to the antifungal screening of the copper-based (nano)formulations as both pristine and ascorbate coated CuO NPs, together with the micronized copper, all of which are compared to the European benchmark for wood preservative; the copper-amine formulation. In this chapter, a specially designed in vitro antifungal screening is presented; this protocol aims to allow the analysis of antifungal effects of NPs in a quicker and less expensive way than the current European standard EN 113 (CEN, 1996).

Chapters 4 and 5 present the gene expression results from the Short-Term Inhalation Study (STIS) in lung, liver and gut tissue (containing Peyer’s patches) and from the Short-Term Oral
Study (STOS) in stomach, liver and gut tissue (containing Peyer’s patches). It has to be stressed that these results, far from being conclusive by themselves, should be considered together with the ones produced by both the RIVM and the KI. Further details can be found in the appendix and via Ilse et al. (2016), and Costa et al. (2017).

Chapter 6 covers all the results obtained testing other NMs prioritised in the SUN Project using C3A cells as the test system. The main endpoints of this testing were the cytotoxicity analysis (Alamar Blue) and cytokine production (Multiplex) elicited by tungsten carbide cobalt-doped nanoparticles (WCCo NPs) compared with hexahydrate cobalt chloride (CoCl₂·6H₂O) as a control, as well as multi-walled carbon nanotubes (MWCNTs), synthetic amorphous silicon dioxide nanoparticles (SiO₂ NPs) and Irgazin Red 254 (diketopyrrolopyrrole nano-form grade). Finally, a genotoxicity test using the Comet assay was performed on the WCCo NPs.

In the last chapter, the conclusions are presented with a final discussion where the main achievements and limitations of this project will be highlighted, and possible future actions will be presented.
Chapter 2: *In Vitro* Investigation of copper-based formulations
2.1 Introduction

The focus of this Chapter is based on the cytotoxicity and inflammatory effect of copper oxide nanoparticles (NPs) as a priority of the SUN project (http://www.sun-fp7.eu), and its comparison with dissolved copper-amine and micronized basic copper carbonate (CuCO₃·Cu(OH)₂).

The aim of the research presented was to evaluate the toxicity exhibited by copper oxide nanoparticles (CuO NPs), nano-free copper-amine formulation and micronized basic copper carbonate (CuCO₃·Cu(OH)₂) to hepatocytes in vitro via assessment of cytotoxicity, cytokine production (as an indicator of pro-inflammatory effects) and genotoxicity.

2.1.1 Copper toxicity: an overview

One of the reasons for the interest in both nano and bulk copper-based formulations is because copper is well-known for its antimicrobial activity, with a range of examples of its use over history (Dollwet and Sorenson 1985; Grace, Chand and Bajpai 2009). Copper materials, for instance, have been used since 2200 BC to produce drink and food storage vessels, which due to their antimicrobial properties prevented spoilage (Dollwet and Sorenson 1985). Then, with the advent of nanotechnology, it was hypothesized that wide use of this technology could be useful in healthcare premises and medical instrumentations, to prevent hospital infections and pandemic diseases especially in the case of microorganisms that have developed a resistance towards conventional disinfecting agents and antibiotics (Ren et al. 2009). The use of CuO NPs in coated orthodontic brackets for preventing Streptococcus mutans infection (Ramazanzadeh et al. 2015) or in novel electrochemical immunoassays for virus detection, such as the one recently developed for the detection of H1N1 in human samples (Li et al. 2012), are just a few practical examples of this new trend.

Copper ions exert their antimicrobial activity primarily through the generation of reactive hydroxyl radicals, known to cause protein oxidation, DNA and RNA cleavage and membrane damage due to lipid peroxidation (Halliwell and Gutteridget 1984; Peña, Koch and Thiele 1998). More specifically, copper ions are hypothesized to interact with negatively charged peptidoglycans on the outside of the bacterial cell wall, stimulating redox reactions that can lead to the production of reactive oxygen species (such as hydrogen peroxide, H₂O₂).
subsequently damaging the cell membrane. Inside the cell, Copper ions can disrupt important biochemical processes by displacing essential metals from their native binding sites in proteins or binding with DNA molecules leading to the disorder of the helical structure by crosslinking within and between the nucleic acid strands (Stohs and Bagchi 1995; Kim et al. 2000).

Several studies have shown that CuO NPs are highly toxic to a range of models including algae (Wang et al. 2011b), bacteria (Bosetti et al. 2002; Gunawan et al. 2011), fish (Zhao et al. 2011) and mammalian cells such as the Caco-2 enterocytes, causing cytotoxicity and inflammation, (Ude et al. 2017) or the A549 human lung epithelial cell line, causing both oxidative stress and DNA damage (Karlsson et al. 2008; Wang et al. 2012). Finally, a strong toxic effect was reported on Wistar rats exposed to CuO NPs both via inhalation (Gosens et al. 2016a) and instillation, assessing the pulmonary toxicity through the analysis of lactate dehydrogenase (LDH), alkaline phosphatase (ALP) and total proteins in bronchoalveolar lavage fluid (Rani et al. 2013). No studies could be identified that investigated the toxic effect exerted by CuO NPs on hepatocytes, despite evidence that the liver is the primary site of NP accumulation in vivo (Sanhai et al. 2008; Yoo, Chambers and Mitragotri 2010; Anselmo et al. 2013).

Within the SUN project, the in vitro assessment of CuO NP toxicity was led by Heriot-Watt University (UK) and the Karolinska Institutet (Sweden). Heriot-Watt University focused on the human C3A hepatocyte cell line, as representative of the liver, primary site of accumulation of NPs and detoxifying organ of the human body. The Karolinska Institute analysed the effects elicited on macrophages (RAW 264.7) as the cell type primarily involved in the uptake of NPs in the lung (Hardy et al. 2013; Gosens et al. 2016a; Kermanizadeh et al. 2016).

The C3A hepatocyte cell line was exposed to a panel of copper-based nanoparticles (NPs) and toxicity assessed via investigation of cytotoxicity and pro-inflammatory responses. Finally, a genotoxicity test through the fpg-modified Alkaline Comet Assay was performed only on CuO NPs and CuSO₄.

2.1.2 Safer by Design (SbyD) Approach

The toxicity of NMs and NPs is closely related to their physicochemical characteristics (Section 1.6) that define their ability to accumulate, persist, and translocate within the environment and the human body (Morose 2010), and the possible routes of exposure consisting of inhalation, ingestion, skin absorption and injection (Section 1.8). Since the use of these NMs is evident in
a vast number of everyday products, nanotechnology might be considered as the next industrial revolution (Rejeski and Lekas 2008). The risk of possible adverse outcomes deriving from the exposure to NPs, even if some fears over the possible dangers may be exaggerated, are not necessarily unfounded (Maynard et al. 2006); for these reasons, novel approaches to ensure nanotechnology is safe are needed. Two possible strategies commonly adopted consist in applying a control step either during the design stage of NPs and products or during later stages, e.g. risk management, of the product life such as processing, manufacturing, use or end of life (Morose 2010).

In order to decrease the toxic effects of several NPs and NMs, specific characteristics (e.g. surface coating) of the material can be altered to modify their biological activity with the aim to maintain the commercial utility of the material (Schulte et al. 2014; Geraci et al. 2015). A practical example of this approach was provided by Sager et al. in a study on how the surface modification of MWCNTs with carboxylic groups (-COOH) decreased the toxicity in vivo in the C57BL/6 mouse model (Sager et al. 2014).

2.1.3 Cytotoxicity

Cell death plays a central role in numerous physiological and pathological processes (Méry et al. 2017). Cytotoxicity or cell death assays are used to rank the relative toxicity of particles and to identify concentrations at which to investigate sub-lethal impacts (e.g. to investigate the mechanism of toxicity).

There are several approaches in analysing cytotoxicity, and countless available cell viability assays (Méry et al. 2017). Some assays are focused on the detection of viability whilst others assess cell death using either viable dyes that can penetrate damaged cell membrane (Trypan blue) or dyes that produce a fluorescent compound once metabolised by the cell, such as the Alamar Blue assay. Other assays assess the presence of intracellular proteins in the supernatant that are discharged from inside the cell due to the loss of membrane integrity, such as the lactate dehydrogenase (LDH) assay. Finally, there are assays designed to assess apoptosis, for example by detecting changes in the lipid membrane, or caspase expression.

In this study, it was planned to use at least two different approaches to analyse the cytotoxic effect of NPs to avoid any misrepresentation of the results due to possible interference caused
by NPs in the assays and to investigate the possible mechanism of toxicity. The approaches used in this study were the Alamar Blue and LDH assays.

2.1.4 Alamar Blue

The Alamar Blue (AB) assay evaluates cell viability by exploiting the natural reducing power of living cells to convert resazurin (the AB reagent) to the fluorescent molecule, resorufin. Resazurin is a non-toxic, cell-permeating compound that is blue in colour and virtually non-fluorescent (Nociari et al. 1998; Al-Nasiry et al. 2007; Rampersad 2012). Upon entering cells, even if the actual mechanism of this process is unknown (O’Brien et al. 2000), resazurin is probably reduced by several mitochondrial enzymes by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes. The metabolic product released by mitochondria is resorufin; this molecule has bright red fluorescence. Since viable cells convert resazurin to resorufin, assessment of fluorescence makes it possible to generate a quantitative measure of viability (Al-Nasiry et al. 2007; Monteiro-Riviere, Inman and Zhang 2009; Rampersad 2012).

2.1.5 LDH assay

Lactate Dehydrogenase catalyses the interconversion of lactate and pyruvate and is located within the cytoplasm of cells. As cells are damaged, and plasma membrane integrity is compromised LDH is released. Therefore, determining LDH release from cells can be used as an indicator of cell death (Chan, Moriwaki and De Rosa 2013). Within the LDH assay, the cell supernatant is collected and the conversion of pyruvate to lactate by LDH is assessed via the use of the dye 2,4-dinitrophenylhydrazine which allows product formation (turning from bright yellow to dark brown) to be quantified via colourimetric measurement.
2.1.6 Inflammation

Since NPs might cause inflammation (Donaldson et al. 2005) via generation of reactive oxygen species (ROS) (Stone et al. 1998, 2016; Fu et al. 2014), and even trigger the immune system (Pallardy, Turbica and Biola-Vidamment 2017) modulating the antigenic response of the antibodies (Ilinskaya and Dobrovolskaia 2016). In vivo inflammatory responses to NMs are characterised by infiltration of immune cells and production of cytokines. The supernatant of treated C3A cells was analysed for the presence of cytokines using an automated multiplex system to measure the response of cells to different copper compounds and NPs. There is evidence that NMs stimulate the production of cytokines (such as IL-8) from hepatocytes (Kermanizadeh et al. 2012c).

Among the cytokines secreted by hepatocytes following exposure to metals and damages caused by oxidative stress, IL-1β, IL-6, IL-8, TNF-α, and RANTES are the most commonly found in in vitro models as well (Dong et al. 1998; Gutiérrez-Ruiz et al. 1999; Barlow et al. 2005; Devhare et al. 2013; Kermanizadeh et al. 2014b; Zhou, Xu and Gao 2016). IL-1β, IL-6 and TNF-α play a pivotal role during the acute inflammatory response activating the functions of inflammatory cells and increasing the vascular permeability (Elsabahy and Wooley 2013) while IL-8 regulates the activation of neutrophils and their recruitment to the site of inflammation together with RANTES (Elsabahy and Wooley 2013; Kermanizadeh et al. 2014b; Fichna et al. 2018).

2.1.7 Multiplex System

The enzyme-linked immunosorbent assay (ELISA) is the test commonly performed to analyse the production of cytokines. In this project was used an automated system that was based on the same principles of functioning of the ELISA. The Bio-Plex™ multiplex system, in fact, is based on magnetic coloured microspheres (beads), each with a distinct colour or spectral address, to allow discrimination of individual group (representing a particular cytokine) within the multiplex suspension. These assays are essentially immunoassays formatted on magnetic beads. Capture antibodies directed against the desired biomarker are covalently coupled to the beads. Coupled beads react with the sample binding the biomarker of interest and are captured to bottom of the well using a magnet. After a series of washes, unbound proteins are removed.
and a biotinylated detection antibody is added to create the sandwich complex. The final detection complex is formed with the addition of streptavidin-phycoerythrin (SA-PE) conjugate as an indicator or reporter (Figure 2.1).

This system not only offers the advantage of a greater sensitivity compared to the manual ELISA present on the market, but it also allows to analyse multiple targets at the same time, reducing time and cost of the whole procedure. Further details about the protocol used are provided in Section 2.2.4.

![Biosen Bio-plex sandwich immunoassay](image)

*Figure 2.1 Bio-plex sandwich immunoassay. Courtesy of Bio-Rad (UK).*

### 2.1.8 Glutathione Assay

Antioxidants are endogenous molecules naturally present in both intracellular and extracellular compartments (Nimse and Pal 2015b) as an immediate defence mechanism to neutralise ROS. However, if the balance is tipped towards oxidative stress due to an increase in ROS production, the cell can upregulate the production of antioxidants (Nimse and Pal 2015a) to help prevent damage (Glantzounis *et al.* 2005a). Glutathione is one of the most common antioxidants produced by the liver, together with enzymes such as glutathione peroxidase, and reductase enzymes superoxide dismutase (SOD), hemeoxygenase (HO), peroxidases and metal binding proteins (Glantzounis *et al.* 2005a, 2005b; Kermanizadeh 2012).

Glutathione is a ubiquitous tri-peptide which primarily functions to scavenge hydrogen peroxide utilising glutathione peroxidise to create glutathione disulphide (GSSG), this oxidized form is then brought back to its reduced form by the enzyme glutathione reductase (Glantzounis *et al.* 2005a). GSH also scavenges other ROS molecules and prevents oxidation of protein
sulfhydryl groups (Diesen and Kuo 2010), and it can be easily depleted by the oxidative action of several NPs (El-Ansary and Al-Daihan 2009).

2.1.9 Genotoxicity

A broad range of NPs have been shown to elicit DNA damage, either through the production of ROS or via physical interaction with cellular components (Gonzalez, Lison and Kirsch-Volders 2008; Lei et al. 2008; Singh et al. 2009; Golbamaki et al. 2015). Evaluating the genotoxicity of NPs is a priority as the accumulation of DNA damage can lead either to carcinogenesis or degenerative conditions (Doak and Dusinska 2017). The most common analyses performed, in order to investigate the genotoxic effects of these materials, are conducted on DNA strand breaks (single and double), DNA repair pathways, gene mutations, chromosomal damage and cell transformation. Recently, other approaches such as epigenetic methods, high-throughput assays, toxicogenomic tools and miniaturisation of classical assays, such as the comet assay, have started to be used in different studies (Doak and Dusinska 2017).

In the SUN project, the assay selected for investigating the genotoxicity of NPs was the fpg modification of the alkaline Comet assay considering both the previous expertise of the Heriot-Watt University and the wider employment of this assay in the literature (Collins 2002, 2004; Karlsson et al. 2015). Due to the time restrictions, additional assays (e.g. micronucleus assay) were not used for data comparison.

2.1.10 Comet assay

The Comet assay, also known as single-cell gel electrophoresis (SCGE), was originally developed by Singh (Singh et al. 1988a) with the aim to find an useful technique to determine DNA damage in a single cell.

In short, after the exposure to a test substance for a specific time, cells are harvested, embedded in low melting point agar and layered on pre-treated microscope glass slides and finally lysed to expose the naked DNA (Figure 2.2).
Then, through a cycle of electrophoresis, the DNA migrates from the original position in accordance with the size of the fragments. The greater the level of damage, the smaller and faster the DNA migrates creating a structure resembling a comet easily observable through DNA staining. The damage is quantified by assessing either the percent of DNA in tail or the tail moment, i.e. the product of the tail length and the fraction of total DNA in the tail (Olive and Banáth 2006). Positive and negative controls, consisting respectively of H$_2$O$_2$ treated and untreated cells, are typically included.

The Comet assay is widely used as it is easy to perform and relatively cheap. However, since its first development, many authors have adapted the original protocol to obtain more reproducible results.

In this study, the Comet assay protocol was compared to other recent publications to optimise and improve the sensitivity towards oxidative damage to DNA. The reason to focus specifically on this kind of effect is linked to the toxic mechanism elicited by metal ions in general and by copper in this particular instance (Bosetti et al. 2002; Karlsson, Holgersson and Möller 2008; Wang et al. 2012).

The alkaline variant of the Comet assay (Singh et al. 1988a; Olive et al. 1990) was applied to assess the toxicity of CuO NPs in this study. Incubation of the naked DNA in an alkaline buffer (pH>13), enhances the unwinding of the DNA and leads to a greater and more pronounced formation of comets. This is possible because a very high pH is needed in order to break all the alkali-labile regions of the DNA such as the AP sites. These sites can be produced either by spontaneous hydrolysis of the N-glycosylic bond or as a consequence of the removal of damaged bases by DNA N-glycosylases and are considered the most frequent spontaneous lesions that occur in DNA (Boiteux and Guillet 2004). In this variation of the assay, an additional step of incubation of the naked DNA with a specific enzyme called formamidopyrimidine DNA glycosylase (fpg) was included. This base-excision repair enzyme firstly isolated from E. coli can identify and remove a broad range of oxidized purines, i.e. 7,8-dihydro-8-oxoguanine, 8-oxoadenine, fapy-guanine and others (Gedik and Collins 2005). If an increase of breakage is observed in the presence of fpg, this indicates that the DNA damage is mediated by an oxidant mechanism.
The literature used to update this protocol was based mostly on the publications of Collins and colleagues, who covered most of the issues highlighted by many researchers, and who lead numerous international collaborations on this specific topic. Moreover, several suggestions made by some international focus groups on the Comet assay, together with the OECD guidelines for in vivo testing were included as well (Tice et al. 2000; Collins 2002, 2004, 2014; Olive and Banáth 2006; Collins et al. 2008, 2014; Azqueta and Collins 2013; Ersson et al. 2013; OECD 2014).

In general, the enzyme-modified alkaline version of the comet assay can be divided into nine operative steps, each one potentially exposed to various external factors able to change the outcome (Figure 2.3).

2.1.11 Influence of light conditions

One of the first recommendations in the literature is that all samples should be handled in dark conditions or in yellow light to prevent DNA damage from ultraviolet radiation (UV) (Nowsheen, Xia and Yang 2012). This is important because artificial lights in the laboratory can generate a small amount of UV (Klein et al. 2009) leading to an increase in the background levels of damaged DNA. For this reason, in this study, experiments were conducted in reduced lighting.
2.1.12 Cell collection

Next, the influence of the method of cell collection on the results obtained for the Comet assay was assessed. A number of enzymatic or mechanical approaches can be used such as trypsinization or cell scrapers, but these, especially trypsin treatment, can cause damage to the cells (Huang et al. 2010). To overcome this, the trypsinization time was limited to not more than 3 minutes. As some cells still adhered to the cell culture plates following trypsinization, the plates were vortexed for a few seconds to help detach the cells. This was found to be a better approach to collecting the remaining cells when compared to mixing with a pipette, as Trypan Blue staining revealed that the excessive mixing using pipetting at this stage caused an increase in damage of the plasma membrane (data not shown).

2.1.13 Agar Concentration

Existing studies have used different concentrations of agar (ranging from 0.5 to 1 %) (Olive and Banáth 2006; Johansson et al. 2010; Collins et al. 2014). This particular aspect is widely cited as an issue influencing the high inter-laboratory variation present in the Comet assay (Collins et al. 2008, 2014; Ersson et al. 2013). In this study, rather than in a microwave the low melting point agar (LMPA) was melted in a water bath in a sealed Falcon tube more than three times (three repetitions of the experiment). This simple method helped to minimize any loss of water keeping the final concentration (0.75%) as close to constant as possible during the subsequent experiment. Then the addition of cell suspension to the LMPA resulted in a final gel of 0.6% LMPA.

Furthermore, using an ultra-low melting point agarose (Sigma, A5030) rather than the usual LMPA (Sigma, A9414) also gives some advantages at this stage. Even if both these products show a close melting point (~50-55ºC), they differ considerably in their transition state having a jelling point at 8-17ºC the first one and 26-30ºC the latter. This difference solves the issue of working quickly during this phase with the consequent detriment of precision in mounting the slide properly. This might help to obtain a more stable gel layer with a more homogeneous distribution of cells among the slide itself.

After the layer is settled, the following step consists of removing the coverslips before placing the samples in lysis buffer. Some colleagues, at this point, will observe gel breakage when they
try to remove the cover slides; in addition, even if the gel does not detach from the slide, the
delicate handling procedure could cause some damage at the integrity of the gel that it is going
to be noticed during the scoring (small cracks, excessive smearing, and halos). The reasons for
such damage are quite simple: since the slides are too thin, when left on ice for a long time a
frosting process may start that “seals” the coverslip; moreover, the difference in temperature
between the slide and the room creates a condensation process that covers with drops of water
the upper side of the slide making it slippery.

Two very effective solutions to avoid this kind of problems consist in just removing the gel
from ice for a couple of minutes before trying to remove the coverslips and to use a tissue for
this action. Of course, ideally, the best solution would be to avoid keeping the slides on ice for
too long, but when managing a large number of samples, this could be difficult.

2.1.14 Lysis

The lysing step is not considered critical and seems to cause not much impact on the results of
the assay (Collins et al. 2014). Some authors have tried lysing for just one hour or up to one
week without a significant difference in the outcome and even obtaining the same comets in
the sample treated with H₂O₂ or methyl methane-sulphonate both with and without lysis
(Collins et al. 2014).

The following stages involve unwinding of DNA and electrophoresis, during these steps
various factors can lead to a significant variability of results. First of all, the DNA, embedded
in the gel after the lysis and washing step in high salts solution, is “naked” of any cellular
structure and proteins; this is the moment where more attention is needed to avoid any rise of
temperature and light exposure.

2.1.15 Tank selection

There are numerous Comet assay products available, which consist of specially designed black
tanks that block the light and that are accessorized with a cooling system that it can be directly
connected to tap water.
This special designed electrophoretic tank was examined and used during the initial first attempts performed during the protocol design of this study, but the benefits were too low to justify the expense. For instance, after closing the tank it is impossible to check the slides until the experiment is finished, consequently, if something wrong happens, there is no chance to rectify the problem. Moreover, the cooling system does not keep the sample at a very low temperature.

As an alternative, a simple polystyrene box, big enough to contain a bed of ice and the electrophoresis tank, was used. With this cheap option, it was not only possible to keep the samples safe from direct light exposure and maintain direct access during the experiment, but also to uphold a very low temperature (~4°C) during the whole experiment.

2.1.16 Unwinding in alkaline buffer

For the unwinding step, some authors have tried different incubation times (from time zero to 60 minutes) reaching the conclusion that the comet tails increase proportionally with incubation time and that a plateau is reached at 40 minutes at low temperature (Azqueta et al. 2011a). A possible explanation for that could be the resilience of the apurinic/apyrimidinic site (AP) sites to be digested even at high pH (>13) (Collins et al. 2008).

2.1.17 Electrophoresis and voltage settings

There is not a unique way to set the instrument parameters during the electrophoresis, and these settings might usually vary between different protocols and for different purposes. For example, Singh et al. (1988a) suggested running for 30 minutes at 24 V and 300 mA. In a publication from Heriot-Watt University using hepatocytes (Kermanizadeh et al. 2012a), the enzymatic variation of this protocol developed by Speit (Speit et al. 2004) was used with 15 minutes at 270 mA and 24 V as default electrophoresis settings.

On the other hand, there is a fruitful debate between scientist over the critical issues of this assay, and it became evident that expressing the power as the total voltage applied during the electrophoresis was conceptually wrong due to the different sizes of tanks used in various laboratories. The best metric to be used to avoid any confusion is V/cm with an optimum between 0.7-1 V/cm (Tice et al. 2000). The measurement used in this calculation should be
the length of the upper surface where the slides are loaded, rather than the distance between the two electrodes. This is because the resistance in the gel is higher than in the rest of the tank. This approach allows comparing the settings used even when running the experiments with different tanks.

For the ampage, probably the habit of running electrophoresis under the 300 mA limit was adopted because this is the limit reached by the most common power supplies, so that using an instrument able to reach greater amperage would be impractical and expensive (Collins et al. 2008).

Singh et al. (1988b) specified leaving a thin layer of buffer on top of the slides. However, almost all the publications analysed did not mention the depth of the buffer to be maintained during the electrophoresis.

If we consider the electrical resistance:

$$ R = \rho \times \frac{l}{A} $$

- $R$ is the resistance in Ohms ($\Omega$)
- $\rho$ is the resistivity ($\Omega \cdot m$)
- $l$ is the conductor’s length (m)
- $A$ is the cross-sectional area of the conductor ($m^2$)

Then, how it is related to the current through the Ohm’s law:

$$ I_R = \frac{V_R}{R} $$

- $I_R$ is the current in Ampere (A)
- $V_R$ is the voltage (V)
- $R$ is the resistance ($\Omega$)

These equations suggest that the cross-sectional area of the conductor leads can influence the results of the electrophoresis and subsequently the migration of DNA. The higher the depth of the buffer on top of the gel, the smaller the influence on DNA migration. Trying to maintain the same ampage during the experiment could be an indirect method of avoiding this effect, but this is difficult the ampage is never completely stable during the electrophoresis. For these reasons, the amperage at the beginning and at the end of every experiment was measured, and the depth of the buffer was kept constant during each experiment (2mm). Finally, a small error in positioning the tank could lead to a huge variation in the readings (Figure 2.4).
Figure 2.4 Position effect of the slide against the working bench: a small tilt of the tank can cause the DNA not running perpendicularly to the gel. The final effect is likely to be a misreading of the length of the tail.

For this reason, a spirit level was used to check the position of the tank after it was placed on ice.

2.1.18 Staining and Imaging

A potentially problematic step in the Comet assay is the staining, imaging and scoring the tails (Forchhammer et al. 2010; Collins et al. 2014). The staining intensity could vary if using a stain such as propidium iodide (Olive et al. 1990), while Hoechst 33342 and DAPI, even if showing minor sensitivities compared to propidium iodide, can give more stable results (Olive et al. 1990). Some studies also suggest that a Mercury vapour lamp (the traditional UV lamp) can be a source of variation if compared with a more modern source of energy like LED systems (Olive et al. 1992; Collins et al. 2014).

2.1.19 Scoring

Comparing the different methods such as visual, manual and automated scoring, Azqueta et al. (2011b) found similar results using the different approaches. The only two significant differences were: the visual scoring, being incapable of detecting low amounts of DNA damage and the automated one, being unable to detect a too high amount of DNA damage. In this study, a blind manual scoring using the Comet IV software for imaging was performed to avoid any subjective interpretation of the results and any involuntary misreading of comet tails due to the
observer expectative. The blind scoring system adopted was performed by a laboratory staff member who was not connected to this study.

Another aspect to consider during the scoring process is the edge effect. It is always good practice to exclude any reading near the corner of the slide and any crack or air bubble embedded inside the gel; cells in this area of the slide often show a high grade of damage (Collins 2004).

The last aspect involved in developing the Comet assay protocol was that the presence of hedgehogs (cells with almost no head and nearly all the DNA present in the tail) need to be considered. Some authors do not include them in the scoring because they consider the hedgehogs as an early form of apoptosis (Olive, Frazer and Banáth 1993; Fairbairn et al. 1996; Kizilian et al. 1999). Other authors highlighted that the size of the DNA fragments present in the hedgehogs would be too big to be the result of an apoptotic process. Moreover, some studies show that after 30 minutes of incubation in fresh medium prior the Comet assay, the same cells were able to repair the damage caused by treatment with H$_2$O$_2$ showing no more hedgehogs-like structures (Tice et al. 2000; Collins et al. 2008). Accordingly, the hedgehogs in this study were considered as highly damaged cells, and consequently, they were included in the analysis.

2.1.20 Interference

One of the most interesting issues related to NM hazard studies is the concept of interference. NMs can interact with organic compounds such as proteins, enzymes, lipids, nucleotides as well as with dyes, substrates and culture media (Kroll et al. 2012). Interactions with organic compounds can be responsible for their biological effects, or they can lead to a misinterpretation of data generating false positive or negative results (Stone, Johnston and Schins 2009). For example, adsorption of cell signalling molecules such as cytokines onto the surface of NMs reduces their ability to be detected by ELISA (Brown et al. 2010). An example of NMs interfering with assay reagents was demonstrated for the MTT assay (Wörle-Knirsch, Pulskamp and Krug 2006), and is the reason that many authors now opt for the WST-1 or Alamar Blue assay to assess metabolic indicators of viability. In regard to the possible generation of false positive or false negative results, genotoxicity tests performed on anatase TiO$_2$ showed these NPs were photo-genotoxic in both Comet assay and chromosome aberration
assay performed on mouse lymphoma L5178Y cells and on CHL/IU (Chinese hamster lung) cells respectively (Nakagawa et al. 1997), while no photo-genotoxicity was observed using the Ames test or with the gene mutation assay performed on mouse lymphoma L5178 tk<sup>+/−</sup> cells (Warheit et al. 2007).

NM interference not only arises due to their size and chemical composition but also to other intrinsic properties such as crystallinity, agglomeration state, coating, shape and micro-porosity (Ibidem), hence the suggestions to verify toxicological data using at least two assays (Monteiro-Riviere, Inman and Zhang 2009). However, these foresights might not be adequate for excluding misleading results and misinterpretations (Kroll et al. 2009, 2012; Monteiro-Riviere, Inman and Zhang 2009) such as false positive or false negative results. Therefore a fundamental aspect of all work performed when assessing the toxicity of NMs in vitro is monitoring their potential to interfere with the assays used.

It is widely demonstrated that NMs can enter a variety of cells; specifically, CuO NPs can reach different cellular compartments including the nucleus (Cronholm et al. 2012, 2013; Gaiser et al. 2013b; Karlsson et al. 2014). Even if this is the driving reason for all the studies involving the genotoxicity of NMs, there could be the chance of direct interactions between the same NMs and the comet assay (Azqueta and Dusinska 2015). Some authors recently started to report the presence of NMs trapped inside the gel during the comet assay (Stone, Johnston and Schins 2009; Karlsson et al. 2015) and, from these observations, the question arising was:

Can NMs trapped in the electrophoresis gel interfere during the assay (i.e. DNA) creating false results?

To answer this question, a specific modification of the protocol was developed. The presence of NPs inside and around the cell was simulated by suspending in the LMPA an equivalent amount of CuO NP to obtain the same concentration used during the exposure (BMD<sub>20</sub>). This suspension was then mixed with C3A cells that had not been treated with any NPs and then, subjected to the Comet assay. This would, therefore, allow NPs in the agarose gel to directly interact with the lysed cells and any DNA present.

The main hypothesis was that in case no interaction occurred between NPs and the Comet assay, it should be possible to obtain almost no tail as in a normal negative control, while if interactions between NPs and DNA occurred, it should be possible to observe comet formation even in ‘untreated’ cells.
2.1.21 Aims

The aim of this research was to assess the toxicity of CuO NPs to hepatocytes *in vitro*. The objectives were to:

1. Assess the cytotoxicity of a panel of copper-based materials to C3A cells.
2. Assess the cytokine production by C3A cells following exposure to the copper-based materials.
3. Detect and quantify glutathione levels in C3A cells treated with CuO NPs.
4. Assess the genotoxicity of CuO NPs to C3A cells.
5. Investigate any interference by CuO NPs in all assays.

2.1.22 Hypotheses

- CuO NPs and micronized copper will elicit greater effects (in terms of cytotoxicity, cytokine induction, glutathione depletion and genotoxicity) in C3A cells compared to equivalent concentrations of copper ions and a Cu-amine solution.
- The safer by design approach can be used as an effective strategy to decrease the toxic effect of CuO.
2.2 Materials and Methods

Unless otherwise stated, chemicals were purchased from Sigma (Poole, UK)

2.2.1 Copper compounds and nanoparticles

Copper oxide (CuO) NPs (Table 2.1), used as wood coating preservative, were supplied from Plasmachem (Germany) within the SUN project. CuO NPs were supplied as a dry black powder with an average primary particle size of 12 nm (as assessed by TEM from the supplier). For a comparison of toxic potency between CuO NPs and Cu ions, copper sulphate (CuSO₄; Sigma) and copper chloride (CuCl₂; Sigma) were included in the hazard studies.

Table 2.1 Summary of the safety data sheet of CuO NPs as provided by Plasmachem (Germany).

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>SUN Code</th>
<th>Batch/Lot</th>
<th>Supplier</th>
<th>Primary size Min- Max (average)</th>
<th>Form / Colour</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper Oxide</td>
<td>CuO_1_NP_SYN</td>
<td>YF131107</td>
<td>Plasmachem</td>
<td>3-35 (12) nm</td>
<td>Dry / Black</td>
</tr>
</tbody>
</table>

According to the SUN project Deliverable D 1.5 (available online at [http://www.sun-fp7.eu](http://www.sun-fp7.eu)), morphological characterization of pristine CuO NPs was performed at Ca’ Foscari University (Italy) and the Institute of Science and Technology for Ceramics within the National Research Council of Italy (CNR-ISTEC) (Appendix).

For the in vitro toxicology study, the selected media were either Minimum Essential Medium Eagle or Leibovitz’s L-15 (Lonza) supplemented with 10% heat-inactivated Foetal Bovine Serum, 1% Penicillin-Streptomycin, 1% L-Glutamine solution, 1% Sodium pyruvate solution and 1% Non-essential Amino Acid Solution, at a concentration of 100 µg/ml. The Dynamic light scattering (DLS) results showed that CuO NPs are unstable in suspension as they agglomerate and de-agglomerate (or dissolve) after incubation for 24 hours at 37°C (Table 2.2) as the DLS main peak, present in fresh preparations, disappear after 24 h of incubation, and two new peaks with a smaller and a bigger size appear. The results were reported as an average of three independent measurements.
Table 2. Hydrodynamic diameter of CuO NPs in cell medium. Measurements (n=3) performed by Ca’ Foscari University (Italy) 1 hour after dispersion (T₀) and after 24 hours (T₂₄). DLS results were expressed as z-average, representing the harmonic intensity-weighted arithmetic average particle diameter, the particle size distribution (PSD) and the polydispersity index (Pdi), a dimensionless measure of the broadness of the size distribution calculated from the cumulants analysis. Blue values represent analysis performed on complete cell medium without NPs. Experiments (n=3). Complete set of data available online at http://www.sun-fp7.eu (Project Deliverable D 1.5).

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Z-aver and PSD (Mean dm in nm ± SEM) Time: 0 h</th>
<th>Pdi Time: 0 h</th>
<th>Z-aver and PSD (Mean dm in nm ± SEM) Time: 24 h</th>
<th>Pdi Time: 24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>CuO NPs</td>
<td>130.98 ± 7.17</td>
<td>0.57 ± 0.09</td>
<td>40.11 ± 10.23</td>
<td>0.53 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>201.23 ± 15.10 (88%)</td>
<td></td>
<td>45.49 ± 6.23 (44%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.24 ± 1.95 (5%)</td>
<td></td>
<td>405.56 ± 111.77 (30%)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4679.31 ± 121.29 (7%)</td>
<td></td>
<td>50.01 ± 0.49 (20%)</td>
<td></td>
</tr>
</tbody>
</table>

The scanning transmission electron microscopy (STEM) analysis to assess NP morphology was performed using a Field Emission-Scanning Electron Microscopy (FE-SEM) instrument (Carl Zeiss Sigma NTS Gmbh, Oberkochen, DE). Briefly, a single drop of 10mg/l CuO NPs suspension was deposited on a film-coated copper grid and air dried, and then, more than 50 particles were analysed to estimate the size distribution of the particle. The results showed the presence of spherical and monodispersed CuO NPs with a primary nanoparticle average diameter of about 12 ± 8 nm in agreement with the description of the supplier (Plasmachem, Germany) (Figure 2.5).

A dissolution test was performed by ISTEC-CNR (Italy) using two physiologically relevant media such as the gambler’s solution, resembling the composition of lung fluids, and the artificial lysosomal fluid (ALF) which simulates the acidic environment of the lysosome. After 1 and 24 h, 15 mL of CuO-ALF or CuO-Gamble dispersion were centrifuged (at 2000 g for 30 min) with a centrifugal filter unit (Amicon Ultra-15, 10 kDa, Millipore) to separate solid particles from the solution. The obtained solution was acidified with 1 ml HNO₃ to determine the soluble Cu concentration by inductively coupled plasma optical emission spectrometry (ICP-OES) analysis. The results showed that the dissolution of CuO NPs was not time dependent but rather linked to the pH; in fact, approx. 1.5% of CuO is dissolved in Gamble solution (pH 7.4) both after 1 and 24 hours while approx. 62% of CuO is dissolved in ALF (pH 4.5) both after 1 and 24 hours (Table 2.3).
**Figure 2.** 5 Electron Microscopy analysis of pristine CuO NPs. STEM image in MilliQ water (A); Shape of the dispersed NPs(B) and their size distribution (C) in MEM at a concentration of 200µg/ml. Statistical analysis performed on more than 50 particles. Experiment performed by Ca’ Foscari University (Italy). Complete set of data available online at [http://www.sun-fp7.eu](http://www.sun-fp7.eu) (Project Deliverable D 1.5).

**Table 2.** Dissolution Rate of CuO NPs in different media. Ions release of CuO NPs during 24 hours incubation time at 37°C in two physiologically relevant media at different pH (n=3). Data from ISTEC-CNR (Italy).  

<table>
<thead>
<tr>
<th></th>
<th>CuO (mg/l)</th>
<th>Cu²⁺ (mg/l)</th>
<th>Cu²⁺/CuO ratio (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gamble</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.4</td>
<td>Reference</td>
<td>122</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>-</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>-</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>ALF</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 4.5</td>
<td>Reference</td>
<td>95.7</td>
<td>76.3</td>
</tr>
<tr>
<td></td>
<td>1 h</td>
<td>-</td>
<td>66.4</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>-</td>
<td>67.8</td>
</tr>
</tbody>
</table>

**Modified CuO NPs**

Within a Safer by Design (SbD) approach, dispersion stability of pristine CuO NPs was assessed and improved by using four different relatively low-hazard stabilizers by ISTEC-CNR.
(Italy), leading to positive (polyethylenimine), negative (sodium ascorbate and sodium citrate) and neutral (polyvinylpyrrolidone) surface charge. The CuO NPs were dispersed and functionalised in a buffer solution to simulate the typical pH of the extracellular fluid (pH=7.4) prior to adding the capping agents. The capping agents used were: Na Citrate (CIT), MW 294.10 (Sigma); Polyvinylpyrrolidone (PVP), MW 29000 (Sigma); Polyethylenimine (PEI), MW 750000 (Fluka); Na ascorbate (ASC), MW 198.106 (Riedel) (Table 2.4 and Figure 2.6). More details about the SbD procedure for modifying the CuO NPs are available online (http://www.sun-fp7.eu) on the Deliverable 7.1.

Table 2.4 Hydrodynamic diameters (nm) and ζ-potential (mV) of functionalised pristine and modified CuO NPs. Experiments (n=3) performed by ISTEC-CNR (Italy). Complete set of data available online at http://www.sun-fp7.eu (Project Deliverable D 1.5). Data from environmentally relevant media omitted.

<table>
<thead>
<tr>
<th></th>
<th>Hydrodynamic Diameter (nm)</th>
<th>ζ-pot (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MilliQ (pH=6.5)</td>
<td>PBS (pH=7.4)</td>
</tr>
<tr>
<td>CuO-pristine</td>
<td>1093 ± 50</td>
<td>2756 ± 347</td>
</tr>
<tr>
<td>CuO-CIT</td>
<td>368 ± 10</td>
<td>271 ± 43</td>
</tr>
<tr>
<td>CuO-ASC</td>
<td>122 ± 1.4</td>
<td>1314 ± 525</td>
</tr>
<tr>
<td>CuO-PEI</td>
<td>247 ± 14</td>
<td>209 ± 16</td>
</tr>
<tr>
<td>CuO-PVP</td>
<td>797 ± 84</td>
<td>2765 ± 432</td>
</tr>
</tbody>
</table>
Figure 2. 6 Average sedimentation velocity data for functionalized pristine and modified CuO NPs. Experiments (n=3) performed by ISTEC-CNR (Italy). Complete set of data available online at http://www.sun-fp7.eu (Project Deliverable D 1.5). Data from environmentally relevant media omitted. In the x-axis: milliQ water (MQ), phosphate buffer solution (PBS), Minimum Essential Medium Eagle (MEM) and Dulbecco modified MEM (DMEM).

Alkaline copper amine solution and micronized copper

BASF (Germany) provided two copper dispersions used as wood preservatives, which work by preventing mould formation. The first dispersion (LP 17206) contained micronized basic copper carbonate (CuCO$_3$·Cu(OH)$_2$) with a copper content of 30% (g/g) and a median hydrodynamic size of 124 nm in mass metrics and 34 nm in number metrics, corresponding to an ensemble surface area of 16 m$^2$/g (data provided by supplier). The second impregnation solution, (LP 17623) containing soluble Cu-Amine and 10% total copper content is the current European gold standard for antimicrobial wood protection.

The endotoxin content of all Cu containing treatments was assessed using the QCL-1000™ Endpoint Chromogenic LAL Assay (Lonza) was performed at the Karolinska Institutet (Sweden). All values obtained were below 0.5 EU/ml, considered the maximum admissible limit for medical devices. Additionally, no interference between the NP samples and the assay substrate were observed as stated by the researcher at Karolinska Institutet who performed this work.
2.2.2 Preparation of NMs and the C3A cell line

Cell culture

The C3A hepatocellular carcinoma cell line (obtained from the American Type Culture Collection; number: CRL-10741™) was grown in Eagle’s minimal essential medium (MEM). The supplements used were: 10% foetal bovine serum (FBS; Sigma), 1% penicillin/streptomycin (Sigma P0781), 1% L-Glutamine solution (200 mM, Sigma G7513), 1% sodium pyruvate solution (100 mM, Sigma S8636) and 1% non-essential amino acid solution (100x, Sigma M7145) (termed complete MEM) in a humidified atmosphere at 37 °C and 5% CO₂. For cytotoxicity analysis, 100µl of C3A cell suspension (concentration of 0.5x10⁶/ml) were transferred to a 96 well plate. For the comet assay and glutathione analysis, 3ml of 0.5x10⁶ cells suspension were seeded in 6-well plates to obtain a layer of approximately 90-100% confluence after 24 hours. The number of cells seeded for both the 96 and the 6 well plates was 1.5x10⁵ cells/cm².

Dispersion protocol

Copper oxide NPs were suspended in 2% heat-inactivated FBS (in Milli-Q water) at a concentration of 1 mg/mL, then vortexed for 20 seconds, according to the ENPRA protocol (ENPRA 2015). The NP suspension was then sonicated for 16 minutes in a bath sonicator (32-38 kHz at 20°C). Following the sonication step, samples were immediately suspended in complete MEM. The same dispersion protocol was used for copper salt compounds (CuSO₄ and CuCl₂), while for the modified CuO dispersions, the micronized copper and the copper amine solutions, 30 seconds high-speed vortex were applied right before use. No further sonication was performed to avoid any possible damage to the capping agents.
2.2.3 Cytotoxicity

*Alamar Blue assay*

The cytotoxic potential of the NPs was initially investigated using the Alamar Blue (AB) cell viability assay. C3A cells were exposed to increasing concentrations of NPs and chemical compounds (from 0.9 µg/ml to 125 µg/ml) (100µl/well) for 24 hours. The concentration of each copper material was standardized in order to expose the cell line to the same nominal amount of copper present in the dispersion/solution. The negative control consisted of viable untreated cells, while the positive control consisted of 100 of 0.01% Triton in MEM added to the cell culture.

After exposure, the supernatants were removed and frozen at -80°C for cytokine analysis (Section 2.2.4). Cells were washed twice with 100 µL PBS and viability was measured using the Alamar Blue assay. For this purpose, 100 µL resazurin salt (10 µg/mL in complete MEM) was added to each well and incubated in a humidified atmosphere at 37 °C and 5% CO₂ for two hours. Then, fluorescence was measured at a wavelength of 570 nm using a Dynex Magellan Biosciences MRX Revelation plate reader. After this first reading, the plate was spun for 5 minutes at 716 g at room temperature, and the supernatant was transferred to a new plate. Fluorescence of both supernatant and cells/NPs pellet was measured again to investigate the presence of any potential interference.

Three repetitions were performed for this study and each time the concentrations tested were changed to obtain 35 different concentrations in triplicate totalling 105 raw data points. These results were used to calculate the Benchmark Dose where 20% effect was detected using the R software with PROAST package (version 38.9). An additional statistical analysis was performed for measuring the EC₅₀ using SigmaPlot (version 13).

*LDH assay*

Part of each supernatant collected from the AB experiment was analysed using the Pierce LDH Cytotoxicity Assay (Thermo Fisher Scientific, Perth) according to the manufacturer’s instructions. In short, 50 µl of supernatant were transferred in a 96-well plate, then 50 µl of the reaction mixture (composed by reaction buffer and a substrate) was added and incubated at
room temperature for 30 minutes. Finally, 50 µl of stop solution were added before measuring absorbance at 490 nm and 680 nm. To assess interference, 3 ml from a suspension of 0.5x10^6 C3A cells/ml were transferred to a 6-well plate and incubated for 24 hours at 37°C (5% CO₂). Then, the cells from the whole plate were lysed to obtain a suspension high in LDH content. The lysate was then transferred to a 96-well plate (40 µl per well) and brought up to a volume of 50 µl by adding 10 µl of treatment as described in Table 2.5. The LDH activity was assessed as described above.

Table 2.5 Sample preparation for LDH interference screening.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cell Lysate</th>
<th>Medium</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl dispersant</td>
</tr>
<tr>
<td>Blank</td>
<td>-</td>
<td>40 µl</td>
<td>10 µl dispersant</td>
</tr>
<tr>
<td>Blank plus CuO</td>
<td>-</td>
<td>40 µl</td>
<td>10 µl from 1 mg/ml CuO</td>
</tr>
<tr>
<td>Blank plus CuSO₄</td>
<td>-</td>
<td>40 µl</td>
<td>10 µl from 1 mg/ml CuSO₄</td>
</tr>
<tr>
<td>CuO 100 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 1 mg/ml CuO</td>
</tr>
<tr>
<td>CuO 200 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 2 mg/ml CuO</td>
</tr>
<tr>
<td>CuO 300 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 3 mg/ml CuO</td>
</tr>
<tr>
<td>CuO 400 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 4 mg/ml CuO</td>
</tr>
<tr>
<td>CuSO₄ 100 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 1 mg/ml CuSO₄</td>
</tr>
<tr>
<td>CuSO₄ 200 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 2 mg/ml CuSO₄</td>
</tr>
<tr>
<td>CuSO₄ 300 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 3 mg/ml CuSO₄</td>
</tr>
<tr>
<td>CuSO₄ 400 µg</td>
<td>40 µl</td>
<td>-</td>
<td>10 µl from 4 mg/ml CuSO₄</td>
</tr>
</tbody>
</table>

2.2.4 Multiplex analysis

The production of cytokines from C3A cells was evaluated at 24 hours post exposure using the Bio-Plex® Multiplex system in the remaining supernatant of cells collected as part of the cytotoxicity testing. The concentrations of the equivalent molar copper present in the BMD_{20} and half the BMD_{20} of CuO NPs were used for comparing the effect of the different copper compounds (Table 2.6).
Table 2. Estimation of nominal copper from CuO BMD$_{20}$. The calculation was performed by subtracting from the CuO amount the mass percentage of oxygen.

<table>
<thead>
<tr>
<th>Sub Lethal Doses</th>
<th>CuO NPs</th>
<th>Equivalent Molar Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD$_{20}$</td>
<td>25.8 µg/ml</td>
<td>20.61 µg/ml</td>
</tr>
<tr>
<td>0.5x BMD$_{20}$</td>
<td>12.9 µg/ml</td>
<td>10.30 µg/ml</td>
</tr>
</tbody>
</table>

The cytokines IL-1β, IL-6, IL-8, TNF-α, and RANTES were analysed according to the manufacturer’s protocol (Bio-Rad, Dalkeith). The experimental setup included a negative control (supernatant of untreated cells) and a blank (complete MEM). Initially, 50 µl of magnetic beads solution was added in each well of a blackened 96-well plate before adding either standards or samples (50 µl per well). After 30 minutes incubation at room temperature shaking at 850 rpm, the beads were captured using a 96 well plate magnetic adaptor and washed 3 times. Then, 25 µl of detection antibodies solution were added before incubating for 30 minutes at room temperature, shaking at 850 rpm protected from light. Beads were captured and washed again as above, and 50 µl of Streptavidin-PE were added and incubated for 10 minutes at room temperature on 850 rpm shaking, covered from light. After the last cycle of washing, supernatants were removed, and 125 µl of assay buffer was added to each well. After another 10 minutes incubation, the plate was read using the Bio-Plex® MAGPIX™ Multiplex Reader.

2.2.5 Glutathione assay

A suspension of 3ml/well from a stock of 0.5x10$^6$ C3A cells/ml was transferred to a 6-well plate and incubated for 24 hours at 37°C (5% CO$_2$) and then, exposed to either CuO NPs or CuSO$_4$ at concentrations of 20.61 µg/ml and 10.30 µg/ml of equivalent molar copper, which were equivalent to the CuO BMD$_{20}$ and half BMD$_{20}$ values respectively from the previous cytotoxicity tests, for 6 and 24 hours. Untreated cells were used as a control for establishing the basal level of glutathione.

The cells were washed twice with 1 mL of PBS and frozen in liquid nitrogen. Immediately after, the cells were thawed in 1.5 ml of ice cold 5% trichloroacetic acid (Sigma) in Redox
Quenching buffer (RQB) (20 mM HCl, 2.15 mM EDTA and 10 mM ascorbic acid), then, the cells were scrapped and resuspended with a pipette before transferring into a clean 2 mL Eppendorf. Samples were centrifuged at 15000g for 5 minutes at 4°C and the supernatant removed and added to an Eppendorf for the GSH and GSH/GSSG quantification following the protocol reported in Senft (Senft, Dalton and Shertzer 2000) with the modification introduced by Kermanizadeh (Kermanizadeh et al. 2012b).

2.2.6 Comet Assay

A suspension of 3ml/well from a stock of 0.5x10⁶ C3A cells/ml was transferred into a 6-well plate and incubated for 24 hours at 37°C (5% CO₂) before being exposed for four hours to either CuO NPs or CuSO₄ at concentrations of 20.61 µg/ml and 10.30 µg/ml of equivalent molar copper, which were equivalent to the CuO BMD₂₀ and half BMD₂₀ values respectively. For analysis of fpg-sensitive sites, microscope slides containing agarose embedded cells were exposed to 100 µL of enzyme buffer containing 0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, and 0.2 mg/mL of albumin from bovine serum. The final pH of the solution was 8. The fpg positive treatments included enzyme (New England Biolabs) at 1:1000 dilution from a frozen stock solution of 8,000 units/ml. Both positive and negative fpg treatments were incubated for 30 minutes at 37 °C protected from direct light. DNA unwinding and electrophoresis time were respectively 40 and 30 minutes followed by 15 minutes in neutralization buffer and a few seconds dehydration in absolute ethanol. The slides were rehydrated and stained with GelRed (Biotium Cat No. 41003-0.5ml). Comets were examined under a fluorescence microscope using an imaging software (Comet Assay IV; Perceptive Instruments, UK) and pictures captured by a stingray (F-033B/C) black and white video camera. In each experiment, the samples were blind-scored evaluating the DNA percentage in the tail of 50 comets/sample for a final amount of 150 comets per treatment. A positive control of 60 µM H₂O₂ (Sigma, 95321-100ML) was added each experiment.
2.3 Statistical Analysis

2.3.1 Benchmark Dose analysis

The Benchmark Dose (BMD) approach (EFSA, 2011) is a relatively new and advanced method of analysing data compared to the No Observed Adverse Effect Level (NOAEL) approach for developing a Point of Departure for risk assessment. The BMD method pre-defines a specific effect, referred to as the Benchmark Response (BMR) and estimates the dose (BMD) associated with the effect we decide to highlight. The BMD is estimated from the complete dose-response dataset by fitting dose-response models. Statistical uncertainties that may be present in the data set are carefully taken into account in the confidence interval around the BMD. The lower limit of which (called BMDL) is the Point of Departure for deriving exposure limits.

The Dutch National Institute for Public Health and the Environment (RIVM) developed a specific package called PROAST for the statistical analysis of dose-response data able to run in the R software. It can be used for:

1) dose-response modelling,
2) deriving a Benchmark Dose in human risk assessment,
3) deriving an effect concentration for ecotoxicological risk assessment.

In PROAST the default assumption for continuous data (i.e. data with a natural lower bound of zero, and with a dimension) is that they are log-normally distributed. Therefore, the analysis is performed on a log-scale, i.e., the data, as well as the dose-response model, is log-transformed, and back-transformed after the statistical analysis. In this way, the dose-response model, and the meaning of its parameters remain as it is. Note, however, that the parameter \( \sigma \) reflects the scatter (residual variance) around the fitted curve on the (natural) log-scale. Further, it is important to observe that the fitted curves in the final plots relate to the median (= geometric mean) at each dose. Similarly, if group means are plotted, these reflect geometric means, not arithmetic means.

This approach uses a generic family of dose-response models for analysing data from toxicity tests:
Model 1: \( y = a \) with \( a > 0 \)

Model 2: \( y = a \exp(x/b) \) with \( a > 0 \)

Model 3: \( y = a \exp(\pm(x/b)d) \) with \( a > 0, \ b > 0, \ d \geq 1 \)

Model 4: \( y = a \left[ c - (c-1) \exp(-x/b) \right] \) with \( a > 0, \ b > 0, \ c > 0 \)

Model 5: \( y = a \left[ c - (c-1) \exp(-x/b)^d \right] \) with \( a > 0, \ b > 0, \ c > 0, \ d \geq 1 \)

Parameter \( a \) represents the background response (response at \( x=0 \)).

Parameter \( b \) reflects the potency of the compound.

Parameter \( c \) is used for curves that level off. Its value is equal to the factor by which the maximum response is larger (if \( c > 1 \)) or smaller (if \( c < 1 \)) when compared to the background response value.

Parameter \( d \) is a shape parameter. It is sometimes argued that the lower bound of parameter \( d \) should be 1 because values of \( d \) smaller than 1 imply that the slope of the curve at dose zero is infinite. This implication is often considered to be biologically implausible. However, when talking about biological plausibility, the log-dose scale is more appropriate. The curve of the model with \( d < 1 \) has slope zero when plotted against log dose. Therefore, all positive values of parameter \( d \) are allowed.

By working with this nested family of models, one can select the one with the optimal number of parameters, i.e. not too few (bad fit), and not too many (over-fit). In general, the decision on the optimal number of parameters may be formally made by the likelihood ratio test. In this way, parameters are only included if such leads to a significantly better fit of the model to the data.

The Hill family of models may be used in addition to the exponential family of models to evaluate a model uncertainty. Model uncertainty refers to the phenomenon that different models pass the goodness of fit test, while the results (such as BMD, BMDL) are not similar. This outcome will happen if the data contain relatively little dose-response information. For
data that contain enough dose-response information, the results from different models will be more similar. Thus, model uncertainty may be evaluated by comparing the results from the (optimal) exponential to the (optimal) Hill model.

Finally, CED is defined as the dose that results in a given (small) percent change in the (continuous) response variable, and it is calculated giving a value for the percent change we consider relevant: Critical Effect Size (CES)

\[
CES = \frac{f(CED) - f(0)}{f(0)} = \frac{f(0)}{f(0)} - 1
\]

The confidence interval for the CED may be calculated by the profile likelihood method also called the likelihood ratio method (Figure 2.7).

Figure 2. 7 Example of calculation of confidence interval performed by R using PROAST package. The upper left was conducted on CuCO₃, upper right on Cu Amine, lower left and right, respectively CuO and CuSO₄.

2.3.2 One- and Two-way ANOVA, and General Linear Model (Graph Pad – Prism v. 6 and Minitab v. 15)

The analysis of variance (ANOVA) is performed to determine whether some significant differences occur between the mean values of three or more sets of data. To use the ANOVA the following assumptions are made: data must be normally distributed, and variances must be homogeneous.
When only an independent variable is present (or analysed) the analysis is referred as “one-way” ANOVA; when the different group analysed are related in some way the analysis is defined as “within the subjects” while if there are no relations between the groups, it is called “between subjects”. When two variables are present, the analysis performed is called “two-way” ANOVA. This analysis using two different variables (time for the glutathione assay and fpg treatment for the comet assay) was performed in this study as well; nevertheless, no significance between the two parameters was found.

Finally, a general linear model (GLM) is an ANOVA procedure in which the calculations are performed using a least squares regression approach to describe the statistical relationship between one or more predictors and a continuous response variable post-hoc Tukey’s honest significant difference test. Predictors can be factors and covariates. GLM codes factor levels using a specific coding scheme (1, 0, -1) as indicator variables, although it is possible to replace this coding scheme with a binary coding scheme (0, 1). The factors can be subdivided into different categories such as crossed, nested, fixed or random. Finally, covariates may be crossed either with each other or with factors or nested within factors. The design may be balanced or unbalanced. GLM can perform multiple comparisons checking the factor levels to find significant differences. As in all the experiment performed, the means of the treatments were compared against the mean value of a control, the Dunnett post hoc test was used.
2.4 Results

2.4.1 Cytotoxicity

*Alamar Blue Assay*

CuO NPs and copper salts (CuCl₂ and CuSO₄) caused a concentration-dependent decline in C3A cell viability 24h post exposure. No changes in cell viability were observed up to a concentration of approx. 10 µg/ml total mass concentration for these treatments; CuO elicited the highest toxic effect with no cells surviving between 50 and 60 µg/ml while both CuSO₄ and CuCl₂ showed a similar effect around 110 µg/ml. In the first instance, the data was presented on the nominal mass of the single compound (Figure 2.8), showing a higher toxicity of CuO nanoparticles when compared to the copper salts (LD₅₀CuO: 32.98µg/ml, LD₅₀CuSO₄: 119.64µg/ml, LD₅₀CuCl₂: 126.56µg/ml). When the concentration of each treatment was expressed as micromoles of copper present in solution (µM), the dose responses shifted closer together, demonstrating almost the same toxic effect for CuO and CuSO₄. It was also noticeable a slightly lower but still significant toxic effect elicited by CuCl₂ (LD₅₀CuO: 0.33µM, LD₅₀CuSO₄: 0.30µM, LD₅₀CuCl₂ 0.45µM) (Table 2.7 and Figure 2.9).
Figure 2. 8 CuO NPs and copper salts cytotoxicity scatter data. Alamar Blue assay data obtained exposing C3A hepatocyte cell line for 24 hours to CuO, CuSO₄ and CuCl₂. Data expressed in µg/ml using PROAST package in R. The data is compiled from 3 separate experiments (n=3), and each data point represents the single replicate conducted on the same day. The large number of concentrations employed, without multiple repeats of each concentration is the starting point for the BMD approach to data analysis. For a better understanding of the quality of the data obtained including a canonical statistic approach, refer to Errore. L’origine riferimento non è stata trovata.

Figure 2. 9 CuO NPs and copper salts molar toxicity. Data obtained from the Alamar Blue assay expressed in total µM of copper. SigmaPlot software. Bars express SD (n=3).
Table 2. CuO NPs and copper salts LD<sub>50</sub>. Results expressed as total mass dose of compound (µg/ml) and as equivalent molar copper content.

<table>
<thead>
<tr>
<th></th>
<th>µg/ml</th>
<th>µM [Cu]</th>
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<tbody>
<tr>
<td>CuO</td>
<td>32.98</td>
<td>0.3298</td>
</tr>
<tr>
<td>CuSO&lt;sub&gt;4&lt;/sub&gt;</td>
<td>119.64</td>
<td>0.2991</td>
</tr>
<tr>
<td>CuCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>126.56</td>
<td>0.4511</td>
</tr>
</tbody>
</table>

Finally, the PROAST analysis performed on CuO NPs showed a BMD<sub>20</sub> of 25.8 µg/ml with a confidence interval between 23.18 and 28.31 µg/ml (Figure 2.10). Rather than using the molar calculation for analysing the data, which takes into account not only the real amount of copper but also the dispersion volumes used during the experiment, the copper amount was expressed as µg/ml of nominal copper rather than as µM, moreover the inflammatory and genotoxic effects comparison between the different copper-based materials were calculated using the BMD<sub>20</sub> of the CuO NPs as reference.

Figure 2. 10 BMD<sub>20</sub> of CuO NPs. Data from the Alamar Blue assay performed on C3A hepatocyte cell line after 24 hours exposure using PROAST package in R software. The data is compiled from three separate experiments (n=3), and each data point represents the mean of three replicates conducted on the same day. The segmented lines show the BMD<sub>20</sub> projection estimated by the software using the Hill model and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of equivalent nominal copper in the dispersion.
LDH Interference

The LDH interference analysis showed that the release of LDH form C3A cell treated with 0.1% Triton was approx. 1600 IU, while the Blank of Medium alone showed an average background of 200 IU. The lysates were treated with increasing amounts of CuO NPs showed a quite strong standard deviation, so that only the 200µg/ml treatment induced a significant decrease in LDH detection compared to the Triton control. On the other hand, all the CuSO₄ treatments induced not only a significant but almost a total inhibition of detectable LDH, even eliminating any background effect in the Blank (Figure 2.11). This suggests that Cu interferes in the LDH assay.

![Figure 2.11 LDH assay performed on C3A cell lysate. On the y-axis, the LDH concentration expressed as IU from three different experiments (n=3); in the x-axis, the different samples tested including Blanks. Bars show S.D. Excel. Confidence interval (Dunnet) analysed with Prism software, v.6.](image-url)
2.4.2 Cytokine Analysis

Initially, IL-1β, IL-6, IL-8, TNF-α and RANTES were analysed in this study. Nevertheless, an increase in cytokine production from C3A cells was only observed for IL-8 after 24 hours treatment with CuO (Figure 2.12) while the other cytokines were below the detection limit. Interestingly, LPS did not induce any cytokine production while the basal amount of IL-8 detected in the untreated samples was usually below 200 pg/ml. Several blanks were also included in the preliminary screening, without finding any significant background or interference between medium and assay (data not shown). At the lowest copper concentration (10.3 µg/ml equivalent copper corresponding to the 0.5x BMD20 of CuO – i.e. 12.9 µg/ml) the NPs were most potent compared to the salt controls. At 20.61µg/ml of equivalent copper (i.e. 1x BMD20) the copper salts induced a higher IL-8 expression while at the highest concentration (i.e. 41.22 µg/ml of equivalent copper or 2x BMD20 CuO), the results showed a decrease in cytokine production, potentially due to the increase of number of dead cells.

![Figure 2.12 CuO NPs and copper salts IL-8 production from C3A cells. Cytokine production from the C3A cell line after 24 hours exposure to 10.3, 20.6 and 41.22 µg/ml of copper content corresponding to 0.5x, 1x and 2x BMD20 of CuO NPs respectively. Results for CuO (A) and copper salt controls, CuCl2 (B) and CuSO4 (C). Data (n=3) expressed as 1000 pg/ml (n=3) Error bar: Standard Deviation.](image)
2.4.3 Glutathione Assay

Glutathione content of C3A cells following exposure to sub-lethal concentrations (10.3 and 20.61 µg/ml of copper) of CuO NPs or CuSO₄ was not significantly affected after 6 hours, with the exception of the highest concentration of CuO NPs. At 24h, all concentrations of CuO NPs and CuSO₄ stimulated a significant reduction in GSH content (Figure 2.13).

Figure 2.13. CuO glutathione assay. Total (red bars) and reduced (blue bars) glutathione production in C3A cells after 6 (A) and 24 (B) hours exposure to CuO NPs and CuSO₄ control salt. Data (n=3) expressed as average (n=3) Error Bars: Standard Deviation. Confidence interval (Dunnet) analysed with Prism software, v.6. Single and double star indicate p ≤ 0.05 and p ≤ 0.01 respectively.
2.4.4 Genotoxicity

According to the Comet assay, CuO NPs caused a significant (p<0.01) increase in DNA damage, as indicated by DNA in the comet tail from treated C3A hepatocytes 4 hours post exposure, with values ranging from a basal level of 5% DNA in the tail in Control to approx. 15-20% in both concentrations of copper (Figure 2.14). The inclusion of fpg did not significantly enhance DNA damage. Metal salts showed a low level of genotoxicity compared to the NPs, with no significant increase in migration of DNA observed (Errore. L’origine riferimento non è stata trovata. A, B). H₂O₂ was able to induce DNA damage enhanced by the inclusion of fpg.

The interference of NPs in the assay was assessed to establish if the DNA damage observed was caused by the nanoparticles during the exposure of cells, rather than as an artefact of the test (e.g. due to an interaction between metal NPs and DNA under an electromagnetic field). The results (Figure 2.15) showed that CuO NPs did not interfere with the assay.

Figure 2.14 Fpg-modified Alkaline Comet Assay. DNA damage caused by CuO NP in C3A hepatocytes after 4-hour exposure in the presence and absence of fpg. Data expressed as average % DNA in tail +/- SEM (n=3). Star indicates a p ≤ 0.001.
Figure 2. Fpf-modified Alkaline Comet Assay Interference. Previous results of DNA damage expressed as DNA in tail normalized by positive control on C3A hepatocytes after 4h exposure in both fpg positive (B) and negative (A) treatments. In x-axis the two sub-lethal concentration of copper (1x and 0.5x BMD20) and the interference representing a CuO total concentration of 20.61 µg/ml in the LMPA.
2.5 Modified CuO: Cytotoxicity and Cytokine Production

The viability tests using the Alamar Blue assay showed a slight increase in cytotoxicity of the uncoated CuO (CuO_101) compared to the pristine CuO NPs. The cytotoxicity analysis of the four modifications produced the following ranking based on the BMD$_{20}$ values standardised on copper content: PVP > no coating > PEI > Citrate > Pristine (unmodified) > Ascorbate (where > means greater than or equal to). (Table 2.8 and Figure 2.16).

Table 2.8 Modified CuO NPs cytotoxicity. Summary of the results using the BMD approach on the raw data obtained with the Alamar Blue assay performed on different modification of CuO NPs (n=3).

<table>
<thead>
<tr>
<th>Ranking</th>
<th>CuO NPs and modifications</th>
<th>BMD$_{20}$</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Polyvinylpyrrolidone (PVP)</td>
<td>16.6 µg/ml</td>
</tr>
<tr>
<td>2</td>
<td>Functionalised (no coating)</td>
<td>18.7 µg/ml</td>
</tr>
<tr>
<td>3</td>
<td>Polyethylenimine (PEI)</td>
<td>20.4 µg/ml</td>
</tr>
<tr>
<td>4</td>
<td>Citrate</td>
<td>25.7 µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Pristine (not functionalised)</td>
<td>33.2 µg/ml</td>
</tr>
<tr>
<td>6</td>
<td>Ascorbate</td>
<td>64.8 µg/ml</td>
</tr>
</tbody>
</table>

The Ascorbate coating was the only capping agent able to show a significant decrease in toxicity against C3A cells compared to the pristine and functionalised NPs and, for this reason, it was selected for further analysis of impacts on IL-8 production. The results obtained showed that the ascorbate coating was able to exert a substantial decrease in cytokine production in C3A cells compared to the pristine CuO NPs (Figure 2.17).
Figure 2. Assessment of cytotoxicity of the Pristine CuO NPs and the five modified CuO NPs assessed by Alamar Blue Assay in the C3A hepatocyte cell line at 24 hours. Exposure concentration is expressed in µg/ml and analysis is conducted using the PROAST package in R software. The data is compiled from three separate experiments (n=3), and each data point represents the mean of three replicates conducted on the same day. The segmented lines show the BMD estimated by the software and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of CuO present in the dispersion.
Figure 2. IL-8 production of ascorbate modified CuO NPs (CuO_105) compared to the pristine CuO. Y-axis indicates the observed concentration expressed as picograms/ml (·1000) while in the x-axis are indicated the different substances analysed. The concentrations tested for each substance were calculated as the total amount of copper contained in the BMD<sub>20</sub> of CuO NPs and while specified, half this concentration (0.5x was used). Bars indicate SD (n=3). Bio-Plex Manager Software 6.1.

2.6 Copper Carbonate and Copper Amine: Cytotoxicity and Cytokine Production

The PROAST analysis performed on the other copper formulations showed that micronized copper was the most toxic copper-based composition with a BMD<sub>20</sub> of 15 µg/ml (Figure 2.18), which was significantly lower than the pristine CuO NPs (22 µg/ml). Copper amine instead, was in the same toxicity range of CuO NPs with a BMD20 of 24.4 µg/ml. CuSO<sub>4</sub>, albeit being more toxic than CuO NPs at LD<sub>50</sub> range value, elicited a lower cytotoxicity than the NPs at the BMD<sub>20</sub>-.
The cytokine data suggest that IL-8 production by C3A cells may be induced by CuCO$_3$ or copper amine. However, IL-8 production (Figure 2.19) by the C3A cells on treatment with the modified materials was highly variable, with no significant changes in viability assessed.

Figure 2.18 Pristine CuO, CuCO$_3$, Copper Amine and CuSO$_4$ (Alamar Blue Assay) in the C3A hepatocyte cell line at 24 hours expressed in µg/ml using PROAST package in R software. The data is compiled from three separate experiments (n=3), and each data point represents the mean of three replicates conducted on the same day. The segmented lines show the BMD$_{20}$ estimated by the software and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of equivalent Cu amount present in the dispersion.
Figure 2. IL-8 production elicited by micronized copper and copper amine compared to CuO NPs and CuSO₄ salt. Y-axis indicates the observed concentration expressed as picograms/ml while in the x-axis are indicated the different substances analysed. The concentrations tested for each substance were calculated as the total amount of copper contained in the BMD₂₀ of CuO NPs and while specified, half this concentration (0.5). Bars indicate SD (n=3). Bio-Plex Manager Software v.6.1.
2.7 Discussion

In this chapter, the toxic effects elicited by unmodified and modified (safer by design) copper oxide nanoparticles to hepatocytes was investigated in vitro. The endpoints analysed were cytotoxicity, cytokine production, glutathione depletion and genotoxic effects accrued by C3A cell model. Metal salts were included as controls to assess the contribution of ion effects to any observed toxicity.

Several studies have assessed the effects of CuO NPs in vitro, with most studies using the human epithelial lung cell line (A549) (Connolly 2010; Chang et al. 2012; Gioria et al. 2016; Wongrakpanich et al. 2016). Recently, other cell lines have also been used such as chronic myeloid leukaemia (CML) K562 cell line (Shafagh, Rahmani and Delirezh 2015), hepatocarcinoma cells HepG2 and macrophages RAW264.7 (Zhang et al. 2016). All these studies show consistent data regarding the ability of CuO NPs to elicit toxicity, causing apoptosis and high ROS formation. Currently, no other research could be identified which specifically investigated the effects of CuO NPs using C3A hepatocarcinoma cell line. Instead previous work performed using this cell line have investigated a panel of several NMs, including zinc oxide and silver NPs (Kermanizadeh et al. 2012a, 2012b, 2014a, 2016; Gaiser et al. 2013a) and both the toxic mechanisms and the cellular response after exposure were consistent with the findings of this study.

Cytotoxicity - Pristine CuO NPs

CuO NPs and the metal salts caused a concentration-dependent reduction in C3A cell viability 24 h post exposure. The cytotoxicity data obtained using the Alamar Blue assay showed that CuO NPs were able to elicit a higher cytotoxicity compared with CuSO₄ and CuCl₂ when the concentration was expressed on a total mass basis. Initially, this result gave the impression that the hypothesis that the toxicity of the NPs was significantly greater than soluble Cu, perhaps due to a Trojan Horse effect as suggested by Cronholm (Cronholm et al. 2012). The Trojan Horse hypothesis suggests that the CuO NP toxicity is mostly driven by the release of copper ions after the NPs are taken up by the cell (Cronholm et al. 2013). Considering the dissolution study performed by ISTEC-CNR as part of the SUN project, it seems that CuO NPs can easily
undergo a significant dissolution process as the pH of the dispersion media decreases. Thus, this dissolution process is more likely to happen inside the lysosome, eliciting an in vitro cytotoxicity similar in amplitude to one of other metals such as silver or zinc (Stanić et al. 2010; Chang et al. 2012; Rai, Yadav and Cioffi 2014).

Comparing the cytotoxic effect by considering only the copper content of the material/compound, the results appeared quite different. The effects on cell viability driven by CuO NPs and CuSO₄ were almost the same while CuCl₂ appeared slightly less toxic, probably due to its low solubility in aqueous solutions (Fritz 1980, 1982).

It is therefore hypothesised that copper ions released from CuO NPs are responsible for their toxicity. The sole Trojan horse hypothesis does not explain entirely the toxic effect observed, since even if there was an uptake of the entire NPs and a subsequent intracellular dissolution, as the NPs more cytotoxic in vitro when compared to high soluble copper salt such as CuSO₄. It would be useful in a subsequent study to investigate the role and the amount of the toxicity driven by the ion effect following differential inhibition of different endocytosis pathways and/or lysosome activity (Qin et al. 2003; Vercauteren et al. 2010; Hsin et al. 2012).

When assessing impacts of NPs on cell viability, it is advantageous to use more than one assay. Selecting the right assays is crucial to determine not only how effective a compound is at eliciting cytotoxicity, but it gives also some insight in regard to the mechanism involved in causing the toxic effect. The most usual established ways in which cell death occur are apoptosis, autophagy and necrosis (Cummings and Schnellmann 2004).

In addition to analysing viability via the mitochondrial ability to metabolise resazurin salt in the Alamar Blue assay, the LDH assay was also employed. This assay involves the quantification of the cytoplasmatic enzyme released in the medium and was selected as an additional assay to assess the impact of CuO NPs on cell viability. The main advantage of this method was to use samples from the same experiment for assessing the cytotoxicity from two different prospectives, as the cell supernatant collected from the Alamar Blue assay could be used. However, Cu interfered in the LDH assay. In fact, Cu(II)-dependent inactivation of LDH has been demonstrated by Pamp et al. (2005). This inhibition is caused by the copper-mediated oxidation of cysteine residues in the active site of this enzyme (Pamp et al. 2005). Recently, it was observed that copper NPs could interact with LDH as well, even if not by a direct binding but rather as a function of their dissolution rate (Han et al. 2011; Holder et al. 2012). The relatively low dissolution of CuO NPs can explain the high standard deviation and the constant
decrease in activity observed without causing the same level of apparent depletion observed with the copper salt.

Interesting alternative methods to the LDH assay to be considered in the future could be the ATP assay. The reason that makes this approach an interesting option is not only the fact the all living cells produce ATP for their survival but also that this method is used to discriminate between necrotic processes (ATP-independent) and apoptosis (ATP-dependent), giving consequently an insight of the processes that are taking place.

**Modified CuO NPs**

In the original planning of the project, the next step would have been the analysis of fragmented CuO nanomaterials originated by cryo-milling from a CuO containing coating in order to represent the Use phase of the product life cycle in which the release of particulate might be likely. The SUN project partners made several attempts without success due to the soft nature of the composite product itself. Thus, we decided to focus on the safer by design modification of CuO NPs. Several capping agents were tested.

It was proposed that ascorbate would have been a novel surface modification to study. Ascorbate is not only negatively charged, a property that would improve the suspension stability, but it is also an antioxidant found naturally in the body, especially in the lung lining fluid; moreover, its antioxidant mechanism is well known in the literature (Beyer 1994; Lü *et al.* 2010; Nimse and Pal 2015a). The initial hypothesis was that the natural occurrence of ascorbate and its antioxidant properties would have helped to mitigate both CuO NPs and ions release induced toxicity. The results obtained in this study clearly confirm that ascorbate-capping agent can decrease the cytotoxic effect of CuO NPs on C3A cells *in vitro*.

In addition, at the latter stage of the Project, other two-novel copper-based formulations produced by BASF (Germany) were tested for the purpose of replacing CuO NPs as a better alternative wood preservative. The result showed that exposing C3A cells to micronized copper led to a substantial increase in cytotoxicity while the same cells exposed to the copper amine solution showed results close to the CuO NP treatments. These first results do not provide enough information for an accurate decision in regard to the best wood preservative formulation without taking into account the data from the antifungal effectiveness detailed discussed in Chapter 3.
Cytokine production

Hepatocytes not only play a central role in different metabolic pathways but can synthesize several cytokines and hormones as well (Kmieć 2001b; Kermanizadeh et al. 2012). Cytokines are widely expressed in liver tissue during inflammation processes, such as IL-1β, IL-6, IL-8, TNF-α, and RANTES (Knight et al. 2005; Kermanizadeh 2012; Devhare et al. 2013; Gaiser et al. 2013b). In this study only IL-8 was detectable, demonstrating that even if C3A cell line is an useful in vitro model for toxicity studies (Kermanizadeh et al. 2012b, 2012c) especially for screening purposes, it may not give complete information about the possible inflammatory mechanisms involved in a specific process. According to other authors (Kermanizadeh et al. 2012c), this specific cell line is not able to produce sustained levels of other cytokines which prove to be too low to be detected by Multiplex instruments (Elsabahy and Wooley 2013). Nevertheless, between all of them, IL-8 (Dong et al. 1998) is one of the most interesting chemokines for mediating the activation and migration of inflammatory cells, being mainly a neutrophil chemoattractant; it, therefore, plays an essential role in initiating the inflammatory response (Puthothu et al. 2006).

In these experiments, C3A cells were not responsive to LPS stimulation as previously described in the literature for HepG2 cell line (Alexopoulos et al. 2010). Nevertheless, further testing with both increasing concentration and time points are needed to confirm this finding.

The different copper treatments induced production of the pro-inflammatory IL-8 by the C3A cells. A significant difference between the higher doses of CuSO₄ and CuCO₃ was not detectable. Nonetheless it was possible to observe a pattern analogous to the results obtained in the cytotoxicity test, where the micronised copper proved to be the most potent copper-based formulation. The ranking score both micronised copper and CuSO₄ as most potent, while pristine CuO NPs and copper amine formulation showed a similar lower effect. Interestingly, when looking at the value obtained at half the BMD₂₀ concentration, C3A cells produced a higher level of IL-8 production when treated with CuO NPs rather than when treated with the other compounds. Finally, ascorbate coated CuO NPs did not induce any cytokine production in vitro at sub-lethal doses. The low inflammagenic property showed by the ascorbate coated version of this NPs might be of particular interest for the industry as the decrease of the hazard might lead to a safer manufacture of the final product (wood paint for external use) with a benefit for the users.
The ability of pristine CuO NPs to deplete glutathione was measured. Two different time points were selected, one at 6 hours and one after 24 hours, to detect any natural fluctuation in glutathione levels (Environment et al. 2001) over the exposure period. It was already demonstrated in the literature that NPs such as ZnO and MWCNTs could deplete glutathione as a result of their ability to induce oxidative stress in hepatocytes at 24 hours (Kermanizadeh et al. 2012b), while the capacity of CuO to deplete glutathione was previously described in Hep-2 airway epithelial cell at 4 hours (Fahmy and Cormier 2009). The results of this study showed that CuO NPs might induce a significant depletion of total glutathione content after 24 hours in the C3A cell line. The relatively low cytotoxicity induced by exposure to CuO NPs at these sub-lethal doses suggests that the depleting action can be ascribed to oxidative stress. Unfortunately, due both to the priorities and the time schedule of the project it was not possible to examine further the oxidative stress hypothesis. Future studies could directly measure ROS production or add antioxidants to assess inhibition of cytokine induction.

Genotoxicity

It was already reported that nanoparticles and CuO, in particular, were able to enter the nucleus of cells such as A549 and BEAS-2B (Cronholm et al. 2012, 2013). In the present study, it was observed that CuO NPs were able to induce DNA damage. Nevertheless, the inclusion of fpg did not significantly enhance DNA damage, suggesting that DNA damage was not mediated by an oxidant mechanism. This lack of oxidative damage is quite unexpected, considering that in literature copper, silver and zinc oxide NPs are known to cause the most toxic effect due their ability to cause high levels of ROS production (Gonzalez, Lison and Kirsch-Volders 2008; Connolly 2010; Kermanizadeh et al. 2012a; Wang et al. 2013; Shafagh, Rahmani and Delirezh 2015; Pallardy, Turbica and Biola-Vidamment 2017). This suggests that the major amount of DNA present in tails was caused by single and double-strand breaks rather than oxidative damage. In contrast, the positive control H$_2$O$_2$ was able to induce DNA damage, and this effect was enhanced by the inclusion of fpg.

The results suggested that even if the cellular effects of these NPs could be ascribed to the intracellular (or extracellular) release of copper ions, the majority of DNA damage is not at least a direct consequence of oxidative stress. Copper ions are known to cause damage to DNA by direct nucleotide cleavage (Sagripanti, Goering and Lamanna 1991; Marín-Hernández et al. 2017).
In fact, the DNA double helix contains two different binding sites for copper with high affinity (*ibidem*).

Moreover, even if the mechanism is still not well known, it has been hypothesized that copper NPs could interact with protein complexes within the nucleus (Galindo-Murillo *et al.* 2015) as they exhibit in other cellular compartments (e.g. with LDH), where they probably inhibited or altered the protein structure and function.

Finally, there is no standard *in vitro* protocol for the Comet assay universally accepted between different laboratories and, since there are considerable differences in the protocol used, often the outcomes are extremely varied as well. At this point, a further analysis using a different approach such as the Micronucleus assay would be ideal to confirm these findings (Golbamaki *et al.* 2015).
2.8 Conclusions

CuO NPs elicited a similar cytotoxicity as CuSO$_4$ to C3A hepatocytes *in vitro*, while CuCl$_2$ showed a slightly lower cytotoxicity, probably due to their different dissolution properties – i.e. it is almost insoluble in aqueous media – and consequently their ability to generate copper ions. This dissolution process probably happens mostly in the media for CuSO$_4$ and inside the cell in association with a strong pH decrease (lysosome) generating the so-called *Trojan horse* effect which, even if might explain the majority of the toxicity of CuO NPs, did not justify entirely the toxic effect observed. Copper-induced effects (from the NP and salts) on the C3A cells included an increase in cytotoxicity, cytokine (IL-8) production, DNA damage and glutathione depletion. Both oxidative stress and IL-8 production can be associated with the induction of inflammation (Rudack *et al.* 2003; Doll *et al.* 2010; Manke, Wang and Rojanasakul 2013). Interestingly, DNA damage seems to be elicited by some direct interaction of the copper with the DNA or its associated proteins, caused mostly by the NPs, rather than from an indirect action of the oxidative stress.

The S$_{by}$D approach, using the ascorbate capping agent, reduced the cytotoxicity and IL8 induction by CuO NPs, suggesting that this could be a good candidate for replacing the pristine NPs as a wood preservative. The micronised Cu formulation showed a higher cytotoxicity when compared with CuO NPs together with a strong production of IL-8 suggesting that it may trigger the inflammatory response in the liver. On the other hand, the Cu-amine formulation did not show any appreciable difference if compared to the other copper compounds. Nevertheless, further analyses are needed: it would be very useful not only to perform some microbiology screenings (refer to Chapter 3 for further details) but also to take into account the environmental impact, the *in vivo* effects (Chapter 4 and 5), and the real human exposure to these products.

In conclusion, these results partially confirm the hypotheses presented at the beginning of this Chapter: both pristine CuO NPs and micronized Cu are more potent *in vitro* than the Cu-amine formulation; moreover, micronized copper is more toxic than copper ions alone. Finally, the S$_{by}$D approach proves to be a useful strategy in decreasing the NPs cytotoxicity and cytokine production.
Chapter 3: Antifungal Studies

Based on publication:

3.1 Introduction

Copper is the most widely used fungicide for treating wood in contact with soil, with no satisfactory alternative available since copper is the only biocide that shows significant effects against soft rot fungi and other soil-borne fungi (Hughes 2004). Copper is also preferred as an antimicrobial wood preservative because it is considered a toxicant with relatively low effect on mammals, including humans (Lebow 1996), even if it shows a relatively high toxicity against aquatic communities (Roales and Perlmutter 1980; Ronald 1990).

Nonetheless, not all fungi are sensitive to copper; in fact, several brown rot fungi can grow at ionic concentrations of up to 1.6 mM (Hughes 2004) or 100 mg/kg (Gadd 2007). This category of moulds causes high economic damage since they are the main reason for the early failure of in-ground timber structures in the US and Europe (Lebow et al. 2003; Bollmus et al. 2012). In Germany alone, copper tolerant fungi cause failures of 1% of wood poles each year, while in Europe the total annual expense for replacing wood poles are estimated to be EUR 36,000,000 (Civardi, Schwarze and Wick 2015).

The high resistance shown by these fungi is one of the key factors driving the continuous development of copper-based formulations and their alternatives such as NPs, since these have shown the ability to bypass some of these resistance mechanisms (Cioffi et al. 2005), and elicit a toxic effect considered low in humans compared to microorganisms (Bosetti et al. 2002), even if recent studies show that CuO NPs are still highly toxic against human cell lines (Karlsson et al. 2008; Chang et al. 2012).

For these reasons, many industries have started to develop formulations using micronized copper that contain a considerable amount of NPs (Mcintyre 2010) able to penetrate into wood (Matsunaga, Kiguchi and Evans 2007; Geers et al. 2014). The use of Cu NPs instead of bulk copper is reported to improve the durability of wood against fungal decomposition (Kartal, Green and Clausen 2009; McIntyre and Freeman 2009; Cookson et al. 2010; Akhtari, Taghiyari and Kokandeh 2013). However, there is still little evidence that Cu NPs can be more efficient than copper salts against soil-borne fungi or Cu-tolerant wood-destroying fungi (Kartal, Green and Clausen 2009; Cookson et al. 2010; Tang et al. 2013).
Copper seems to exert its toxic effect primarily through the generation of reactive hydroxyl radicals (Cervantes and Gutierrez-Corona 1994). These are known to cause protein oxidation, DNA and RNA cleavage (Stoehs and Bagchi 1995; Kim et al. 2000) and membrane damage due to lipid peroxidation (Halliwell and Gutteridge 1984; Peña, Koch and Thiele 1998). While the toxic mechanism of copper ions is known, there is still not sufficient information about the exact toxic mechanism of CuO NPs (Civardi et al. 2015). It seems reasonable to assume that copper-based nanomaterials can elicit their toxic action through both direct particle interaction and ion release (Cioffi et al. 2005; Ivask et al. 2010; Wu et al. 2010; Cioffi and Rai 2012). Copper NPs, in particular, are highly reactive due to their larger surface area (Oberdörster 2000; Chen et al. 2006) which causes the production of ROS leading to oxidative stress (Saliba et al. 2006; Heinlaan et al. 2008) and DNA damage. Also, they may lead to conformational changes in protein structure including their active site, causing inhibition or neutralisation of their biological activity (Borkow and Gabbay 2009). Moreover, they can influence the production of lignocellulose-degrading enzymes (Shah et al. 2010), damage to mitochondria (Chang et al. 2012), cell wall (Heinlaan et al. 2008; Shah et al. 2010) and impaired homeostatic processes (Chang et al. 2012).

In the present chapter, the anti-fungal effects of a range of different copper formulations are tested and compared to copper sulphate used as an ion-releasing control and against the current European benchmark wood preservative treatment - the Cu-amine impregnating formulation.

The main hypotheses were the following:

- Copper nano-enabled formulations (CuO NPs and micronized Cu) will elicit a greater antifungal effect compared to non-nano formulations (Cu-amine and CuSO₄).
- The safer by design modification of CuO NPs even after showing a decrease of toxicity to C3A cells, will maintain its antifungal activity against a selected panel of wood destroying basidiomycetes.

The European Committee for Standardization (CES), and subsequently the British Standard Institution (BSI), developed and adopted some official guidelines for testing the antifungal activity of wood preservatives. Specific protocols for assessing the effectivity of these products can be found in the CES document: “Wood preservatives. Test method for determining the protective effectiveness against wood destroying basidiomycetes. Determination of the toxic values” (EN 113:1996).
The elective method for testing the antifungal activity in a laboratory consists of weighing and exposing directly treated blocks of wood – e.g. Scots pine (Pinus sylvestris L.) – to the direct action of wood destroying basidiomycetes in a Kolle flask for 16 weeks. After this period the sapwood specimen can be removed, brushed free of the mycelium and weighed again to compare the mass lost during the fungal attack. Additional testing can be performed at this point such as biomass production or enzymatic assays.

This procedure offers the advantage of testing the product effectivity directly on a wood sample, taking into account any modifications that usually undergo the wood treatment; on the other hand, the same procedure is highly time-consuming and often needs many repetitions due to the high variability of the results.

The main aims were, therefore:

- To develop a quicker and efficient in vitro protocol to the current European standard method for determining effectiveness against wood destroying basidiomycetes (EN 113:1996).
- To simulate in vitro the effectiveness of two methodologic approaches used in the wood preservative industry to assess if either concentrating a greater amount of Cu at the surface of the sample – i.e. the acrylic paint enriched with CuO NPs coating – or distributing homogeneously the copper content through the sample – i.e. the micronized Cu impregnation system – might increase the protection against wood-destroying fungi.
3.2 Materials and Methods

3.2.1 Fungal models

The European standard for analysing antifungal property of wood preservatives (EN 113:1996) advises using three species of filamentous Rot Fungi from a suggested panel. The selected strains were obtained from the Belgian co-ordinated collection of micro-organisms, more specifically from the Mycothèque de l'Université Catholique de Louvain in test tubes as living cultures.

Coniophora puteana (Schumacher: Fries) P. Karsten (strain MUCL 20565), Gloeophyllum trabeum (Persoon: Fries) Murrill (strain MUCL 11353) and Trametes versicolor (Linnaeus: Fries) Pilát (strain MUCL 11665) were cultured in 90mm Petri-dishes (AlphaLab, UK) containing malt extract agar (MEA, Sigma, UK) in an incubator at 20°C and subcultured every two weeks by plug-transfer in new Petri-dishes. All procedures were performed using a Bunsen burner to avoid sources of environmental contamination and, for the same purpose, several stock cultures were prepared each time.

3.2.2 Cu nano-enabled formulations and Cu compounds preparation

The pristine CuO NPs, as well as the CuSO₄ salt, was prepared according to the ENPRA protocol (ENPRA 2015).

As previously mentioned (Chapter 2), both the pristine NPs and the Cu salt were suspended in Milli-Q water with the addition of 2% heat-inactivated FBS at a concentration of 1 mg/mL and then, vortexed for 20 seconds. Both solutions and suspensions were sonicated for 16 minutes using a sonicating water bath (32-38 kHz at 20°C) and used within 30 minutes. Micronized Cu, Cu-amine formulation and the safer by design modification of CuO NPs dispersion were vortexed for 20 seconds before use. No additional sonication was performed to these products due either due to the stability of the first two products dispersion or to avoid the risk to alter/damage the latter.
3.2.3 Fungal exposure protocol

Since the percentage of copper differs in each test sample, both experiments and dilutions were adjusted to allow comparison of the amount of copper present as previously done for the *in vitro* experiment on C3A cells (Chapter 2).

*Layered Agar: coating model preparation*

Fresh MEA was prepared according to the manufacturer’s instructions and poured into sterile Petri-dishes at a final volume of 15ml, left to settle at room temperature (R.T.) until complete solidification and then and kept in the fridge at 4-6°C for 24 hours.

The following day, the Petri-dishes left at R.T. for 30 minutes before use. Then, 2ml of freshly made dispersion (or solution) containing either 2mg or 1mg equivalent copper was transferred to a 50ml falcon tube, and 18ml of freshly made MEA (previously autoclaved and kept in water bath at 66°C) were added to a final volume of 20ml containing either 200 or 100μg of nominal copper.

After mixing gently to prevent any bubble formation, 5ml of copper-containing agar was transferred onto the Petri-dish containing the settled 15ml of simple MEA creating a two-layer agar (Figure 3.1). The Petri-dish were gently tilted to help the new layer to cover homogeneously the previous one. The agar was left at R.T for 30 minutes before being transferred to the fridge at 4-6°C for 24 hours.

*Figure 3.1 Two-layer MEA agar. 5ml of copper-containing MEA agar was spread onto a pre-settled layer of simple MEA.*
Agar Dispersion: impregnation model preparation

One millilitre dispersion (or a solution) containing either 2mg or 1mg equivalent copper, was transferred to a 15ml falcon tube in triplicate. Subsequently, 9ml of freshly made MEA (previously autoclaved and kept in water bath at 66ºC as described above) was added to reach a final volume of 10ml containing either 200 or 100μg of nominal copper per ml.

The agar was immediately poured on a plate where it started to solidify in less than 30 seconds due both to its small volume and the handling procedure that involved a gently mixing with the particle suspension without any bubble production (Figure 3.2). This quick setting prevented any deposition of NPs to the bottom of the Petri-dish.

Finally, control agar plates were prepared using 9ml of MEA and 1ml of water containing 2% of heat-inactivated FBS. Each dispersion/solution left at R.T. before being placed in fridge at 4-6ºC. All media were used after 24 hours.

![Figure 3.2 Agar Dispersion. MEA containing a homogeneous dispersion of copper content.](image)

Exposure

A 10mm plug containing the mycelium was obtained by gently pressing the back of a 1000µl sterile standard tip on the active growing sub-margin of a 2-week-old culture and then, was immediately transferred onto the surface of the agar preparations. Two experiments were performed in triplicate for all three species of Fungi.

After a one-week incubation at 20ºC, the diameter of each colony was measured, and the data analysed via a One-way ANOVA followed by Dunnett’s multiple comparisons test using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.
3.4 Results

The pilot study indicated potential fungal growth inhibition with the dispersed agar, while with the layered one it was not possible to observe any inhibition of growth, even against the most copper sensitive *G. trabeum* (Figure 3.3).

Following these findings, the subsequent experiments were performed in detail using only the dispersed agar model.

![Figure 3.3 G. trabeum growing on CuO NP layered agar. Fungal growth after one-week incubation; no visible inhibition of growth was noticeable even at high exposure concentrations.](image)

*Figure 3.3* G. trabeum growing on CuO NP layered agar. Fungal growth after one-week incubation; no visible inhibition of growth was noticeable even at high exposure concentrations.

*C. puteana* was the most copper-resistant fungus (Figure 3.4). Control samples grew to a size of 4.67 (±0.2) cm in the absence of Cu treatment while all treatments at a concentration of 100 µg of nominal copper per ml did not significantly inhibit the growth of the fungi (P>0.05). In contrast treatments with 200 µg/ml of copper from micronized copper, copper amine, SbyD (ascorbate coated) CuO and CuSO₄ inhibited fungal growth significantly (p<0.01, <0.001, <0.5 and <0.5 respectively) with the ranking: Cu-amine > micronized Cu > CuSO₄ = CuO-ascorbate. No significant inhibition was observed (p>0.05) with 200 µg/ml copper from pristine CuO NPs.
Figure 3.4 C. puteana growth after 1-week incubation at 20°C on malt extract agar supplemented (via dispersion) with different concentrations of copper from each treatment. The Y-axis represents the diameter of each fungal colony measured in centimetres, with 1 cm representing the size of the original fungal plug (red line). Each bar represents the mean data from two repetitions (n=2), each conducted in triplicate. Data were analysed using GraphPad Prism version 6.0 and one-way ANOVA (Dunnett).

Also, a visual inspection of the fungal cultures showed that the micronized Cu, the CuSO₄ salt, and the safer by design CuO NPs coated with ascorbate were able to induce pigmentation of the fungus (Figure 3.5). Moreover, a physical modification to the colony shape with a beginning of primordium growth in the sample exposed to the ascorbate modification was noticeable (Figure 3.6).
Figure 3. 5 Pictures of *C. puteana* cultured on copper dispersed agar after 7 days of growth. On the left (A, C, E, G) pictures taken from above, while on the right (B, D, F, H) the same culture dishes from underneath showing the undergoing pigmentation process.
For the fungi *T. versicolor*, growth occurred in concentric circles; the inner circle was constituted by growth above and underneath the surface of the agar (4.7 ±0.1 cm after one-week incubation), while the outer circle was formed by only the hyphae growing deep underneath the surface (6.63 ±0.05 cm after one-week incubation).

The fungus, even if more sensitive to copper when compared to *C. puteana*, did not undergo much inhibition of growth by the action of both pristine and modified CuO NPs, even if some statistical significance was recorded for both the treatments (p<0.05). Again, the higher inhibiting effect was obtained by the Cu-amine impregnating solution (p<0.0001 in both 100 and 200 µg/ml nominal copper treatments) followed by micronized Cu (p<0.05 and <0.001 for inner and outer circle of growth respectively at 200 µg/ml nominal copper) and CuSO₄ salt (p<0.01 both treatments for only the outer circle) (Figure 3.7).
The visual inspection did not show any morphological change nor pigmentation on the treated T. versicolor fungus; the only recordable effect was the decrease in diameter of the colony after a week of incubation (Figure 3.8).
Finally, *G. trabeum* (growing up to 3.66 ±0.05 cm after 1 week of incubation) proved to be the most sensitive species to copper showing a clear and significant effect on fungal growth caused by every treatment analysed (p<0.0001) (Figure 3.9). Cu-amine was the most potent substance against the fungus being able not only to decrease the fungal growth at 100µg/ml of relative copper but also to kill the fungus growing onto the plug at 200µg/ml (Figure 3.10, D). The final ranking for these copper-based compounds was: Cu-amine > micronized copper > CuO-ascorbate > pristine CuO > CuSO₄.

Again, no morphological changes or pigmentation were visible (Figure 3.10).
Figure 3. 9 G. trabeum growth after 1-week incubation at 20°C on MEA supplemented with different concentrations of copper carbonate (CuCO₃), copper-Amine, ascorbate-coated copper oxide NM, pristine copper oxide NM or copper sulphate (CuSO₄). The Y-axis represents the diameter of each fungal colony measured in centimetres, with 1 cm representing the size of the original fungal plug. Each bar represents the mean data from two repetitions, each conducted in triplicate (n=2). Data were analysed using GraphPad Prism version 6.0 and one-way ANOVA (Dunnett).

Figure 3. 10 Pictures of G. trabeum on MEA supplemented with different concentrations of copper from a panel of different copper materials: CuO and CuO-ascorbate (A), CuSO₄(B), CuCO₃(C) and Cu-Amine (D).
3.5 Discussion

Metal and metal-oxide NPs are used as additive in timber industry due to their ability to increase the protective effect and decrease the environmental release of the biocides applied to wood (Liu et al. 2001; Fufa and Hovde 2010). Nevertheless, even if their exploitation increased over the last two decades, there is still a lot of uncertainties about the environmental effects of the release of NPs from timber (Fufa and Hovde 2010). Furthermore, samples of wood treated with the same pristine CuO used in this study were able to release both particulate and ions whilst the micronized copper formulation released only ions (Pantano et al. 2017). This result suggests that, when analysing the impact of NPs on the ecosystem, the general environmental effects of ion toxicity must be taken into account as well.

The aim of this study was not only to test the antifungal effectiveness of the different copper formulations prioritised in the SUN project but also to develop a novel method for in vitro testing for assessment of anti-fungal properties that could imitate the main procedures used in treating wood. Two approaches were considered in this study which attempted to simulate the procedures of impregnation and coating which are routinely used to treat wood with antimicrobial copper formulations. The impregnation is most widely used and is part of the European standard EN113:1996, and allows the preservative to penetrate deeply into the wooden structure under high pressure, creating a homogeneous gradient through the wood. The coating procedure concentrates the antifungal agent into a thin layer on the surface of the wood.

Following the pilot study, it was clear that the layer agar model was less effective at inhibiting fungal growth compared to the dispersed protocol. Only a limited number pilot studies were conducted, making it difficult to identify the reason for the ineffectiveness of the layered model. Nonetheless, a hypothesis can be formulated. It is possible that in the layered model, the fungal hyphae can penetrate the outer layer containing the antifungal agent enough to reach the inner layer and extract the nutrients to growth with minor effects (Figure 3.11, A). If this hypothesis is true, the reason of why the hyphae can penetrate the layer could be ascribed either to a possible initial copper tolerance of the fungus which could be enough to challenge the copper presence for a few millimetres or to an inhomogeneous distribution of the copper through the layer. In this scenario, the copper formulation can be deposited within the agar creating micro-islands with different gradients of ions; the fungal hyphae can consequently
avoid the copper island “sensing” the gradient of copper. In the dispersed model, on the contrary, the fungus could be incapacitated due to the inability to access agar lacking copper (Figure 3.11, B). It should be stressed that these are only hypotheses and it was not a project priority to test and verify them. Nevertheless, it could be an interesting analysis to be performed in the future.

![Figure 3.11 Comparison of both layered and dispersed agar models. The possible hypothesis of fungal growth showing why the layered agar representing the coating model (A) was not effective as compared to the dispersed one (B) representing the impregnation system. The red dots represent the NPs, while the pink circle and the blue ramifications represent the agar plug and the hyphae respectively.](image)

*Coniophora puteana*, also known as cellar fungus, is considered a common element of brown rot wood decay for both soft and hardwood and it is widely accepted to be a copper tolerant species (Cioffi and Rai 2012). The results generated confirmed this observation in that it was the only species to show high tolerance against all copper formulations tested. A significant inhibition of growth was recorded only at the highest exposure concentration of CuCO$_3$, Cu-Amine, and to some extent to CuSO$_4$. No significant effect was observed using the pristine
CuO NPs while the safer by design formulation did show some small antifungal properties. This effect is probably ascribable to the ascorbate coating which is known to elicit a toxic effect in some Fungi – e.g. *Candida sp.* – through the production of hydroxyl radicals via the Fenton reaction (Avci *et al.* 2016).

Imaging of *C. puteana* revealed that pigmentation of the fungal growth occurred on both sides of the Petri-dish for all treatments except the ascorbate-coated NPs treatment, for which the pigmentation was only visible beneath the colony. For this treatment, the colony surface develops a complex structure of air mycelium suggesting a vertical increase of growth (or primordium) rather than horizontal growth. A clear decrease of growth together with a strong pigmentation and a morphological change of the culture shape was clearly observable in samples treated with CuCO$_3$ and CuSO$_4$.

Brown Rot Fungi have been shown to accumulate metals during decay processes (Hastrup, Jensen and Jellison 2014), which may account for the pigmentation observed. In fact, melanisation appears to be a well-known defensive mechanism against the toxic effect of heavy metals (Gómez and Nosanchuk 2003).

The fungus *Trametes versicolor*, commonly known as turkey tail, is a white rot Fungus able to produce oxalate crystals in the presence of high concentration of metals (Jarosz-Wilkolazka and Gadd 2003). This fungus demonstrated a dense growth near to the original plug with a more diffuse/lighter growth at the periphery. For this reason, the results are presented as internal and external diameter respectively. The Cu-amine solution showed the most potent effect on fungal growth. All other copper formulations inhibited the growth of the filamentous fungus compared to the control according to the statistical analysis, although the impacts observed were relatively small and unlikely to be of use in a real-world scenario. No melanisation nor morphological changes were shown.

Finally, *Gloeophyllum trabeum* is a common brown rot copper sensitive Fungus prevalent in coniferous forest. This fungus was found to be extremely sensitive to almost every treatment tested, even at lower concentrations. Both CuO NMs and the CuCO$_3$ eliciting a higher toxicity than the CuSO$_4$ and copper-amine treatments, resulting not only in complete inhibition of growth but also the death of the fungal plug at higher doses.

In addition to this *in vitro* testing, more work was performed by the Empa – *Swiss Federal Laboratories for Materials Science and Technology* (Gallen, Switzerland) – and at the Institute
for Building Materials (Zurich, Switzerland) as part of the SUN project. The results of this testing can be found in Pantano et al. (2017). Briefly, the sapwood samples were at first either covered by a thin layer of acrylic paint containing CuO NPs or impregnated with the other copper formulations according to EN113. Then, the wood specimens were placed on a glass support inside several Kolle flasks entirely covered by C. puteana and stored at 22°C and 70% relative humidity for 16 weeks. After incubation, the wood blocks were removed from the flasks, brushed free of mycelium and oven dried at 103±1 °C. The percentage mass loss was calculated from the dry mass before and after the test.

Again, pristine CuO NPs embedded in acrylate did not prove to be a suitable candidate for the wood protection industry. The reason for that can be that the nanoparticles are not easily released by the acrylate matrix to be subsequently available for the fungus, or that the acrylate layer can crack during the ageing process producing a small exposed area that can be attacked by the fungus (Pantano et al. 2017). Several attempts to analyse the safer by design modified CuO using the wood blocks were tried without success, as it was impossible to obtain a homogeneous dispersion of the NPs through the acrylate matrix. For the European benchmark impregnation solution (Cu-amine) and the micronized copper formulations, the results were consistent with the in vitro testing performed at Heriot-Watt University.

Finally, characterisation of the NPs within the in vitro model was considered. The pH of the agar medium used in this study was 5.4, is in line with the average pH of wood between 4 -5.9 (Feldman 1985). Since acidic conditions may influence the NP stability, BASF performed a dissolution analysis of both CuO NPs and micronized copper at different pH. This study showed that the nano-enabled formulations released a percentage of ions approx. 3-4% after three days at the same level of acidity (Pantano et al. 2017). These results suggest that the majority of the antifungal effect recorded was due to the presence of NPs rather than by ions, justifying the development of these novel formulations by the wood preservative industry.
In conclusion, these results suggest that the most potent treatment against the three different species of wood decay fungi – the brown rot copper tolerant *C. puteana*, the white rot copper tolerant *T. versicolor* and the brown rot copper sensitive *G. trabeum* – was the European benchmark already available on the market, together with the micronized copper formulation. The pristine CuO NPs did not show the same level of effectiveness against the selected fungi. Nevertheless, a slightly greater effect than the pristine NPs was obtained using the modified CuO formulation coated with ascorbate probably due to the coating formulation.
Chapter 4: *in vivo* Studies

Based on publications:


4.1 Introduction

As already mentioned in Chapter 1, both NPs and NM s can enter the human body through four major routes: inhalation, ingestion, skin absorption and parenteral administration (Yah, Iyuke and Simate 2012; Ellenbecker and Tsai 2015). The respiratory system represents a unique target for NPs not only because it is the main entry route of airborne particles (Elsaesser and Howard 2012), but also for the potential exposure to any NPs that, after entering the body from other routes, may result in systemic distribution and reach the lungs via blood (Card et al. 2008).

The toxic effect derived from NP exposure is determined by their physicochemical characteristics (Klein et al. 2012). Size, for example, is one of the key elements to consider as it was widely demonstrated that materials, such as TiO$_2$, can elicit a toxic effect which is higher for materials in the nanoscale compared to the same material in micro-sized form (Oberdorster, Ferin and Lehnert 1994; Bermudez et al. 2004; Roller 2009). In addition, NP surface chemistry and crystal phase form can influence the toxicity of NPs (Warheit, Reed and Sayes 2009). Moreover, the bio-persistence related to NPs and NM s is another fundamental factor to consider as their toxic effect can be related either to their prolonged presence in the lung or to the toxicity of the material released in the lung by deposited material (i.e. water-soluble materials) (Klein et al. 2012). Finally, NM s with high aspect ratio such as rods and fibres, can exhibit asbestos-like fibre toxicity, especially if with the dimensions of less than 5 µm of diameter or 3:1 aspect ratio (Donaldson et al. 2006; Poland et al. 2008), with the exception of some multi-walled carbon nanotubes (MWCNTs) that do not follow this toxic pattern (Klein et al. 2012). Since all these parameters might influence the potency, the duration and eventually the regression of the inflammation derived by either NP or NM exposure, it is difficult to correlate the toxicity with the properties of a specific material and it follows that it is still needed to assess their toxicity case by case (Klein et al. 2012).

In view of the fact that studies performed in human or in large animals are limited, rodents represent the main source to obtain enough data for risk assessment in regard to inhaled NPs (Kuempel et al. 2006; Geiser and Kreyling 2010); thus, within the European FP6 project NanoSafe2 and the German BMBF project nanoCare, Ma-Hock et al. developed a first short-term inhalation study (STIS) for NM s using rats as a model (Ma-Hock et al. 2007), that detected
relevant and predictive changes after 5 days exposure and after 3 weeks of recovery (Ma-Hock et al. 2007), and it is now an OECD guideline (OECD 2009a).

Regardless of the effort made in the last few decades to decrease and even eliminate the need to use in vivo testing in scientific research, animals are still widely used around the world. Animal testing is still fundamental, not only for ameliorating and protecting people’s health but also for animal and environmental safety (Stokes, 2015). Nevertheless, increasing concerns of the public about animal welfare in science have led to the production of many laws and policies designed to decrease and avert animal suffering and distress (Stokes, 2015).

In line with these policies, an in vitro screening tests (Chapter 2) was used to identify concentrations required to induce toxicity and to rank the relative toxicity of the NPs and NMs of interest prior to the animal experimentation. This in vitro section included the results obtained at Heriot-Watt University with the C3A hepatocellular model but also the results obtained at the Karolinska Institutet (Norway) using the Raw 264.7 murine macrophage cell line (Appendix). Both absence of pre-existing data about CuO NPs and in vitro toxicity assessment led to the choice of CuO as a priority for in vivo studies.

An first STIS using pristine CuO NPs was performed at The Netherlands National Institute for Public Health and the Environment (RIVM – The Netherlands) with the aim to define the hazard resulting from the inhalation of CuO NPs, originate a series of dose-response data for the evaluation of the subacute effects (Gosens et al. 2016b) and the generation of a conclusive risk assessment. It was hypothesized that CuO NPs could elicit a strong toxic effect in lungs as already reported in the literature for other copper-based NMs (Chen et al. 2006; Yokohira et al. 2008; Cho et al. 2010; Kim et al. 2011b) and that the NPs could be able to translocate to other organs (Geiser and Kreyling 2010).

A second STIS was performed using ascorbate coated (negatively charged) and polyethylenimmine (PEI) (positively charged) CuO NPs produced by the Institute of Science and Technology for Ceramics (ISTEC) at the National Research Council of Italy (CNR) through the Safer by Design (SbyD) approach. These modifications were selected accordingly with the in vitro screening results. This time, it was hypothesized that the positive surface charge would enhance the particles’ toxicity compared to the unmodified ones, and that the coating with negativ charge would decrease the toxic effect. In fact, a particle with positive charge, such as the PEI coated NPs, is likely to be recognized more easily by macrophages, while the ascorbate coating would decrease the cellular uptake due its surface charge (Fröhlich
Furthermore, ascorbate is a natural antioxidant of the lining lung fluid and it is able to protect against oxidative stress (Kelly and Tetley 1997), reducing Cu$^{2+}$ ions (that are linked to the toxicity) to Cu$^{0}$, or forming a complex with the Cu$^{2+}$ ions.

The results from these studies were analysed using the Benchmark Dose (BMD) approach previously described in Chapter 2, as a more advanced and statistically consistent (Hardy et al. 2017) alternative compared to the less precise (Kalantari et al. 2017) no observed adverse effect (NOAEL) approach. Between the advantages of this procedure, it should be considered the opportunity to establish a more reliable reference point (Hardy et al. 2017) and the chances to decrease the number of animals per experiment (EFSA 2011). Moreover, since this approach can generate more precise and significant information compared to the NOAEL approach (Slob 2014a), it could be possible decrease the number of animal used (Slob 2014a) according with the principle of Replacement, Refinement, and Reduction – 3Rs – (William and Burch 1959) in animal studies (Slob 2014b).

Since it has been shown that different types of NMs and NPs can translocate from primary exposure sites into the body (Sadauskas et al. 2009), and that as a secondary exposure site the liver is extremely important for its ability to accumulate either NPs and NMs at much higher quantities compared to other organs (Semmler-Behnke et al. 2008; Sadauskas et al. 2009), livers of the exposed animals were isolated and analysed for both biodistribution and toxicological studies including cytokine production and gene expression performed at Heriot-Watt University. Furthermore, during exposure to the aerosol, a that substantial accumulation of NPs occurred onto the buccal mucosa of the rats, suggesting a possible accidental ingestion of the same during the experiment; for this reason, also gut samples containing Payers patches (a component of gut-associated lymphoid tissue) were analysed.

The following genes were analysed during the gene expression performed in this study: the chemokine (C-X-C motif) ligand 2, Interleukin-1β, Tumour Necrosis Factor α, the Cluster of Differentiation 46, Interleukin-10, Glutathione Peroxidase, Fas-ligand, the Intercellular Adhesion Molecule 1 and several Metallothioneins (MT1A, MT3 and MT4).

The chemokine (C-X-C motif) ligand 2 is a small cytokine belonging to the CXC chemokine family also called macrophage inflammatory protein 2-alpha (MIP2-alpha). CXCL2, together with other GROs family members such as CXCL1 and CXCL3, are known as powerful neutrophil chemoattractants (Rudack et al. 2003; Dyer et al. 2017). The same genes are also involved in metastatic development (Doll et al. 2010), angiogenesis, and wound healing (Zaja-
Recent reports also suggest that CXCL2 could play an important role in asthma severity (Goleva et al. 2008; Fitzpatrick et al. 2010) and asthmatic airway remodelling (Imaoka et al. 2011; Al-Alwan et al. 2012, 2013).

Interleukin-1β is also a pro-inflammatory cytokine involved in several immune responses (O’Neill 2000; Subramaniam, Stansberg and Cunningham 2004) that plays an important role in cellular homeostasis by modulating the apoptotic cascade (Friedlander et al. 1996), lowering pain threshold and damaging tissues (Dinarello and van der Meer 2013). Its receptor (IL-1RI) plays an important role for the initiation and maintenance of diverse activities of the immune system (Oelmann et al. 2015) eliciting an agonistic modulating effect against inflammation (Chamberlain et al. 2014).

Tumour Necrosis Factor α (TNFα), is a well-documented pro-inflammatory cytokine produced by macrophages/monocytes during acute inflammation (Lesur et al. 2010) and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis (Idriss and Naismith 2000) and it is especially involved in protection against pathogens and as a mediator in autoimmune diseases (Pfeffer 2003).

Cluster of differentiation 46 (CD46) is a type I membrane protein and is a regulatory part of the complement system. The encoded protein has cofactor activity for inactivation of complement components C3b and C4b by serum factor I, which protects the host cell from damage by complement (Sherbenou et al. 2016). CD46 activation also promotes cell growth and accelerates repair mechanism in intestinal epithelial cells (Cardone, Al-Shouli and Kemper 2011).

Interleukin 10 (IL-10) is an anti-inflammatory cytokine that can block the synthesis of other inflammatory cytokines and is a potent suppressor of the functions of macrophages (Moore et al. 1993); moreover, it promotes the regenerative healing of wounds (Peranteau et al. 2008), and it seems to be involved in inhibiting autophagy in fibroblasts (D 2016).

Glutathione peroxidase (GPx) is an intracellular enzyme able to reduce hydrogen peroxide into water, exerting a function of modulation of cellular oxidative stress and redox-mediated responses (Lubos, Loscalzo and Handy 2011); its involvement in the redox cellular homeostasis is a key element in cell proliferation and even, tumour growth (Jin et al. 2015).

Fas ligand (FasL) is a member of the TNF family of type 2 membrane proteins that provokes a rise in inflammatory response and decrease in microvascular endothelial cell
hyperpermeability (Sawant et al. 2013); in addition to that, it can lead to the Fas-mediated apoptosis (Wise et al. 2013).

The intercellular adhesion molecule (ICAM-1) plays a pivotal role mediating the interactions between leukocytes and activated endothelial cells (Liu, Liu and Xin 2017), facilitating the adhesion of leukocytes and enhancing their migration after an injury (Mohamadi Yarijani et al. 2017); even if it is considered a marker for inflammation in tissue (Mohamadi Yarijani et al. 2017), recent studies hypothesise that it may also play a role in the immunosuppression of T cells by mesenchymal stromal cells in case of chronic inflammatory status (Rubtsov et al. 2017).

Finally, metallothioneins are cysteine-rich low-molecular-mass intracellular proteins involved in intracellular storage, transport and metabolism of metal ions (Gumulec et al. 2014) and, for this reason, they play an essential role in both toxicokinetic and biochemistry of essential and toxic metals in the human body (Nordberg and Nordberg 2000). They allow the detoxification from heavy metals (Miles et al. 2000) and oxidative stress (Krizkova et al. 2012) preventing cytotoxicity (Namdarghanbari et al. 2011), and they are widely investigated as a marker for the various type of cancers (Gumulec et al. 2014).

It was hypothesized that pristine CuO NPs could elicit a strong inflammatory effect on different tissues following translocation and that the modified NPs could elicit a different effect to the animal model depending of the coating (higher toxicity for PEI coated NPs and lower toxicity for ascorbate coated NPs). The aim of this chapter is to help verifying these hypotheses using the gene expression approach and identifying the appropriate markers for inflammation and metal homeostasis.
4.2 Materials and Methods

Both STIS studies were performed at the RIVM. In addition, RIVM conducted the toxicokinetic study while lung, liver and gut (containing Payers patches) tissues were sent to Heriot-Watt University for cytokine and fibrosis analysis and gene expression and to the Karolinska Institutet, at first, for epigenetic study, then, in a second phase of the project, for OMICS study (microarray).

The experimental part performed at Heriot-Watt University was organized as follows:

- The gene expression was performed as part of this PhD project
- The cytokine and fibrosis analysis were performed by Dr David Brown. For a comprehensive view of the results, please refer to the Appendix.

4.2.1 STIS of Pristine CuO NPs

The first STIS used rats exposed via nose inhalation to 10mg/m³ pristine CuO NPs following different exposure times per day (in order to generate different deposited doses), while control animals were exposed to clean air (Table 4.1). (OECD 2009b) Animals were exposed repeatedly for 5 days.

<table>
<thead>
<tr>
<th>Group</th>
<th>Target Concentration (mg/m³)</th>
<th>Exposure Time (min)</th>
<th>Toxicology - mg/m³</th>
<th>Kinetics - mg/m³</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Measured Concentration (SD)</td>
<td>6 hours-concentration equivalent</td>
</tr>
<tr>
<td>1</td>
<td>Clean air</td>
<td>360</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>18</td>
<td>11.3 (6.5)</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>36</td>
<td>13.7 (11.4)</td>
<td>2.4</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>90</td>
<td>13.0 (1.3)</td>
<td>3.3</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>180</td>
<td>12.7 (2.1)</td>
<td>6.3</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>360</td>
<td>13.2 (1.5)</td>
<td>13.2</td>
</tr>
</tbody>
</table>
The tissue samples were collected at days 6 and 28 (respectively 24 hours and after three weeks of recovery post exposure) for both toxicological and kinetic analysis. Tissues from the rat lungs, livers and ileum (containing 5 to 6 Peyer’s patches) were immediately frozen in liquid nitrogen and maintained at -80°C until the delivery to Heriot-Watt University for gene expression and cytokine analysis.

### 4.2.2 STIS of modified CuO NPs

A second STIS was performed using both the ascorbate and PEI coated copper oxide NPs produced at ISTEC-CNR following the SbyD approach. The same protocol was applied at RIVM with the aim of maintaining the concentration of exposure in the same range of the previous experiment (Table 4.2). Again, tissues from lung, liver and ileum were frozen and sent to Heriot-Watt for both gene expression and cytokine analysis.

#### Table 4.2 Second STIS aerosol exposure. Cumulative exposure and duration of the different SbyD modifications.

<table>
<thead>
<tr>
<th>Group</th>
<th>Modification</th>
<th>Cumulative Exposure</th>
<th>Exposure Time min</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>Clean air</td>
<td>180</td>
</tr>
<tr>
<td>2</td>
<td>PEI</td>
<td>0.6</td>
<td>7</td>
</tr>
<tr>
<td>3</td>
<td>PEI</td>
<td>1.7</td>
<td>20</td>
</tr>
<tr>
<td>4</td>
<td>PEI</td>
<td>5</td>
<td>60</td>
</tr>
<tr>
<td>5</td>
<td>PEI</td>
<td>15</td>
<td>180</td>
</tr>
<tr>
<td>6</td>
<td>Ascorbate</td>
<td>0.77</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>Ascorbate</td>
<td>2.2</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>Ascorbate</td>
<td>6.7</td>
<td>60</td>
</tr>
<tr>
<td>9</td>
<td>Ascorbate</td>
<td>20</td>
<td>180</td>
</tr>
</tbody>
</table>

### 4.2.4 RNA extraction

RNA was extracted from tissues using the Mag MAX total RNA isolation kit (Ambion) according to the manufacturer's instructions. First, samples up to 5 mg animal tissue were homogenised directly in 100 µl of lysis/binding solution from the kit and the binding beads were magnetically captured and washed to remove impurities. Then, the samples were
incubated with 50 µl of turbo DNase for 15 min at room temperature to remove possible traces of genomic DNA that could interfere with the qPCR results. After the incubation step, 100 µl of rebinding solution were added, and the binding beads captured again and washed twice. The beads were captured and completely dried prior adding 50 µl of the final eluent.

The RNA content was measured using a NanoDrop 2000 (Thermo Scientific) and adjusted to the required final concentration of 200 ng RNA per ml. Using the High Capacity RNA-to-cDNA kit (Applied Biosystems), cDNA was produced, according to the manufacturer's protocol, by adding 9 µl sample in a PCR Eppendorf tube containing 10 µl of 2x Buffer mix and 1 µl 20x enzyme mix to a final volume of 20 µl per reaction. Then, the samples were incubated at 37°C for 60 min to allow the retro-transcription of RNA. Finally the reaction stopped by warming up the samples to 95°C for 5 min. After the cDNA synthesis, all the samples were frozen at -25°C for less than a week prior use.

4.2.5 Real-Time PCR

PCR experiments were conducted in quadruplicate on a 7900 RT fast PCR system, in 384 well plates and using SDS 2.3 software (Applied Biosystems). Each reaction consisted of 10 µl of 2x Master mix, 1 µl of 20x gene expression assay and 9 µl of RNase-free water containing the cDNA sample to a final volume of 20 µl. The thermal cycling conditions consisted of 40 cycles, each one alternating 15 seconds at 95°C and 1 minute at 60°C. Kits consisted of TaqMan® Master mix and the following primers: Rn00562055 m1 (tumour necrosis factor, TNFα), Rn00580432 m1 (interleukin 1 beta, IL1β), Rn00565482 m1 (interleukin 1 receptor, IL-1RI), Rn00586403 m1 (chemokine cxcl2, MIP-2), Rn00569886 m1 (complement regulatory protein, MCP-1 or CD46), Rn00563409 m1 (interleukin 10, IL10), Rn00577994 g1 (glutathione peroxidase, GSHpox), Rn00563754 m1 (FAS ligand, FASL), Rn00564227 m1 (ICAM-1), Rn00821759 g1 (Metallothionein 1A, MT1A), Rn00588658 g1 (Metallothionein 3, MT3), Rn01531734 g1 (Metallothionein 4, MT4) and Rn01479927 g1 (Ribosomal Protein - Large P2, Rplp2) as housekeeping gene. Rplp2 was selected as part of a validated panel of control gene suggested by TaqMan® for rat tissue showing a high stability in several rat tissues (Li et al. 2014) while the Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) did not show a high grade of stability throughout the different tissues (Hernández, Curi and Salazar 2015). The fold increase for each gene relative to Rplp2 was calculated as ΔΔCt using the system software.
4.2.6 Statistical Analysis

The data from all experiments were analysed using the Graph Pad Prism (version 6) statistical package with analysis of variance (ANOVA) followed by Dunnett post hoc test. PCR results were analysed using SDS 2.4, RQ Manager 1.2.1, DataAssist 2.1 and ExpressionSuite Software 1.0.3 (all Applied Biosystems). A 1.8-fold increase or decrease in gene expression was used as cut-off value as reported in other similar studies (Cheng et al. 2007; Gaiser et al. 2013c).
4.3 Results

4.3.1 STIS of Pristine CuO NPs

Pristine copper oxide NPs induced a dose-dependent upregulation of CXCL2 and IL-1β in lung tissue at day 6 at both 2.5 and 10 mg/m³ dose exposures, while no significant effect was detectable at 0.5 mg/m³. TNFα was upregulated only at the highest dose (10 mg/m³) (Figure 4.1). IL-10, IL-1IR, ICAM-1, FasL, GPx, CD46 and MT1A, 3 and 4 did not show any significant changes in expression in lung tissue at day 6. For the liver tissue, a -1 and -1.9-fold down-regulation of MT1A expression was detected at doses of 0.5 and 2.5 mg/m³, and a 1.5-fold upregulation at the highest dose (10mg/m³). However, only the downregulation occurring at 2.5 mg/m³ was both statistically significant and physiologically relevant (1.8-fold change boundary) (Figure 4.2). CXCL2, IL-1β, TNFα, IL-10, IL-1IR, ICAM-1, FasL, GPx, CD46 and MT3 and 4 did not show any significant change in expression in liver tissue at day 6. In addition, no physiologically relevant alteration in gene expression was observed in the gut, with the exception of CD46, that even though the decrease was statistically significant (-1.49-fold change), it was below the physiological limit of 1.8-fold change (Figure 4.3). Some of the genes analysed did not show any statistical significance, even if expression increased by over the cut-off value of ±1.8 fold (Table 4.3). This was due to the high variability of the basal expression in the control group.
Figure 4. 1 Gene expression of rat lung tissue exposed to pristine CuO NPs (day 6). Values are expressed as fold change compared to the control ± standard deviation (n=3). Statistical analysis via ANOVA (Dunnett) was performed, and a cutoff limit of ±1.8 fold change was used to indicate physiological significance.

Figure 4. 2 Gene expression of rat liver tissues after inhalation of pristine CuO NPs (day 6); MT1A results expressed as fold change. Error bars represent the standard deviation (n=3). Statistical analysis via ANOVA (Dunnett) was performed, and a cutoff limit of ±1.8 fold change was used to indicate physiological significance.
Figure 4. Gene expression of rat ileum tissues after inhalation of pristine CuO NPs (day 6); CD46 results expressed as fold change. Error bars represent the standard deviation (n=3). Statistical analysis via ANOVA (Dunnett) was performed, and a cutoff limit of ±1.8 fold change was used to indicate physiological significance.

Table 4.3 Synthesis of results that fulfilled both statistical and cut-off restrain values.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Genes</th>
<th>Groups</th>
<th>Fold Change</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>CXCL2</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>2.688</td>
<td>(P&lt;0.025)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>3.718</td>
<td>(P&lt;0.01)</td>
</tr>
<tr>
<td></td>
<td>IL-1β</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>3.013</td>
<td>(P&lt;0.025)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>4.109</td>
<td>(P&lt;0.001)</td>
</tr>
<tr>
<td></td>
<td>TNFα</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>-1.9</td>
<td>(P&lt;0.025)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>-1.49</td>
<td>(P&lt;0.025)</td>
</tr>
<tr>
<td>Liver</td>
<td>MT1A</td>
<td>2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1.9</td>
<td>(P&lt;0.025)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Ileum</td>
<td>CD46</td>
<td>6</td>
<td>-1.49</td>
<td>(P&lt;0.025)</td>
</tr>
</tbody>
</table>
4.3.2 STIS of Modified CuO NPs

The modified CuO NPs showed no statistically significant changes in expression of any of the genes investigated in both liver and ileum tissues. No recognisable dose dependence pattern of expression was identified in the samples. Only in lung tissues were any changes in gene expression detected, in which TNFα increased 20-fold after exposure to 0.6 and 5 mg/m³ cumulative dose exposures for the PEI coated NPs at day 6 (Figure 4.4). It was not possible to obtain data for the highest dose (15 mg/m³) probably due to potential issues occurred during packaging and/or transport. Ascorbate coated CuO showed a 7-fold upregulation of TNFα at day 6 at 0.77 mg/m³, but again it was not possible to record any change in expression at exposure concentrations of 6.7 and 20 mg/m³. A possible explanation could be linked to the possibility that some samples were compromised either during the delivery or storage.

![Figure 4.4: Gene expression of lung tissue tissues after inhalation of modified CuO NPs (day 6) treated with modified PEI and Ascorbate CuO NMs. TNFα results are expressed as log of the relative quantification (RQ) or fold change increase compared to the control ± standard deviation (n=3). Statistical analysis was conducted using ANOVA (Dunnett). Cut off limit ±1.8-fold change used to indicate physiological significance.](image-url)
4.4 Discussion

4.4.1 STIS of Pristine CuO NPs

These results demonstrate that copper oxide induced a dose-dependent upregulation of CXCL2, IL-1β and TNFα in lung tissue 24 hours after the 5 days inhalation exposure to CuO NPs. All of these genes are involved in orchestrating a pro-inflammatory response and in the pathogenesis of acute lung inflammation (Inoue et al. 2006) and correlates well with the changes in neutrophils observed by Gosens et al. (2016a) in the lung. TNFα is a key regulator of the inflammatory process (Bradley 2008) inducing the secretion of chemotactic molecules with consequent migration of macrophage (Barlow et al. 2005), and it is usually upregulated or produced by NPs both in vitro and in vivo generating oxidative stress (Khanna et al. 2015) like other metal oxides such as ZnO (Kermanizadeh et al. 2012a; Bashandy et al. 2017) and Ag NPs (Kermanizadeh et al. 2014a; Seo et al. 2017) or even by ultrafine particles such as carbon black (Brown et al. 2004). CXCL2 upregulation is associated to lung and tissue damage (Serebrovska et al. 2017), and it was already reported in other studies to increase after exposure to silica NPs (Higashisaka 2014; Marzaioli et al. 2014) and MWCNTs (Howard and Murashov 2013). In addition to that, there is interesting evidence suggesting that IL-1β gene expression is increased during inflammation following exposure to silver and TiO2 NPs (Trickler et al. 2010; Yazdi et al. 2010).

In liver tissue, metallothionein 1A was downregulated at the lowest and median dose exposure (respectively 0.6 and 2.5mg/m³), while it was up-regulated at the highest dose (10 mg/m³). MT1A has a high content of cysteine residues that bind various heavy metals, it is transcriptionally regulated by both heavy metals and glucocorticoids and is usually induced when serum and hepatic copper levels increase (Jiang et al. 2011). These proteins increase the resistance of cells to high levels of copper (Tapia et al. 2004) and abnormalities in their expression are usually associated with various diseases. For example, low levels of MT1A and in general, downregulation of MT isoforms are found in human lung cancers, especially in malignant tumours compared with cancer-surrounding tissues (Liang et al. 2013). On the contrary, the increase in expression of metallothioneins is usually found as a consequence of oxidative stress to protect cells against cytotoxicity and DNA damage (Ruttkay-Nedecky et al. 2013). Finally, in ileum samples containing the Peyer’s patches only the complement
regulatory protein, also known as cluster of differentiation 46 significantly decreased by 1.49-fold but this downregulation was not physiologically relevant.

In synthesis, the gene expression results for the pristine CuO exposure at day 6 suggested the existence of a general inflammation process in lung tissue with some possible cross-linked effect also on liver tissue, while it was not possible to detect any inflammation markers in the ileum. These results are consistent with the findings obtained by the other project partners. In fact, scientists from the RIVM were able to detect signs of alveolitis, bronchiolitis, vacuolation of the respiratory epithelium and emphysema in the lung starting at 2.4 mg/m$^3$, together with the degeneration of the olfactory epithelium in the nose twenty-four hours after exposure to 6.3 and 13.2 mg/m$^3$. The cytokine analysis performed at Heriot-Watt University showed a significant increase in IL-1$\alpha$, IL-1$\beta$ and MIP-2 (also known as CXCL2) together with a small variation of IL-12 and TNF$\alpha$ in lung tissue (even if no change in liver tissue were detectable). The epigenetic study performed by the Karolinska Institutet did not produce any significant result as the methylation was highly variable and below 1%; on the other hand, the OMICS study performed using the microarray technology showed that more than 1000 genes involved in inflammatory pathways were dysregulated. Since gene expression studies on acute inflammation are not useful after prolonged periods of recovery (Noël et al. 2016), the only available data after 28 days were the histopathology and kinetics results together with the microarray. These results showed that even if copper was completely cleared from the tissue and returned to the baseline levels a limited inflammation was still present in the lungs with no sign of fibrosis; the microarray results showed that almost all the genes returned to the baseline values. A brief summary of the project partners’ result is presented in Figure 4.5, 4.6 and 4.7; for more detailed information refer to Appendix.
Figure 4. 5 Summary of the histology and toxicokinetic data obtained at RIVM.

Histology and Toxicokinetic

Day 6

Alveolitis, bronchiolitis, vacuolation of the respiratory epithelium and emphysema in the lung starting at 2.4 mg/m³

Limited inflammation was still observed, but only at the highest dose of 13.2 mg/m³

Olfactory epithelium in the nose degenerated twenty four hours after exposure to 6.3 and 13.2 mg/m³

Restored

No histopathological changes were detected in the brain, olfactory bulb, spleen, kidney and liver

The measured total load for the highest exposed group was 0.085 mg per lung

Cu completely cleared from the lung and levels returned to baseline

Figure 4. 6 Summary of the cytokine analysis performed at Heriot-watt University.

Cytokine Analysis

No clear dose effect

Lung:

IL-6 or IL-13 no detectable
IL-1α, IL-1β, MIP-2 significantly increased
IL-12 and TNF-α variation but no significant increase

Liver:

No significant increase

Figure 4. 7 Summary of the epigenetics and microarray data obtained by Karolinska Institutet and Health Canada.

Epigenetics

Gene methylation were overall low and highly variable

A relation between epigenetic regulation of gene expression and the reported endpoints related to inflammatory response in the exposed animals could not be shown

Microarray

Upregulation of 1000 genes mostly related to cell proliferation and inflammation at high dose exposure at day 6 while less than 20 were still dysregulated after 28 days

No evident correlations were found between the epigenetics results and transcriptomics results
4.4.2 STIS of Modified CuO NPs

Interestingly, TNFα was the only cytokine showing a significant difference in the lung of the animals treated with the modified CuO NPs. For the ascorbate coated NPs, only the lowest dose, corresponding to 0.66 mg/m³ cumulative exposure, induced a 7-fold increase in TNFα suggesting that at day 6, even in the presence of a natural antioxidant as ascorbate, CuO NPs were able to elicit an inflammatory effect even at low doses. The PEI coated NPs were able to generate a TNFα upregulation of around 20-fold change in both 0.6 and 5 mg/m³ cumulative dose exposure as expected.

Since PEI coating is widely used as an alternative to viral gene delivery (Pathak, Patnaik and Gupta 2009) due to its ability to enter the cell membrane and its intrinsic endosomolytic activity (Di Gioia and Conese 2008), the high increase of inflammatory response in lung tissue could be explained with possible enhanced uptake effect driven by the PEI coating itself. In contrast, the result obtained using the other coated NPs showed that ascorbate, even if it was not visible any protective effect on day 6, after the recovery period elicited an enhanced healing effect as expected since ascorbate is historically known for its antioxidant properties (Halliwell 1990; Beyer 1994; Jomova et al. 2010) preventing oxidative damage led by metal ions including copper (Gaetke and Chow 2003).

On the other hand, it must be stressed that the histopathological results from RIVM showed that the inflammation caused by ascorbate coated NPs in rat lungs at day 6, completely disappeared even at the highest doses after the recovery period unlike what happened with the pristine form of CuO NPs, suggesting that the ascorbate coating could play a role in helping the recovery of the damaged tissue (refer to Appendix). A brief summary of the project partners’ result is presented in Figure 4.8 and 4.9; for more detailed information refer to Appendix.
Figure 4. Summary of the histology and toxicokinetic results for both Ascorbate and PEI coated CuO NPs obtained at RIVM.

**Histology and Toxicokinetic**

Day 6

- Interstitial/alveolar inflammation
- Hypertrophy/hyperplasia of bronchioles/alveoli
- Alveolar (cellular) debris in the lung
- Paracortical histiocytosis in the mediastinal lymph node

Day 28

- No inflammatory cell accumulation at day 28 for any exposure concentration

Similarly for what has been found for the pristine CuO NPs, no Ascorbate or PEI coated NPs were observed other than in the lung and the Cu was cleared after the recovery time.

Figure 4. 8 Summary of the histology and toxicokinetic results for both Ascorbate and PEI coated CuO NPs obtained at RIVM.

**Cytokine Analysis**

Clearer dose effect pattern

**Lung:**
- IL-6 or IL-13 no detectable
  - Ascorbate: No increased cytokine release except for MIP-2, which was significantly greater than the control at high dose
  - PEI: increase in IL-1α at the 1.8 mg/m³ dose and a significant increase in IL-1β at the 0.6 and 17.3 mg/m³ doses. Similarly, MIP-2 increase at the highest dose of 17.3 mg/m³.

**Liver:**
- Both Ascorbate and PEI: Significant increase in IL-1β

Figure 4. 9 Summary of the cytokine analysis performed at Heriot-Watt University.
4.5 Conclusions

According to the STIS results, inhalation of pristine CuO NPs caused an acute reaction in the lung tissue associated with inflammation, including both activation and mobilization of immune white cells. These observations were backed up by the up-regulation of genes for pro-inflammatory mediators as well as cytokine proteins. Coating these NPs with PEI seemed to enhance the inflammatory effect of the material, suggesting that PEI coated CuO is likely to be relatively toxic. Interestingly, no significant increase of TNFα was detected during the cytokine analysis, nevertheless both a small increase in IL-1α at 1.8 mg/m³, a significant increase in IL-1β at 0.6 and 17.3 mg/m³ doses and an increase in MIP-2 (CXCL2) at 17.3 mg/m³ were detected confirming the pro-inflammatory activity of PEI coated CuO.

Although the histopathological data confirmed the presence of inflammation and degeneration of tissues, no clear effects on gene expression of lung tissue were observable after exposure to ascorbate coated materials; furthermore, the ASC modified CuO NP did not cause any increased cytokine release in the lung except for MIP-2 (CXCL2), which was significantly greater than the control at the dose of 21.8 mg/m³. No further data about microarray in regard to either PEI or ascorbate coated CuO NPs are still available from Karolinska Institutet and Health Canada. In conclusion, analysing the results obtained in the light of the fact that after the recovery period the inflammation was almost resolved, the study seems to confirm the hypothesis according to which the ascorbate coating might be a promising strategic alternative to the pristine CuO NPs.

Probably the exposure to pristine CuO NPs could lead to a mild increase of plasma level of both CuO translocated and Cu²⁺ released by damaged lung tissues, affecting the homeostasis of metals in the liver and causing a change in expression of metallothioneins involved in metal regulation; moreover, the kinetic data showed that in both STIS, after the recovery period there was no presence of copper over the physiological level. During the inhalation exposure (aerosol phase), a small fraction of nanomaterials is usually accumulated between the nose and the mouth of rats, and it can be easily ingested by the animals itself leading to a small oral exposure (Fröhlich and Salar-Behzadi 2014). Nevertheless, no suggestion of any possible pro-inflammatory gene expression of the intestinal tissue was identified.
Chapter 5: *in vivo* Oral Study
5.1 Introduction

After inhalation, ingestion represents the second route of exposure to NPs and NMVs via both direct and indirect exposure (Bergin and Witzmann 2013). Possible examples of this direct ingestion are due to food contamination i.e. eating fish and shellfish that, filtering water to feed on microorganisms, can accidentally ingest NPs dispersed in the environment leading to a phenomenon called bioaccumulation (Gaiser et al. 2009). Some application in food industry such as additive (i.e. anti-caking agent), pigments and health supplements (Bouwmeester et al. 2009; Fröhlich and Roblegg 2012) can also lead to possible exposure. On the other hand, products such as Ag NPs coated toothbrush or food and drink containers and even food-related childcare products like feeding bottle and pacifier can lead to a potential indirect ingestion exposure (Benn et al. 2010).

Finally, another possible route of exposure is represented by the secondary unintentional ingestion of NPs cleared from the respiratory tract either via mucociliary escalator in the respiratory tract (Bergin and Witzmann 2013) or via alveolar macrophage clearance of the distal part of the lung (Geiser et al. 2008; Gustafson et al. 2015). Mucociliary clearance is the first defence mechanism of the respiratory tract and consists in the action of cilia that, beating in coordinate fashion, propel mucus outside the lung to the final expulsion through coughing (Möller et al. 2006). Macrophages can help this clearing process by phagocyting those particles deposited inside the alveoli and, by active migration, reaching the mucociliary escalator (Barlow et al. 2008).

No current studies address the oral toxicity of either micronized copper or pristine CuO NPs. Other copper NPs (not oxide) were severely toxic at 5000 mg/kg in an oral study performed on rats (Lee et al. 2016) and their toxic effect seemed to be mainly driven by highly reactive copper ions after their dissolution due to gastric juices (Bahadar et al. 2016; Lee et al. 2016). In a similar way, also the micronized copper formulation is able to release toxic amount of ions in acidic environment as highlighted in a study of dissolution in synthetic stomach fluid (Santiago-Rodríguez et al. 2015).

Both copper-based formulations are widely used either as paint additive or as impregnating solution in the timber industry for their antifungal properties against wood destroying basidiomycetes. Occupational exposure to substances used in wood impregnation such as
copper, arsenic and chromium is historically known (Nygren, Nilsson and Lindahl 1992), and together with the occupational exposure to paint (de Oliveira et al. 2011; IARC 2012) may represent a possible safety risk that needs to be investigated. The Work Package 5 of the SUN project highlighted that occupational ingestion exposure can occur during the consumption of food or beverages which contain or are contaminated with nanomaterials, swallowing NPs cleared by ciliated airways of the respiratory tract (both already mentioned) or touching the mouth and perioral region with contaminated hands and/or objects. During this contact, in fact, NPs can be transferred from the hand directly to the oral mucosa (http://www.sun-fp7.eu; Deliverable 5.2). This inadvertent form of exposure is generally defined either as hand-to-mouth or object-to-mouth, and it has been estimated that in the sole UK, 16% of working population is exposed to this kind of accidental ingestion (Cherrie et al. 2006).

The main hypothesis of this study was that the ingestion of either pristine CuO NPs or micronized copper formulation following oral exposure can elicit an inflammatory response in vivo. The hypothesis was tested using a new short-term oral study (STOS) protocol (OECD 2008a) based on the existing STIS protocol and conducted by RIVM. The STOS was designed to evaluate whether a 5-day oral exposure could be indicative of (sub)acute toxicity. The aim of this study was to assess whether there was any relationship between exposure to the copper oxide by the oral route and the range of adverse effects observed in rats and gene expression markers of inflammation. The endpoints of the STOS consisted of molecular, histological and biochemical analysis of tissue responses, including intestine as an organ of exposure and liver as the primary location of NMs accumulation and the gene expression. While the latter is presented in this chapter as part of the PhD study, the additional results generated by project partner are presented in Appendix.
5.2 Materials and Methods

This STOS was performed at the RIVM, with tissues provided to Heriot-Watt University for the gene expression and the cytokine analysis.

5.2.1 STOS

Male rats (RjHan:WI, pathogen-free) of 8-9 weeks old, obtained from Janvier Labs (Le Genest-Saint-Isle, Saint Berthevin, France) were treated with either pristine CuO NPs or micronized copper formulation on five consecutive days and the autopsy was performed at day 6, 24 hours after the last oral administration (Table 5.1). In addition to that, a post-exposure period of 3 weeks was included in the experiments to evaluate either recovery or possible persistence of the NPs in the body and ongoing pathology. An autopsy of the recovery groups was performed at day 26. A first pilot study with CuO NPs administered by oral gavage was performed at 64 mg/kg, then, both CuO NPs and micronized copper were administered as 0.1 ml per 20 g (1 ml per 200 g) during the experiment. The doses of exposure were chosen based on information in the literature for soluble CuSO₄ which indicated a no observed adverse effect level (NOAEL) of 16.3 mg/kg (Hebert, 1993). The doses were chosen according to the OECD Test no. 425 (OECD 2008b); these guidelines suggest to treat a single animal with a single dose of toxicant. If no toxicity is observed the dose should be increased in the next animal until a toxic response is noted, if the animal dies, the dose should be decreased accordingly. When a single animal shows a toxic response, the same dose can be administered to 4 additional animals. The highest dose chosen for the CuO was 512 mg/kg and for CuCO₃ 128 mg/kg (Table 5.2).

Both CuO NPs and micronized copper were prepared by either dispersion or dilution in Milli-Q water with subsequent 16 minutes sonication in a water bath (Elmasonic S100) at room temperature to optimize dispersion quality. Before each administration, the NPs dispersion was evaluated by CPS Disc Centrifuge™ (CPS Instruments Europe, Oosterhout, The Netherlands) to determine nanoparticle size and size distribution. Both CuO NP and micronized copper dispersions remained stable up to 24 and 72 hours respectively, after preparation of the dispersion.
Animals were killed by exsanguination from the abdominal aorta during anaesthesia by isoflurane (3.5% in oxygen) inhalation. General post-mortem examinations were performed on all animals. Additional data regarding the tissue collected and the related findings are available in Appendix.

The gene expression analysis was performed on liver, stomach and ileum at the highest dose exposure of 32 mg/kg for CuO NPs, and for the micronized copper at the doses of 32 mg/kg and 128 mg/kg. Stomach, liver and ileum were chosen for their role as the main organs exposed to the action of both NPs and micronized copper formulation and for consistency with the previous gene expression performed in both STIS.

Table 5. 1 Experimental Design for STOS on CuO NPs. Autopsy and consequent tissue collection were performed at day 6 for the acute inflammation analysis and at day 26 for post-recovery analysis assessing ongoing pathology or recovery. Group 8 (red) represented the pilot study, and group 9 (green) was an extra high dose exposure group.

<table>
<thead>
<tr>
<th>Group (CuO) Exposure (mg/kg)</th>
<th>Autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 6</td>
</tr>
<tr>
<td>1</td>
<td>n=4</td>
</tr>
<tr>
<td>2</td>
<td>n=4</td>
</tr>
<tr>
<td>3</td>
<td>n=4</td>
</tr>
<tr>
<td>4</td>
<td>n=4</td>
</tr>
<tr>
<td>5</td>
<td>n=4</td>
</tr>
<tr>
<td>6</td>
<td>n=4</td>
</tr>
<tr>
<td>7</td>
<td>n=4</td>
</tr>
<tr>
<td>8</td>
<td>n=2</td>
</tr>
<tr>
<td>9</td>
<td>n=4</td>
</tr>
</tbody>
</table>

Table 5. 2 Experimental Design for STOS on CuO NPs. Autopsy and consequent tissue collection were performed at day 6 for the acute inflammation analysis and at day 26 for post-recovery analysis assessing ongoing pathology or recovery.

<table>
<thead>
<tr>
<th>Group (Micronized Cu) Exposure (mg/kg)</th>
<th>Autopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 6</td>
</tr>
<tr>
<td>1</td>
<td>n=4</td>
</tr>
<tr>
<td>2</td>
<td>n=4</td>
</tr>
<tr>
<td>3</td>
<td>n=4</td>
</tr>
<tr>
<td>4</td>
<td>n=4</td>
</tr>
<tr>
<td>5</td>
<td>n=4</td>
</tr>
<tr>
<td>6</td>
<td>n=4</td>
</tr>
<tr>
<td>7</td>
<td>n=4</td>
</tr>
</tbody>
</table>

5.2.2 RNA extraction, Real-Time PCR and Statistical Analysis

For further information on these methods, refer to Chapter 4 (STIS Studies) sections 4.2.4, 4.2.5 and 4.2.6.
5.3 Results

In liver samples, the only detectable gene to be altered in expression both statistically (p<0.001) and according to the physiological boundaries (-2.5-fold change) was CXCL2 at day 6 post-exposure (Figure 5.1). This alteration took the form of a down-regulation of this gene following exposure to micronized copper at the dose of 32 mg/kg. Treatment with 32mg/kg of CuO NPs did not modify the expression of this gene in liver tissue at day 6.

Contrary to the STIS, neither TNFα nor MT1A gene expression was observable in the liver tissues at day 6 following the STOS. Gene expression results from stomach samples were inconclusive due to either the high variability between the animals or possible degradation of the samples. Micronized copper-induced a significant (p>0.05) down-regulation of MT1A in ileum; nonetheless, being the downregulation of -1.4-fold change, it was below the 1.8 limit used in this study (Figure 5.2).

![Figure 5.1](image1.png)  
*Figure 5.1* Gene expression of CXCL2 in liver tissue from STOS (day 6) treated with CuO NPs and CuCO$_3$ NMs. Values are expressed as log of the relative quantification (RQ) or fold change increase or decrease compared to the control ± standard deviation (n=3). Statistical analysis was conducted using ANOVA (Dunnett). Cut off limit ±1.8 fold change used to indicate physiological significance.
Figure 5. Gene expression of MT1A in ileum tissue from STOS (day 6) treated with CuO NPs and CuCO₃ NMs. Values are expressed as log of the relative quantification (RQ) or fold change increase or decrease compared to the control ± standard deviation (n=3). Statistical analysis was conducted using ANOVA (Dunnett). Cut off limit ±1.8 fold change used to indicate physiological significance.
5.4 Discussion

These results, far from being conclusive on their own, should be interpreted in the light of both results from RIVM and the cytokine analysis presented in their entirety in the Appendix.

Briefly, in the STOS performed with the pristine CuO NPs, while there was no evident correlation between dose and cytokine production, only the increase of few parameters was recorded. These ones included liver markers, such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the total copper content in liver and mesenteric lymph node and the white cell count. These findings indicated an occurring liver toxicity at the lower doses (16 and 32 mg/kg) confirmed by histopathological analysis only at the dose of 512 mg/kg. On the other hand, in the STOS performed with the micronized copper there was a significant weight loss in all the animals exposed to 128 mg/kg together with a substantial alteration of several parameters for both 64 and 128 mg/kg dose exposure. The production of liver enzymes such as AST, ALT, alkaline phosphatase (ALP) and lactate dehydrogenase (LDH) increased together with the number of neutrophilic granulocytes while the relative percentage of lymphocytes decreased. There were severe lesions in the gastrointestinal tract with inflammation in the stomach, a sign of apoptosis in duodenum and ileum, and ulcerative lesions/erosions in the large intestines (cecum, colon, rectum). Moreover, the liver showed inflammation, hepatocellular hypertrophy, hepatocellular necrosis and single cell necrosis, especially in the two high dose groups (64 mg/kg and 128 mg/kg). In the lymphoid organs spleen and thymus, a severe lymphocyte depletion was present. Finally, the analysis of cytokine production showed an increase in IL-1α in the duodenum, liver and colon samples while there was no significant increase in IL-1β in any of the tissues. Furthermore, a significant decrease in IL-12, which is known to stimulate both innate and adaptive immune effector cells production (Liu et al. 2005), was observed at the two highest doses in liver tissue.

The gene expression results did not show any correlation with histology or cytokine production of the different tissues. The data was too variable in order to identify a statistically significant change and where this change occurred, it was below the physiological boundary established for this study. Liver tissues did not show any observable modification of gene expression values following exposure to CuO NPs, while the micronized copper-induced two different responses related to the exposure dose. First, it produced a significant down-regulation of CXCL2, one of the principal mediators of inflammation in acute liver injury (Qin et al. 2017), at 32 mg/kg.
Then, it caused a small up-regulation of the same gene at 128 mg/kg suggesting a change in trend that, even if still not significant, was still consistent with the other findings that emphasized a principle of inflammation in liver tissue. Finally, the ileum showed a down-regulation of MT1A in both doses; the down-regulation of metallothioneins in general in the different intestinal tracts are often associated with diarrhoea and intestinal inflammation (Tronstad et al. 2015).
5.5 Conclusions

The different markers analysed in the STOS – i.e. blood and liver parameters, histopathology and cytokine – showed a clear sign of inflammation subsequent exposure to both CuO NPs and micronized copper. The sole gene expression analysis, on the other hand, was not able to highlight any suitable marker of inflammation in both stomach and liver of the treated rats probably due to the high variability observed between the different animals. Moreover, an investigation of more time point might have been helpful to screen if the selected genes were or not a suitable choice for all the tissues analysed. However, the results obtained from the ileum indicated that the metallothioneins, even if not a direct indicator of inflammation, could be considered as one of the possible markers for analysing the response to the copper-containing formulation of the gastrointestinal tract. Furthermore, MT1A showed an indicative pattern even at doses that do not produce other observable effects with other analysis.
Chapter 6: Other Nanoparticles
6.1 Introduction

After analysing the different copper formulations, also the toxicity of other NPs was investigated in accordance with the prioritization criteria established within the project’s consortium. The criteria adopted for developing this strategy included considerations such as the effective volume of product on the market which feeds into the potential for exposure, the lack of available information about risk and the potential for toxicity based upon chemistry.

Following this approach, tungsten carbide cobalt-coated (WCo) NPs, NANOCYL™ NC7000 NMs consisting of multi-walled carbon nanotubes (MWCNTs), silicon dioxide (SiO₂) NPs and Irgazin, a nanoform of the organic pigment diketopyrrolopyrrole (DPP) coded as Pigment Red 254, were selected for both cytotoxicity and cytokine production (Table 6.1).

Table 6.1 List of the case study NPs and NMs

<table>
<thead>
<tr>
<th>CASE STUDY</th>
<th>NOAA Composition</th>
<th>SUN CODE</th>
<th>Primary nm size Min- Max (average)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WCCo Coatings</td>
<td>Tungsten Carbide &lt;88% Cobalt (Co) &lt;12%</td>
<td>WCCo-1-NP-SYN</td>
<td>23-1446 (170)</td>
</tr>
<tr>
<td>CNT for lightweight conductive plastic and for antifouling coating</td>
<td>Multi-walled short tangled carbon nanotubes 90% Other additives 10%</td>
<td>CNT_1_NP_SYN</td>
<td>Ø: 4-16 (8) L: 575-3462 (1543)</td>
</tr>
<tr>
<td>SiO₂ Food additive</td>
<td>NM202 powder (SiO₂)</td>
<td>SiO2_1_NP_SYN</td>
<td>3-27 (11)</td>
</tr>
<tr>
<td>Organic Pigment in plastic</td>
<td>diketopyrrolopyrrole C18H10Cl2N2O2 (Pigment Red 254)</td>
<td>Pigment_1_NP_SYN</td>
<td>14-151 (43)</td>
</tr>
</tbody>
</table>

WCCo NPs were produced by MBN Nanomaterialia through a High Energy Ball Milling, a process that leads to their combination and their reduction in size. The synthesis was made in an inert atmosphere during which the process of milling results in the transfer of mechanical energy to the raw WC and Co powder. Isopropyl alcohol is added as a process control agent and later removed by spray drying.
The pristine WCCo NPs are then deposited as a thick coating by Thermal Spraying by MBN’s customers onto the final product. Specifically, for WCCo, High-velocity oxy-fuel spraying (HVOF) is used, this process involves high temperatures and high-velocity particle jet flow. These WCCo sintered ceramics are applied for enhancing toughness and strength of a wide variety of mechanical part of industrial machinery (Bastian et al. 2009).

MWCNTs (NANOCYL™ NC7000) were produced by Nanocyl S.A. through the Catalytic Chemical Vapor Deposition (CCVD) process in a facility with tons-scale capacity. The final usage of this materials would be either the production of lightweight conductive plastics for automotive industries (Figure 6.1) or as antifouling coatings for naval purposes.

In the first scenario, car bumpers are produced by combining the MWCNTs with the polypropylene in a master batch by extrusion with a concentration of approx. 2.5% and subsequent moulding (Hambali et al. 2010).

For the antifouling coating, a mixture of 0.6% of MWCNTs with epoxy resin was manufactured by milling and mixing processes. The MWCNTs were dispersed in the paint to form the LioCyl HPT TOPCOAT HS. This coating aims to help drastically increase the hydrophobicity of motorboats while immersed, allowing an overall enhancement of the boat’s performance. The coating also reinforces the hulls mechanical properties, increasing its corrosion and scratch resistance (Yakobson and Avouris 2001).
Nano-SiO₂ (NM202) has been dispersed in a powdered pancake matrix an anti-caking agent to prevent the powder forming lumps (Figure 6.2). Aiming at a homogeneous dispersion of NM202 in the matrix, four different mixing technologies were applied by the RIKILT Institute of the Wageningen University (The Netherlands), including several head-over-head mixing strategies.

![Picture of commercial pancake mixture](image1.png)

*Figure 6.2 Commercial pancake mixture with homogeneously dispersed NM-202. Picture from deliverable D 1.3, available online at [www.sun-fp7.eu](http://www.sun-fp7.eu).*

Diketo-pyrrolo-pyrroles (DPPs) are a new class of high-performance pigments brought to the market in the eighties due to their high-performance properties such as fastness, lightness, heat and weather resistance, *etc.* Due to these reasons, these pigments cover a wide range of industrial applications including painting dispersants, colour coatings, printing inks and general plastic applications (Hofmann *et al.* 2016).

Considering the wide employment of these pigments, the fact that they are produced, transported and used as an ultrafine dry powder, raises some concerns about their possible health effects, especially in occupational sectors where they are handled in large quantities (Hofmann *et al.* 2016).
Organic pigment Red 254 is a very transparent and saturated DPP, well suited for automotive effect finishes produced by BASF (Figure 6.3). It is marketed as a transparent pigment in the sense that it is possible to achieve high transparency with low light scattering (as is normally achieved only for molecularly dissolved dyes), but still to retain the colour performances and light stability advantages of particulates (pigments).

![Figure 6.3](image)

*Figure 6.3* Photograph of formulated pigment in plastic presently available as granulates and plates. Picture from deliverable D 1.3, available online at [www.sun-fp7.eu](http://www.sun-fp7.eu).

**Aim**

The aim of this chapter was to screen the toxicity of WCCo, MWCNTs, SiO2 and Red 254 investigating both cytotoxicity (Alamar Blue) and IL-8 production (Multiplex) in C3A cells. Moreover, for the sole WCCo, as established in the project prioritization, also the genotoxicity was investigated using the Comet assay.
6.2 Materials and Methods

All NPs and NMs of this study were screened for endotoxin detection. This test was performed by our partner at Karolinska Institutet (Norway). The procedure followed the Nanotechnology Characterization Laboratory protocol (NCL Method STE-1.1) using the assay QCL-1000™ Endpoint Chromogenic LAL Assay (Lonza). All values obtained were below 0.5 EU/ml, which is considered the maximum admissible limit for medical devices. Additionally, no interference between the NM samples and the assay substrate were observed suggesting that the data was reliable.

6.2.1 Tungsten Carbide Cobalt-doped NPs

The WCCo NPs series J300 were provided by MBN Nanomaterialia S.p.A. (Italy). This product is a heterogeneous mixture of fine tungsten carbide product with cobalt binder (Table 6.2) that it functions as a metal matrix, for this reason, it is also known as cemented carbide. It is widely used as a thermal spray and in the fabrication of diamond cutting tools.

<table>
<thead>
<tr>
<th>Product / ingredient</th>
<th>Wt.%</th>
<th>CAS Number</th>
<th>EINECS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tungsten Carbide (WDC)</td>
<td>&lt;88%</td>
<td>12070-12-1</td>
<td>235-123-0</td>
</tr>
<tr>
<td>Cobalt (Co)</td>
<td>&lt;12%</td>
<td>744-48-4</td>
<td>231-158-0</td>
</tr>
</tbody>
</table>

The hydrodynamic diameter of WCCo NPs suspended in DMEM medium (supplemented with 10% FBS) at a concentration of 125 µg/ml was analysed by DLS analysis by colleagues at Ca’ Foscari University of Venice (Italy).

The dispersions were analyzed immediately after the preparation and after 24 hours of incubation at 37°C. According to DLS analysis, the hydrodynamic diameter of WCCo was 315 nm with a PDI of 0.680 at time zero. A slight increase of the PDI and a slight decrease on the Z-average occurred after 24 hours of incubation (Table 6.3 and Figure 6.4).
Table 6. WCCo: Dynamic light scattering (DLS) measurements of hydrodynamic diameter in DMEM with 10% FCS at time 0 (T₀). Data from deliverable D 1.4, available online at www.sun-fp7.eu.

<table>
<thead>
<tr>
<th>NPs</th>
<th>PDI (polydispersity index)</th>
<th>Z-Average (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T₀</td>
<td>T₂₄</td>
</tr>
<tr>
<td>WCCo_1_NP_SYN</td>
<td>0.641</td>
<td>0.680</td>
</tr>
</tbody>
</table>

Figure 6. WCCo: DLS size distribution measurements at T₀ and T₂₄ for CuO, WCCo and SiO₂ dispersed in DMEM culture medium supplemented with 10% FBS. Picture from deliverable D 1.4, available online at www.sun-fp7.eu.
6.2.2 Synthetic Amorphous Silicon Dioxide (NM 202)

The NM202 powder was provided by the Fraunhofer Institute of Molecular Biology and Applied Ecology (IME, Germany) and all the information about characterization were already available from the European Commission’s Joint Research Centre Repository (Figure 6.5).

Particle sizes, measured on TEM, range from 5 to 30 nm and they are reported to usually form agglomerates having a size ranging from 10 nm up to 600 nm (Institute for Health and Consumer Protection, JRC, 2013) (Figure 6.6). XRD analysis suggests a very low presence of impurities of Böhmite (γ-AlO(OH)).

![Figure 6.5 SiO2 (NM 202) Characterization. BET analysis results on the top table; TEM primary particle size and aggregates formation (A); XRD (B) and DSL in Milli-Q water (C and table on the bottom). Pictures and data from the European Commission’s Joint Research Centre Repository (Rasmussen et al. 2013b).](image)

DLS analysis performed by Veneto Nanotech (Italy) on SiO₂ NPs dispersed in serum-supplemented medium revealed a similar slight increase of the polydispersity index (PDI) and a decrease of the Z-average after 24h of incubation compared to the one seen in WCCo (Table 6.4).
Table 6. Dynamic light scattering (DLS) measurements in DMEM. Data from deliverable D 1.4, available online at www.sun-fp7.eu.

<table>
<thead>
<tr>
<th>NPs</th>
<th>PDI (polydispersity index)</th>
<th>Z-Average (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$T_0$</td>
<td>$T_{24}$</td>
</tr>
<tr>
<td>SiO$_2$-1_NP_SYN</td>
<td>0.491</td>
<td>0.506</td>
</tr>
</tbody>
</table>

Figure 6. SiO$_2$: DLS size distribution measurements at $T_0$ and $T_{24}$ for CuO, WCCo and SiO$_2$ dispersed in DMEM culture medium supplemented with 10% FBS. Data from deliverable D 1.4, available online at www.sun-fp7.eu.
6.2.3 Multiwalled Carbon Nanotubes

The MWCNTs used in this study are part of the commercially available NANOCYL™ NC7000 series.

According to the manufacturer, they are produced via the catalytic carbon vapour deposition (CCVD) process and are described as “tube-shaped materials, composed of carbon atoms, having a nanometric diameter” (http://www.nanocyl.com/product/nc7000) (Figure 6.7); characterization data were provided alongside the product by the manufacturer (Table 6.5).

![Figure 6.7 NC7000™ multiwall carbon nanotubes – scale: 100 nm – TEM. Source: Nanocyl safety data-sheet NC7000-V11.](image)

Table 6.5 Specific characterization of NC7000™. Data provided by Nanocyl SA.

<table>
<thead>
<tr>
<th>PROPERTIES</th>
<th>UNIT</th>
<th>VALUE</th>
<th>METHOD OF MEASUREMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average diameter</td>
<td>μm</td>
<td>9.5</td>
<td>Transmission Electron Microscopy (TEM)</td>
</tr>
<tr>
<td>Average length</td>
<td>μm</td>
<td>1.5</td>
<td>Transmission Electron Microscopy (TEM)</td>
</tr>
<tr>
<td>Carbon purity</td>
<td>%</td>
<td>90</td>
<td>Thermogravimetric analysis (TGA)</td>
</tr>
<tr>
<td>Transition Metal oxide</td>
<td>%</td>
<td>&lt; 1%</td>
<td>Inductively Coupled Plasma Mass Spectrometry (ICP-MS)</td>
</tr>
<tr>
<td>Amorphous carbon</td>
<td></td>
<td>*</td>
<td>High resolution Transmission Electron Microscopy (HRTEM)</td>
</tr>
<tr>
<td>Surface Area</td>
<td>m²/g</td>
<td>250-300</td>
<td>BET surface area analysis</td>
</tr>
<tr>
<td>Volume resistivity</td>
<td>Ω cm</td>
<td>10¹¹</td>
<td>Internal test method (resistivity on powder)</td>
</tr>
</tbody>
</table>

*Pyrolytically deposited carbon on the surface of the NC7000
6.2.4 Irgazin – Diketopyrrolopyrrole nano-form grade – Red 254

Organic Pigment Red 254 was provided by BASF (Germany). Hofmann et al. (2016) in their Short-Term Inhalation Study (STIS) performed a detailed characterization of a wide range of the most common commercially available DPPs including the Red 254. These NPs showed an average size of 43 nm (TEM d50 estimation) with a surface BET of 94 m²/g with a Zeta potential of -16 mV (Figure 6.8) (data from the Deliverable D 1.4 available online at www.sun-fp7.eu).

![Figure 6.8 Organic Pigment Red 254. TEM image of the nano-form grade of Irgazin pigment (a generic chemical formula is presented on the bottom left of the picture) while a summary of the Characterization data is presented on the right side. Source: Hofmann et al., 2016.]

6.2.5 Cytotoxicity and Cytokine Analysis

The cytotoxic potential of the nanomaterials was investigated using the Alamar Blue cell viability assay accordingly to the protocol used for the copper study (Section 2.2.3).

Cells were exposed to increasing concentrations of either NPs or corresponding chemical compound controls (from 0 µg/ml to 125 µg/ml) for 24 hours. The resulting raw data were collected using standardized SUN Excel templates in order to allow storage of the data within
a data repository. The templates included calculation of the Benchmark Dose where 20% of effect ($BMD_{20}$) was measured using R software with PROAST package (version 38.9).

After exposure, cell medium was removed and frozen for cytokine analysis. Cells were washed twice with 100 µL PBS and the viability was measured using the Alamar Blue assay. For this purpose, 100 µL of complete medium supplemented with resazurin salt (Sigma R7017) (10 µg/mL in MEM) was added in each well and incubated in a humidified atmosphere at 37 °C and 5% CO$_2$ for two hours.

The samples were then transferred to a microplate reader and the fluorescence measured at a wavelength of 570 nm. Supernatants were transferred to fresh plates to decrease the potential interference of the NPs during the measurement of the fluorescence.

The inflammatory response was evaluated using the Bio-Plex® Multiplex system in the supernatant of cells exposed to WCCo, CoCl$_2$, SiO$_2$ and Red 254 NPs as already described in Section 2.3.4. No further analysis was performed for MWCNTs in order to meet the project priorities and deadline. The measurements were performed after 24 hours of exposure to concentrations corresponding to the $BMD_{20}$ and half the $BMD_{20}$.

The experimental setup included negative controls (culture medium only) and a blank for background examination. The possible interference of NPs with the cytokine detection was also evaluated testing the interaction between the assay and any NPs involved in this study.

For the C3A cell line, IL-8 was the only cytokine assessed since the ones previously analysed (IL-1β, IL-6, TNF-α, and RANTES) were not detectable.

**6.2.6 Genotoxicity of WCCo**

Since in the literature other tungsten carbide NPs were reported to exert DNA damage (Moche et al. 2014, 2015; Paget et al. 2015), the genotoxicity of the project’s WCCo NPs was analysed. For this study, the same protocol as developed in Chapter 2 (section 2.2.6) was used. C3A cells (5x10$^4$ per well) were seeded in 6 well plates and incubated for 24 hours at 37°C and 5% CO$_2$ prior to exposure. After checking that the confluence reached more than 90%, the cell layer
was washed and exposed for four hours to WCCo or cobalt chloride hexahydrate salt (CoCl$_2$ · 6H$_2$O) at concentrations of 134 µg and 67 µg (corresponding to the BMD$_{20}$ and half BMD$_{20}$ of WCCo).

For analysis of \textit{fpg}-sensitive sites, microscope slides containing agarose embedded cells were exposed to 100 µL of enzyme buffer. This enzyme buffer contained 0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES, and 0.2 mg/mL of albumin from bovine serum. The final pH of the solution was 8. The \textit{fpg} positive treatments included enzyme at 1:1000 dilution. Both positive and negative \textit{fpg} treatments were incubated for 30 minutes at 37 °C protected from direct light. DNA unwinding and electrophoresis time were respectively 40 and 30 minutes followed by 15 minutes in neutralization buffer and a few seconds dehydration in absolute ethanol. The slides were rehydrated and stained with GelRed (Biotium Cat No. 41003-0.5ml). Comets were examined under a fluorescence microscope using an imaging software (Comet Assay IV; Perceptive Instruments, UK) and pictures were captured by a stingray (F-033B/C) black and white video camera. In each experiment, the samples were blind-scored evaluating the DNA percentage in the tail of 50 comets/sample for a final amount of 150 comets per treatment. A positive control of 60 µM H$_2$O$_2$ (Sigma, 95321-100ML) was added each experiment. All the reagents were purchased from Sigma unless differently stated.

As already attempted in Chapter 2 (2.1 \textit{Interference}), the same final concentration of WCCo was added to the LMPA to simulate the presence of NPs inside and around the cell. A suspension containing untreated C3A cells was added to the modified agar to test any interference within the assay. For any details about the statistical analysis involved in the following study, please refer to Section 2.3.2.
6.3 Results

6.3.1 Cytotoxicity screenings

The Alamar Blue analysis showed that the toxicity elicited by the cobalt salt was three times higher if compared to WCCo NPs (Figure 6.9) on a total mass concentration basis, showing an estimated BMD$_{20}$ of 47.2 and 134 µg/ml respectively. All the remaining NPs showed a low toxic potency, reaching a BMD$_{20}$ of 249 µg/ml for the SiO$_2$ NPs (Figure 6.10), 240 µg/ml for the Organic Pigment Red 254 (Figure 6.11) and 180 µg/ml for the MWCNTs (Figure 6.12).

![Figure 6.9 WCCo cytotoxicity. Alamar Blue Assay on C3A hepatocyte cell line at 24 hours expressed in µg/ml using PROAST package (version 38.9) via R software. The data is compiled from three separate experiments (n=3), and each data point represents the value of every single replicate. The segmented lines show the BMD$_{20}$ estimated by the software using the Hill model and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of either WCCo or CoCl$_2$ present in the dispersion.](image_url)
Figure 6. 10 SiO₂ cytotoxicity. Alamar Blue Assay on C3A hepatocyte cell line at 24 hours expressed in µg/ml using PROAST package (version 38.9) via R software. The data is compiled from three separate experiments, and each data point represents the value of every single replicate. The BMD estimated by the software using the Hill model and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of NPs.

Figure 6. 11 Organic Pigment Red 254 cytotoxicity. Alamar Blue Assay on C3A hepatocyte cell line at 24 hours expressed in µg/ml using PROAST package (version 38.9) via R software. The data is compiled from three separate experiments (n=3),
and each data point represents the value of every single replicate. The segmented lines show the BMD$_{20}$ estimated by the software using the Hill model and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of pigment.

Figure 6. 12 MWCNTs cytotoxicity. Alamar Blue Assay on C3A hepatocyte cell line at 24 hours expressed in µg/ml using PROAST package (version 38.9) via R software. The data is compiled from three separate experiments (n=3), and each data point represents the value of every single replicate. The segmented lines show the BMD$_{20}$ estimated by the software using the Hill model and at the bottom end of the legend the upper and lower bounds of the confidence interval. In the y-axis, the data are expressed as fluorescence reading (raw data) according to the PROAST analysis while the x-axis expresses the dose as µg/ml of MWCNTs.
6.3.2 Cytokine analysis

As mentioned in Section 2.4.2, since the production of IL-1β, IL-6, TNF-α and RANTES were below the detection limit, the cytokine analysis was performed only on IL-8. None of the NPs and NMs analysed induced the production of this pro-inflammatory cytokine after 24 hours (Figure 6.13). LPS did not elicit any effect on C3A cells.

Figure 6.13 NPs induced cytokine production. IL-8 detected in C3A cell line after 24 hours exposure to 0.5x and 1x BMD20 value of WCCo, Organic Pigment Red 254, SiO2 NPs and CoCl2 salt. On the y-axis, the concentration of IL-8 expressed as pg/ml (x100). Error bar: Standard Deviation (n=3).
6.3.3 Genotoxicity of WCCo

According to the Comet assay, WCCo NPs exhibited a significant (p<0.01) increase in % DNA in the Comet tail, suggesting DNA damage in C3A hepatocytes at 4 hours post exposure (Figure 6.14). The inclusion of fpg did not significantly enhance DNA damage, suggesting that the majority of the DNA damage was elicited by single and double-strand breaks rather than oxidative reactions. H₂O₂ induced DNA damage which was enhanced by the inclusion of fpg indicating that the assay was sensitive to oxidative DNA damage.

The interference of NPs in the Comet assay, as already described for CuO NPs (Section 2.1 interference), was assessed to establish if the DNA damage observed was caused by the impact of the NPs during the exposure of cells, rather than as an artifact of the test (e.g. due to an interaction between metal NPs and DNA under an electromagnetic field). The results (Figure 6.15) showed that the effect elicited by WCCo NPs is entirely due to their interaction with the DNA during the Comet assay rather than an intrinsic genotoxicity.
Figure 6. 14 WCCo genotoxicity. DNA damage in C3A hepatocytes after 4-hour exposure in the presence and absence of fpg. Data expressed as average % DNA in tail +/- SEM (n=3). Pristine WCCo NPs increase the percentage of DNA in the comet tail. There is not observable dose-effect and enhancement by fpg.

Figure 6. 15 WCCo NPs interference. DNA damage results expressed as DNA in tail normalized by positive control on C3A hepatocytes after 4h exposure in both fpg positive (B) and negative (A) treatments.
6.4 Discussion

This study demonstrated that WCCo, NC7000 MWCNTs, SiO$_2$ and Pigment Red 254 caused a cytotoxic effect only at high doses exposure without any significant production of IL-8 on C3A cells. Based on the Alamar Blue assay, WCCo NPs were the most toxic particles, followed by the MWCNTs and SiO$_2$ and Red 254 together, eliciting a dose-dependent cytotoxicity to the C3A cells.

**WCCo**

Comparison of the impacts of WCCo NPs on hepatocytes and macrophages (Appendix) demonstrated that the hepatocyte cell line was three times less sensitive than the RAW264.7 cell line, having a BMD$_{20}$ in the region of 134 µg/ml for the C3A cells and 48.1 µg/ml for the RAW264.7. Even if the real reason for this effect is not yet established, some authors (Paget et al. 2015) hypothesised that the main reason could be ascribed to the different endocytic abilities of the cell lines (i.e. higher in macrophages than in hepatocytes). For highly phagocytotic cells, such as macrophages, the uptake of WCCo NPs is higher than in other cell line and, since these NPs can stimulate a high production of ROS (Zhao et al. 2013; Paget et al. 2015), the cytotoxicity and inflammatory effects produced are higher here compared to other cells.

In a study on the toxic effect of tungsten carbide (WC) and WCCo NPs, Bastian et al. reported that while WC NPs did not exert a significant toxicity on mammalian epithelial (CaCo-2, HaCaT, A549), neuronal (primary cells from Wistar rat foetuses), and glial cells (OLN-93) in vitro (Bastian et al. 2009), the doping with Co markedly increases the cytotoxicity of these NPs. Busch et al. (2010), testing hypoxia-like effect on HaCaT cells, examined WC and WCCo NPs with CoCl$_2$ as cobalt ionic control finding that while the sole WC exerted very little effect on the transcriptomic level after 3 hours and 3 days of exposure, the addition of the cobalt doping made the WCCo NPs able to interact with hypoxia-inducible factor 1-α (HIF1α) similarly to cobalt salt (Busch et al. 2010).

Cobalt toxicity is widely reported in the literature as it is a well-known hypoxia mimetic agent (Jeon et al. 2014). In fact, ions deriving from the dissolution of CoCl$_2$ are able to accumulate
within human cell inducing cytotoxicity in both time and dose dependent manner – e.g. in HaCaT keratinocytes (Ermolli et al. 2001). Moreover, this salt is able to induce apoptosis in P12 cells (a cell line derived from a pheochromocytoma of the rat adrenal medulla) via AP-1 (activator protein 1) after increasing the intracellular ROS production (Zou et al. 2001) and to induce release of the proinflammatory molecules prostaglandin E2 (PGE2), interleukin 6 (IL-6), and interleukin 8 (IL-8) in TR146 cells from the buccal mucosa (Schmalz, Schweikl and Hiller 2000).

Previous studies on occupational exposure to micrometric cobalt-containing dust showed its ability to elicit bronchial asthma, alveolitis, and lung cancer in human (Lison 1996; Moulin et al. 1998). Furthermore, cobalt seems able to exert a toxic effect in its nanoform as well; a significant cytotoxicity together with an increase in ROS generation, lipid peroxidation and decrease of intracellular glutathione was reported on MCF-f breast cancer cell line, probably due to dissolution with the release of Co²⁺ (Akhtar et al. 2017). Even if the ion release elicited a significant part of the toxic effect of cobalt NPs on mammalian cell, another study, performed exposing osteoclast (derived from RAW 264.7 macrophages) to both cobalt NPs and CoCl₂ salt, showed as the cytotoxicity is higher in cell treated with NPs rather than with salts (Liu et al. 2015).

Data on the cytokine production on hepatocytes exposed to WCCo is not available in literature, and these NPs did not show to enhance the production of a pro-inflammatory cytokine in C3A cells. On the other hand, the toxicity testing performed at Karolinska Institutet (details in Appendix) showed that WCCo induced an increase of TNF-α, IL-6 and IL-12 and a decrease of, IL-10, MCP-1 (MCAF; CCL2), MIP-1β (CCL3), MIP-1α (CCL4), RANTES (CCL5), while KC (IL-8, CXCL8) and IL-1β were not detectable. It is possible that the toxic mechanism exerted by WCCo in vivo might be dependent more on the activation of the immune cell rather than by a direct action on a specific tissue as suggested in a study performed on a co-culture of lung epithelial cells and macrophages (Armstead and Li 2016).

WCCo is classified as group 2A material, described as “probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC, 2006). Although some older studies suggest WCCo did not show high genotoxic power in its microparticulate form (De Boeck, Lison and Kirsch-Volders 1998), when uptaken as nanoparticulate other studies suggest WCCo can cause damage to DNA on several in vitro models, i.e. A549 (lung), Hep3B (liver), and Caki-1 (kidney) (Moche et al. 2015), using tests such as the Micronucleus test (Organization
For Economic Cooperation And Development 2010) in combination with fluorescence in situ hybridization (FISH) and the fpg-modified Comet assay (Moche et al. 2014). Moreover, ions release might play an important role in regulating the genotoxicity of these NPs as cobalt ions are able to inhibit the incision and the polymerization step of nucleotide excision repair as observed in human fibroblasts (Kasten, Mullenders and Hartwig 1997).

In this study, WCCo treatment induced Comet formation in C3A cells. Nevertheless, when the interference assay was performed adding WCCo NPs directly in the LMPA containing untreated cells, the results showed a Comet production with a similar length and DNA content as in the treated samples. Even if the presence of Comet formation in untreated cells could be ascribed to a possible interaction between the NPs and the naked DNA during the assay, the mechanism is not clear nor when these interactions occur. It is important to stress at this point that the fact that WCCo can interfere with the comet assay, it doesn’t imply necessarily that there is no genotoxic effect elicited by these NPs but rather that the comet assay is not effective in detecting it and that best laboratory practice in the future could be to run this assay alongside with another genotoxicity test – i.e. the Micronucleus assay – together with a suitable variant to check eventual interference.

MWCNTs

Toxicity of MWCNTs is affected by different factors such as size, shape, impurities and functionalization (Madani, Mandel and Seifalian 2013). Even if they are considered less active if compared to SWCNTs (Jia et al. 2005), increasing studies suggest that MWCNTs can cause inflammation and fibrosis (Allegri et al. 2016).

In vitro exposure to MWCNTs (NM400 and NM402) of mouse primary lung fibroblasts, human fetal lung fibroblasts (HFL-1), mouse embryonic fibroblasts (BALB-3T3) and mouse lung fibroblasts (MLg) showed that long MWCNTs can directly stimulate fibroblast proliferation in a dose-dependent manner and even induce lung fibrosis in vivo (Vietti et al. 2013), probably through the prolongation of ERK 1/2 signalling pathway (Vietti, Lison and van den Brule 2015) that is involved in the regulation of a large variety of processes such as adhesion, cycle progression, migration, survival, differentiation, metabolism, proliferation, and transcription (Roskoski 2012). Furthermore, MWCNTs can induce cell cycle arrest and increase apoptosis/necrosis of human skin fibroblasts (Liu et al. 2009) and IL-8 increase in
primary human 3D liver microtissue model (Kermanizadeh et al. 2014b) and in both C3A and primary hepatocytes (Kermanizadeh et al. 2012c) exposed to MWCNTs (NM400). In vivo inhalation exposure of Wistar rats to MWCNTs from the Nanocyl NC7000 series, show that while this material produced no systemic toxicity, it was possible to observe an increase in lung weights and inflammation of the respiratory tract (Ma-Hock et al. 2009).

In this study, the Nanocyl NC7000 series did not show high cytotoxicity (BMD$_{20}$ of 180 µg/ml) nor IL-8 production if compared to other MWCNTs such as NM400 that showed an estimated LC$_{20}$ around 5 mg/cm$^2$ (equivalent to approx. 15 µg/ml) for both C3A cells and primary hepatocytes (Kermanizadeh et al. 2012c), probably as consequences of differences in their physico-chemical properties such as their shape (clustered) and size (1.5 µm long).

SiO$_2$

The toxicity of silicon dioxide in vitro is size, dose and cell-dependent (Murugadoss et al. 2017). In a study on NIH-3T3, A549 and HEP-G2 using silicon NPs ranging from 20 up to 200 nm, Kim et al. (2015) found that the toxicity of these NPs was also highly cell type dependent (Kim et al. 2015). Accordingly, another study showed that smaller NPs (20nm) were able to elicit a stronger cytotoxicity together with an increase of ROS production compared with larger NPs (100nm) on keratinocytes in vitro (Park et al. 2013). Furthermore, exposure to these NPs on air-blood barrier models in vitro induced an increase of IL-8 production (Kasper et al. 2015) and TNFα (Farcal et al. 2012). Finally, chronic studies in vivo performed on rats showed that SiO$_2$ NPs did not cause any local or systemic effects upon subacute oral administration (Buesen et al. 2014) or even at doses up to 975.9 and 1000 mg/kg (Yun et al. 2015) probably because rats excreted most of the ingested silica through their faeces (Ryu et al. 2014); on the other hand, several inhalation studies described NM-202 as inflammogenic when inhaled (OECD WPMN ENV/JM/MONO(2016)23 2016). Even if another study performed on Hep-G2 cells showed that 7–20 nm silica NPs could cause oxidative stress and apoptosis (Lu et al. 2011), the SiO$_2$ analysed in this study showed a cytotoxic effect only at high doses, unrealistic in a real-life scenario (a BMD$_{20}$ of 249 µg/ml), whitout effect on IL-8 production. A possible explanation for this lack of effect in vitro could be linked to their tendency to form small aggregates that decrease the availability to the cell.
Pigment Red 254

There are no studies performed on the toxic effects of Pigment Red 254 NPs in the literature *in vitro*, and the results of this study showed that this material elicited cytotoxicity only at high doses (BMD<sub>20</sub> at 240 µg/ml) with no pro-inflammatory cytokine production on C3A cells. Nevertheless an *in vivo* study was recently performed by Hofmann *et al.* (2016) on Wistar rats; in this study, the authors highlighted the presence of pigment deposits and phagocytosis by alveolar macrophages, a slight hypertrophy of the bronchioles and alveolar ducts, but without evidence of inflammation (Hofmann *et al.* 2016).

The results of this chapter confirm that this subset of the panel of NPs and NMs analysed were relatively low in toxicity, justifying the decision for them not to be a priority for further testing *in vivo*.
6.5 Conclusions

The results obtained, showing that the panel of NPs and NMs analysed was not able to induce a significant toxic effect in C3A cells, demonstrated that it is always good practice to have different representative models for an in vitro study – e.g. macrophages representing the lung exposure and the hepatocytes representing the major organ of accumulation – or analysing an endpoint by using at least two different assays.

Moreover, this good practice should be implemented developing suitable and standardise strategies for testing interferences.

Finally, even if no toxicity was observed within a reasonable range of exposure concentrations, it is worth stressing the relevance of a null.
Chapter 7: General Discussion
7.1 Hypotheses of the Study

This thesis was developed to assess the two main hypotheses presented in the first chapter of this study. The first one was that NPs, and CuO as one of the main priority of the SUN project and case study in this thesis, can show changes in their toxicity related to the specific stage of their life cycle. The second hypothesis was that Safer by Design approach could be used to decrease the toxic effect of CuO NPs in both in vitro and in vivo models maintaining its antifungal property. Moreover, the aim of this study was to evaluate the toxicity of a panel of NPs and NMs proposed in the SUN project using both in vitro and in vivo models.

It was not possible to verify the first hypothesis of this study due to the difficulty found by other project partners to artificially produce an ultrafine fraction of the final composite product containing CuO NPs. Using a cryogenic milling method (mechanical grinding at low temperature in order to avoid overheating which could alter the material), the fraction produced was not useful either because it was too big in size to be successfully inhaled or because, due to the low density of the polymer used as final embedding product, the fraction was not dispersible, and it ended up floating on the medium, not interacting with the cells. Furthermore, after repeated cycles of cryomilling and filtration, the number of nanoscale products was too low to justify a real scenario of human exposure. The second hypothesis, according to which the SbyD approach could have led to a substantial decrease of CuO NPs potency in both in vitro and in vivo models, was verified. The SbyD approach concept is already in use in several industrial sectors as an integral part of the management of the innovation process (Micheletti et al. 2017) and its role in the safe manufacturing of NMs is crucial. Specifically, in the case of the CuO NPs used in this study, four surface capping agents were chosen: positively charged (PEI); neutral (PVP); negatively charged (sodium citrate) and negatively charged with strong anti-oxidant properties (sodium ascorbate). Then, the effects arising from the different surface chemistries and charges were tested to identify possible promising design alternatives. The aim of modifying the surface was, at first, to control surface charge and the electrostatic interaction with cell membranes; secondly, to test the antioxidant property against free radicals of both citrate and ascorbate. The study highlighted that SbyD can be a useful approach in developing new strategies for NMs manufacturing and that sodium ascorbate coating can be a promising alternative to pristine copper formulation due to its lower toxic effects against mammalian cells and the enhanced effect against copper-resistant Fungi. Finally, the in vitro model used (C3A
cell line) was able to predict the effects provoked by CuO NPs exposure in animal models (e.g. inflammation and oxidative stress), confirming the hypothesis that \textit{in vitro} testing can be, far from being a possible substitute of the \textit{in vivo} testing, a useful method for an initial toxicological screening that might allow to reduce significantly the use of animals during the experiments.
7.2 Overall Considerations

Within the panel of NPs and NMs analysed in this study, the different copper formulations, both nano (CuO NPs and micronized Cu) and nano-free (Cu-amine), were able to elicit a greater cytotoxic effect on the C3A cell line compared to WCCo, MWCNTs, SiO₂ and Pigment Red 254; moreover, all copper formulations were able to induce production of the pro-inflammatory cytokine IL-8 suggesting that they may trigger an inflammatory response in liver. CuO NPs were also tested for their impact on hepatocyte glutathione content and capacity to induce DNA damage. Interestingly, while the antioxidant levels started to drop after 24 hours with both CuO NPs and CuSO₄ treatments suggesting that an oxidative damage might occur and could be related to the action of the copper ions, the DNA damage was high only in cells exposed to CuO NPs rather than copper salts. These results suggest that the genotoxic effect might be linked mostly to a possible interaction between the NPs with either the DNA or with protein complexessuch as histones, rather than be caused by ions; moreover, the fpg (formamidopyrimidine [fapy]-DNA glycosylase) incubation showed that the damage was attributed principally to a direct action of the NPs in lieu of the indirect effect of oxidative stress. This hypothesis is confirmed by several studies showing that undissolved CuO NPs can be actively uptaken by epithelial cells (A549 and BEAS-2B) and elicit DNA damage measured with the Comet assay (Karlsson et al. 2008, 2009, Cronholm et al. 2012, 2013). The majority of the studies about the genotoxicity of Cu NPs and CuO NPs used the Comet assay to evaluate their genotoxicity, nevertheless a few studies employed different approaches such as the gene expression analysis of cell cycle checkpoint and DNA repair pathways (i.e. p53, Rad51 and MSH2) (Ahamed et al. 2010) or the micronucleus assay (Perreault et al. 2012). Even if in all these studies CuO NPs elicited genotoxicity, some authors described these effects as a consequence of the oxidative stress induced by the NPs while others attributed the same to a direct interaction between NPs and DNA. In this study, the fpg analysis showed that there was not a significative increase of oxidative events, ruling in favour of the second hypothesis. While the nano-free copper amine formulation showed a toxic effect in range with the pristine CuO NPs, micronized copper was ranked as the most toxic between the copper compound, suggesting that the cost/benefit aspects should be carefully examined prior to using it.

Antifungal studies performed to better understand the potential capacity of the different copper formulations to behave as a wood preservative suggested that the pristine CuO NPs were not a
better antifungal agent than copper amine (as the European benchmark treatment). The SbyD modification with ascorbate coating did not only enhance the antimould effect of CuO but was also able to decrease both cytotoxicity and cytokine production in C3A cells. Furthermore, the protocol developed in the section of the antifungal study, when compared to the OECD guidelines for testing a preservative against wood destroying basidiomycetes (CEN, 1997), proved to be able to produce more stable results in a shorter period of time (Pantano et al. 2017).

The in vivo data, consisting of the gene expression results from the inhalation study with pristine CuO and both PEI and ascorbate coated CuO NPs and from the oral study with CuO NPs and micronized copper respectively, when examined in the light of the other partner’s findings (fully presented in Appendix), confirmed the results obtained from the in vitro study, that evaluated pristine CuO NPs and micronized copper as both toxic and inflammagenic, together with the ascorbate coated CuO NPs that induced an inflammation effect similar to the pristine form after 5 days exposure, since the recovery of animals in the long term period was not statistically significant.

Another interesting aspect of this thesis was the development of a strategy for testing interference during the comet assay, a test that already proved to be subjected to a relatively high variability (Ersson et al. 2013; Collins et al. 2014). This method showed that almost all of the apparent genotoxic effect of WCCo might be ascribed to possible interactions between the NPs and DNA during the assay rather than a direct effect taking place during the cell exposure.

Finally, the findings obtained in vitro from the panel of both NPs and NMs were used, at first, both for refining animal studies (with the aim to reduce the number of animals used) and for making decisions for strategic development, modification and testing of nanomaterials. Then, together with the animal studies, the same results were used for risk assessment as well as developing tools and guidelines for sustainable manufacturing within the SUN project.
7.3 Future Work

As this study was part of a greater European project, any potential room for manoeuvre from the project deliverables was quite limited. An interesting follow-up for this study would be extending some of the endpoints to other materials to obtain a more transversal comparison of them; in particular, it would be useful to perform the comet assay for the other copper formulations analysed in this thesis (i.e. copper amine and micronized copper). Since the main use of these copper formulations is for external wood coating, both human and environmental exposure are likely to happen; a more in-depth analysis of these formulations would be useful not only for risk assessment but also for helping the correct decision management at the industry level. Moreover, to obtain more robust results in regard of the genotoxicity of these and other materials, a second approach (e.g. the micronucleus assay) in conjunction with the comet assay would be needed to confirm the findings. Furthermore, since the CuO NPs can also elicit oxidative stress (Zhang et al. 2012; Karlsson et al. 2014), it would be extremely interesting to investigate the cellular ROS production with Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay.

All the copper-based formulations of this study could compromise cell viability; even if a model of toxicity for copper NPs (i.e. CuO) consisting in the trojan horse effect already mentioned in Chapter 2, was proposed by Cronholm and Karlsson in their studies (Karlsson, Holgersson and Möller 2008; Cronholm et al. 2012, 2013; Karlsson et al. 2014), it would be stimulating to perform a more detailed analysis of the mechanisms involved in their toxic action. Furthermore, it would be interesting to discriminate between apoptotic and necrotic cells using either flow cytometry, Propidium Iodide uptake or Annexin V binding.

Finally, particular attention is required for developing and validating more specific testing strategies to analyse possible interactions between NPs and the assay chosen for the study as they proved to be able to interact with several proteins, for example altering the correct activity of enzymes (Wang et al. 2011a; Sanfins et al. 2014) or absorbing cytokines on their surface (Piret et al. 2017).
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