D4.6 – Report on human and environmental hazard evaluation

Project Information

<table>
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<th>Grant Agreement Number</th>
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<td>Nanomaterials for conservation of European architectural heritage developed by research on characteristic lithotypes</td>
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<td>Project Acronym</td>
<td>NANO-CATHEDRAL</td>
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<tr>
<td>Funding scheme</td>
<td>NMP-21-2014 Materials-based solutions for protection or preservation of European cultural heritage</td>
</tr>
<tr>
<td>Start date of the project</td>
<td>June, 1 2015</td>
</tr>
<tr>
<td>Duration</td>
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</tr>
<tr>
<td>Project Coordinator</td>
<td>Andrea Lazzeri (INSTM)</td>
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<td>Project Website</td>
<td><a href="http://www.nanocathedral.eu">www.nanocathedral.eu</a></td>
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Deliverable Information

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<tr>
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<td>Report on human and environmental hazard evaluation</td>
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<td>WP no.</td>
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<td>WP Leader</td>
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<td>Contributing Partners</td>
<td>KIT, COLOR, OWS, INSTM, ISCR, WIED, Diputacion Foral de Alava</td>
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<td>Report</td>
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</tr>
<tr>
<td>Reviewers</td>
<td>Andrea Lazzeri (INSTM)</td>
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<tr>
<td>Contractual Deadline</td>
<td>September 30th 2017</td>
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<td>Delivery date to EC</td>
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Dissemination Level

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<td>Oleksandra Fokina</td>
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1 Aim

The aim of this report is to provide a general evaluation of potential risks for human health and environment for nanomaterials developed and used in the Nano-Cathedral project. Though extensive data is available for various nanomaterials, as presented in the D4.1. Report on state of the art on Nano-technology impact on environment and health in the field of stone preservation, the relevance of these studies for the Nano-Cathedral project is limited. There are several general concerns regarding the literature-available about evaluation of nanoparticle (NP) impact on environment and human health:

- Studies are mostly performed with particle overload to achieve evaluable data sets, not at doses relevant to normal occupational or environmental exposure conditions
- Lack of nanoparticle-specific test methods and systematic studies - controversial results due to variations in NPs concentration, time of exposure, assay techniques and test systems
- Lack of long-term in vivo studies

As a part of Nano-Cathedral project an experimental comparison of the applied NPs was intended, to allow an evaluation of their hazardous potential under the same conditions using the same methods. Test for in vitro cytotoxicity was performed to determine, whether the nanoparticles used in Nano-Cathedral project were toxic for mammalian cells under laboratory conditions. Additionally, Ames test with bacterial cultures was performed to determine possible mutagenic effects under native conditions and in the presence of human-like metabolic activation agent in the laboratory. These methods provide information on the possible short-term health effects in living organisms of the exposure to selected NPs in case of local release of the materials.

Following NPs were tested in the current study:
Consolidants – NC-25C, NC-27CP, NC-12C, NC-29C
2 Introduction

Engineered nanoparticles have various applications from textile industry and catalysis to medicine and architectural heritage preservation (Salata, 2004; RS/RAE, 2004; Buzea et al., 2007; Som et al., 2011). The field is rapidly growing and the safety of NP-based materials and products for human and environment became a subject of various studies. Though fortunately no incidents involving NPs releases that posed an acute threat to the environment or public health have been documented to date, the issue remains important, especially when dealing with novel NP materials or formulations. The reason is primarily the small size of the NPs (1-100 nm), which allows them to easily enter living organisms and penetrate natural biological barriers inside them, reaching internal organs (Bahadar et al., 2016). In some cases, engineered nanomaterials have been shown to have cytotoxic, cancerogenic or mutagenic effect (Seemayer et al., 1990; Seaton et al., 2010). Potential toxic risks associated with NP applications depend on their unintentional release in the environment and their bioavailability (Nanowerk, 2012). Possible transmission pathways for NPs to humans, but also microorganism, plants and animals are through contaminated air, water and soil (RS/RAE, 2004; Bernhard et al., 2010). The exposure can happen through inhalation (air), skin contact (water, soil) or ingestion (water). NPs differ in their bioavailability and therefore in their potential hazardous risks, depending on their chemical nature, size and structure. The nanomaterials used in Nano-Cathedral project include metal oxide-based, silica-based and carbon-based polymer NPs.

2.1. Silica-based nanoparticles

Human toxicity

Toxicological studies with silica-based NPs were mainly focused of the toxicity of the “natural” crystalline NPs of 0.5 -10 µM size (Napierska et al., 2010). These crystalline NPs can accumulate in lungs and cause several diseases like for example silicosis. The real-life studies with amorphous NPs are difficult to interpret due to the high contamination levels of material with the crystalline form NPs. However, high doses of amorphous silica may result for example in acute pulmonary inflammatory reactions (Rosenbruch, 1992). The risk assessment studies use in vitro assays with cell lines, exposure through inhalation or skin contact. (Jaganathan and Godin, 2012; Frujtier-Pölloth, 2012; Bahadar et al., 2016). Though the results of in vitro and inhalation studies show different results, mostly due to the lack of standardization, skin surface application of NPs is generally safe. High concentrations of NPs in mg/l concentrations cause different inflammatory or cytotoxic effects. However, amorphous silica used in engineered nanomaterials are considered less toxic than crystalline NPs.

In the environment

Silica-based NPs generally dissolve in water, but their aggregation properties can be influenced by pH, salinity, water hardness or presence of organic matter (Frujtier-Pölloth, 2012). In the environment under normal conditions SiO₂ particles are inert and can form aggregates around 100-200 nm size. They can locally supersaturate and remain mainly in soil/sediment, weakly in water (Depasse and Watillon, 1970). Bioavailable forms of silica are dissolved silica [Si(OH)₄], silicic acid and silicates.

Silica-based NPs are often used as a negative control in NP toxicity studies, due to their low toxicity to microorganisms (Som et al., 2011; Frujtier-Pölloth, 2012). However, some studies show toxic effect at mg/l concentrations, which are highly above the expected environmental exposure concentrations.
In the review by the OECD (2004) and the ECETOC (2006), no acute toxicity was reported for fish and daphnia, even after exposures to extremely high concentrations of silica-based NPs.

2.2. TiO$_2$-based nanoparticles

*Human toxicity*

Main human exposure pathway for TiO$_2$-based NPs is inhalation, though the amount translocated NPs in the lungs is very low, compared to the exposure concentrations (Som et al., 2011). No penetration of intact human skin was shown for TiO$_2$-based NPs (Final report Nanoderm: http://www.uni-leipzig.de/~nanoderm/). Several studies show no or negligible effects of NPs on living cells (Som et al., 2011). However, others show toxic effects on DNA, immune system, liver and other organs (Som et al., 2011; Shi et al., 2013; Bahadar et al., 2016). Up to date there are no studies showing cytotoxicity at doses relevant to normal occupational or environmental exposure conditions. Apart from Recommended Exposure Limits (RELs) by National Institute for Occupational Safety and Health (NIOSH) - no occupational or environmental exposure limits have been set by any other regulatory agency.

*In the environment*

Photostable TiO$_2$-based NPs are considered inert and can form agglomerates and sediments in the environment with no threat to environment even at g/l concentrations (Som et al., 2011). In the case of surface coatings intended to eliminate the photocatalytic reactivity of the material, TiO$_2$-based NPs are more toxic for some microorganisms, algae, fish and crustaceans in maritime environments at concentrations around 1 mg/l (Hund-Rinke and Simon, 2006; Auoja, 2009; Auffan et al., 2010).

2.3. ZnO-based nanoparticles

*Human toxicity*

ZnO-based NPs have been shown to decrease metabolic activity, cause oxidative stress and inflammatory response through *in vitro* studies with different cell lines (NanoCare, 2009; Som et al., 2011). Also genotoxic effects of Nano-ZnO have been shown *in vivo* and *in vitro* studies (Bahadar et al., 2016).

Primary exposure routes for Nano-ZnO are inhalation or instillation that lead to systemic distribution of NPs in liver, spleen, lungs and others (Vandebriel and De Jong, 2012). Nano-ZnO do not, or only to a minimal extent, cross the intact skin.

*In the environment*

Nano-ZnO is present in waste waters in concentrations around 1 µg/l (Gottschalk, 2009), it is water soluble and is rapidly removed from the environment.

ZnO-based NPs are often won from plant leaves or other biogenic sources and show antimicrobial activity against Gram-negative (*Salmonella paratyphi, Escherichia coli, Vibrio cholerae*) and Gram-positive (*Staphylococcus aureus*) bacteria (Ramesh et al., 2015). Fungal species like *A. fumigatus* or *A. aculeatus* are also inhibited by Nano-ZnO (Baskar et al., 2013).

2.4. ZrO$_2$-based nanoparticles

*Human toxicity*

There is not much research available on the toxicity of ZrO$_2$-based NPs. They are used in skin care products and have been shown to show toxic and inflammatory effect (HCN, 2002; NanoCare, 2009).
However, other studies showed no evidence of toxicity of these NPs (Takamura et al., 1994; HCN, 2002; 122).

In the environment
Generally, no data on environmental stability of Nano-ZrO$_2$ is available (Seabra and Duran, 2015). Some inhibitory effect on microorganisms was shown for Gram-negative bacterium *E. coli*, but not Gram-positive *Bacillus subtilis* or fungi *Aspergillus nidulans* (Jangra et al., 2012). ZrO$_2$-based NPs showed also no inhibitory effect on algae and zebrafish (Jemec et al., 2015).

2.5. Ag-based nanoparticles

Human toxicity
Silver in metallic or ionic form has low toxicity in humans and has been used as disinfectant or preservative agent for water for generations (Wijnhoven et al., 2009). However, Nano-Ag may probably reach cells that are normally not influenced by Ag$^+$ ions, and therefore has potential negative effect on human physiology (Som et al., 2011). Some studies suggest that Nano-Ag affects mitochondria or is cytotoxic, but it is unclear, whether the Nano-Ag or Ag$^+$ is responsible for the observed effect (Hsin et al., 2008).

In the environment
Nano-Ag (in form of Ag$^+$) is highly toxic for microorganisms, but also for fish, algae and crustaceans in maritime environments (Asharani et al., 2008; Wijnhoven et al., 2009; Yin et al., 2011). However, recent studies suggest that under normal environmental conditions Nano-Ag is rapidly converted to silver sulfide and becomes non-toxic (Choi et al., 2009; Kim et al., 2010).

2.6. Carbon-based nanopolymers

Human toxicity
Carbon-based polymeric NPs represent a heterogeneous group of nanomaterials and are considered non-toxic, non-immunogenic and non-inflammatory and bio-compatible (Bahadar et al., 2016). However, there have also a report of NPs toxicity toward macrophages, if applied on surfaces (Grabowski et al., 2015).

In the environment
Carbon-polymer nanoparticles differ considerably in chemical reactivity, surface charge, length, structure, surface chemistry, solubility and agglomeration behavior, therefore general characteristics of this class of NPs are difficult to determine (Cha et al., 2017). The environmental stability, bioavailability and toxicity depend strongly on very distinct chemical and physical properties of these NPs.
3 Experimental procedures

The experimental procedures described in the following were performed to determine the potential risks connected to the NPs used in the Nano-Cathedral project. General toxicity of the NPs for mammalian cells was measured using the in vitro cytotoxicity assay CellTiter-Blue® Cell Viability Assay from Promega Corporation (Madison, WI, USA). Possible risks connected to different mutagenic effects of the NPs for humans and environment were tested using Ames test (Ames et al., 1971).

3.1. Test for in vitro cytotoxicity

The CellTiter-Blue® Cell Viability Assay is a fluorometric method for viable cells detection in multiwell microtiter plates using the metabolic indicator dye resazurin. Viable cells are capable to reduce the dye to fluorescent compound resorufin, but nonviable cells without the metabolic activity are not able to perform this reaction. The spectral properties of the dark blue resazurin change as the result of the metabolic reaction to the pink resorufin with a strong fluorescence emission maximum at 584 nm. The resulting fluorescence signal (560 nm excitation/590 nm emission) is therefore proportional to the number of viable cells and can be directly used as a measure of cells viability.

In the in vitro assay performed in this study CHO-K1 (ATCC® CCL-61™) cells from ATCC (Manassas, VA, USA) were used. The CHO-K1 (chinese hamster ovary) cells from the hamster Cricetulus griseus are an epithelial cell line with the biosafety level 1, often used in genetic and toxicological studies or for recombinant protein production. The fluorometric assay was performed in 96-well assay plates according to the manufacturer’s instructions (Promega Corporation, Madison, WI, USA). The cells were first precultivated in F12K culture medium (Gibco™, Thermo Scientific™, Schwerte, Germany) with 10% fetal bovine serum supplement at 37 °C. To measure the toxicity of the NPs, they were incubated with the cells for 0, 24, 48 and 72 hours at standard culture conditions. Test substances were added in 1:10, 1:100 and 1:1000 dilution/suspension (v/v in water) to the cells to the final volume of 100 µl per well. Water and 10% isopropanol were used as negative controls. After the incubation period at 37°C 20 µl of CellTiter-Blue® Reagent was added to each well and mixed for 10 sec, followed by 1 hour incubation under standard cell culture conditions. The fluorescence at 560/590 nm was recorded using a plate reader to determine the cytotoxicity of the samples.

3.2. Ames test

The Ames test is a mutagenicity assay, which is based on the inability of certain mutants of Salmonella typhimurium and Escherichia coli to grow without histidine/tryptophan supplement. These mutations can be reverted under the influence of the certain mutagens, allowing the bacteria to grow on histidine- or tryptophan-deficient media. Different strains are sensitive to different types of reversion events like base substitution, extragenic suppressors, transitions, transversions, small deletions or frameshifts. The strains used in this study have biosafety level 1 and were acquired from Xenometrix (Allschwil, Switzerland). The strains and their genotypes are listed in Table 1.
Table 1: Strains used in Ames test mutagenicity assay

<table>
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<tr>
<th>Bacterial strain</th>
<th>His-/Try-mutation</th>
<th>Genotype¹</th>
<th>Reversion events</th>
<th>Reference</th>
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<td><em>S. typhimurium</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA 98</td>
<td>hisD3052</td>
<td>∆gal, rfa, ∆uvrB, ∆bio, ∆chlD</td>
<td>-1 base pair frameshifts</td>
<td>Isono et al., 1974</td>
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<tr>
<td></td>
<td></td>
<td>pKM101</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TA 1535</td>
<td>hisG46</td>
<td>∆gal, rfa, ∆bio, ∆uvrB, ∆chlD</td>
<td>Base pair substitutions, Extragenic suppressors</td>
<td>Barnes et al., 1982</td>
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<tr>
<td>TA 1537</td>
<td>hisC3076</td>
<td>∆gal, rfa, ∆bio, ∆uvrB, ∆chlD</td>
<td>+1 base pair frameshifts near -CCC-</td>
<td>Ames et al., 1973</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>WP2 uvrA</td>
<td>trpE</td>
<td>uvrA</td>
<td>Transitions, transversions, small deletions</td>
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The Ames test procedure was adapted from Ames et al. (1973) and Mortelmans and Zeiger (2000) according to the OECD guideline for testing of chemicals (1997). The cells were precultivated in LB culture medium (Bertani et al., 1951) overnight at 37°C. Prior to the chemical exposure the cells were harvested at OD₅₇₈=1 and washed in sterile saline (0.95% NaCl w/v in water). The exposure was performed using preincubation method with 100 µl bacterial suspension, 50 µl chemical sample (1:10, 1:100, 1:1000 v/v dilution/suspension in water) and 500 µl saline/buffer or 10% S9 fraction mix were incubated at 37°C, 500 rpm for 20 min. Rat liver S9 Aroclor 1254 fraction (Xenometrix, Allschwil, Switzerland) is a post-mitochondrial fraction from rat liver and served as a metabolic activation system to simulate possible chemical reactions that the NPs could undergo in human organism. After the incubation the cells were mixed with top agar and put on M9 low His- or Try-agar plates (Harwood and Cutting, 1990) and cultivated for 2-4 days at 37°C depending on the strain used. The number of revertant colonies was counted after the cultivation and used as the measure of NPs toxicity. Water, 10% Isopropanol and 10% Dimethyl sulfoxide (DMSO) were used as negative controls. Different chemicals were used as positive controls for each strain to cause different revertant mutations in the strains. The chemicals used as positive controls are listed in Table 2.

Table 2: Positive control chemicals used in Ames test

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<tr>
<th>Bacterial strain</th>
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<th>With S9 mix (µg/plate) [CAS No.]</th>
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<tr>
<td>TA 98</td>
<td>4-Nitro-o-Phenylendiamine (2) [99-56-9]</td>
<td>2-Aminoanthracene (5) [613-13-8]</td>
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<tr>
<td>TA 1535</td>
<td>Sodium azide (5) [26628-22-8]</td>
<td>2-Aminoanthracene (5) [613-13-8]</td>
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<td>TA 1537</td>
<td>9-Aminoacridine (25) [90-45-9]</td>
<td>2-Aminoanthracene (5) [613-13-8]</td>
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<tr>
<td>WP2 uvrA</td>
<td>4-Nitroquinoline-N-oxide (2) [56-57-5]</td>
<td>2-Aminoanthracene (5) [613-13-8]</td>
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4 Results and Discussion

The evaluation of potential hazardous risks from nanoparticles used in Nano-Cathedral project were performed as a part of the WP4 task 4.3 Human & Environmental Hazard Evaluation of applied Nanoparticles. It combined analysis of in vitro cytotoxicity of NPs towards mammalian cell line CHO-K1 and Ames test assay of NPs mutagenic effect on bacterial strains *Salmonella typhimurium* TA 98, TA 1535, TA 1537 and *Escherichia coli* WP2 uvrA.

4.1. Evaluation of consolidants

**NC-25C**

NC-25C is a consolidant with photocatalytic properties, based on ethyl ester of silicic acid at 70% with Nano-TiO$_2$, diluted in isopropanol. According to the manufacturer instruction it has low toxicity for man and environment. In the in vitro cytotoxicity assay no toxic effect was observed for NC-25C in 1:10, 1:100 and 1:1000 suspensions in water, compared to the negative control measurement with water (Fig. 1). Since isopropanol is used as a solvent in NC-25C formulation, it was also used as a control and showed no negative effect on cell viability.

![Graph](image)

**Fig. 1:** Results of the in vitro cytotoxicity test for NC-25C consolidant. Formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test different bacterial strains were applied to test various possible mutations. Water, isopropanol and dimethyl sulfoxide were used as negative controls, because they are used as solvents for NPs and positive control substances in the test. Number of revertants with NC-25C were similar to the water negative control, except for TA 98 strain (Fig. 2 and 3). The amount of revertants in *S. typhimurium* strain TA 98 without S9 metabolic activation was approximately doubled, compared to negative controls in all diluted dispersions (1:10, 1:100, 1:1000) (Fig. 2A). However, the number of revertants was much lower than in the positive control. The fact that the effect was not concentration-dependent suggests that it could be an experimental artefact. Further studies are required to understand, whether this effect was caused by the mutagenic influence of the tested formulation. No mutagenic effects were observed in other bacterial strain, as shown in Fig. 2 and 3. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains (Fig 3D). However, according to the suppliers manual (Xenometrix, Allschwil, Switzerland) this strain generally reacts weakly to 2-Aminoanthracene.
Fig. 2: Results of the Ames test with consolidant NC-25C without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylenediamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 3: Results of the Ames test with consolidant NC-25C with S9 fraction. Isopropanol – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
NC-27CP is a consolidant with a high photo-catalytic activity. It is based on ethyl silicate and alkyl silanes with nano-TiO$_2$ in isopropanol dispersion.

In the *in vitro* cytotoxicity assay no toxic effect was observed for NC-27CP in 1:10, 1:100 and 1:1000 suspensions in water (Fig. 4), compared to the negative control measurements with water or isopropanol.

![Fig. 4: Results of the in vitro cytotoxicity test for NC-27CP consolidant.](image)

NC-27CP formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test water, isopropanol and dimethyl sulfoxide were used as negative controls, because they are used as solvents for NPs and positive control substances in the test. Number of revertants with NC-27CP was similar to the negative control, therefore no mutagenic effects were observed in any bacterial strain, as shown in Fig. 5 and 6. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 6D).
Fig. 5: Results of the Ames test with consolidant NC-27CP without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylendiamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 6: Results of the Ames test with consolidant NC-27CP with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
NC-12C is a consolidant based on Nano-SiO$_2$ suspension in water and ethanol. In the *in vitro* cytotoxicity assay no toxic effect was observed for NC-12C in 1:10, 1:100 and 1:1000 suspensions in water (Fig. 7), compared to the negative control measurements.

**Fig. 7:** Results of the *in vitro* cytotoxicity test for NC-12C consolidant. NC-12C formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-12C was similar to the negative control (Fig. 8 and 9), except for the TA 98 strain with metabolic activation (Fig. 9A). Here the number of revertants was doubled in 1:10 sample. The effect was also concentration-dependent and the number of revertants was reduced to the negative control levels in 1:100 and 1:1000 samples. For other strains and conditions no mutagenic effects were observed. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 9D).
Fig. 8: Results of the Ames test with consolidant NC-12C without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylendiamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 9: Results of the Ames test with consolidant NC-12C with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
NC-29C
NC-29C is a Nano-ZrO$_2$-based consolidant solved in water. In the in vitro cytotoxicity assay no toxic effect was observed for NC-29C in 1:10, 1:100 and 1:1000 dilutions, compared to the negative control measurements (Fig. 10).

![Graph](image_url)

**Fig. 10: Results of the in vitro cytotoxicity test for NC-29C consolidant.** NC-29C formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-29C was similar to the negative controls, as shown in Fig. 11 and 12, except for the TA 98 strain without metabolic activation (Fig. 11A). Here the number of revertants was doubled in all samples. However, the effect was not concentration-dependent and could be an experimental artefact. For other strains and conditions no mutagenic effects were observed. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 12D).
Fig. 11: Results of the Ames test with consolidant NC-29C without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylenediamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 12: Results of the Ames test with consolidant NC-29C with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
4.2. Evaluation of protectives

**NC-21P**
Photocatalytic water repellent NC-21P is based on Nano-TiO$_2$ and alkyl silane oligomers solution in water and has low volatile organic compound content according to the manufacturer. In the *in vitro* cytotoxicity assay no toxic effect was observed for NC-21P in 1:10, 1:100 and 1:1000 dilutions (Fig. 13), compared to the negative control measurements.

**Fig. 13:** Results of the *in vitro* cytotoxicity test with protective NC-21P. NC-21P formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-21P not higher than in the negative control, therefore no mutagenic effects were observed in any bacterial strain, as shown in Fig. 14 and 15. However, NC-21P showed an inhibitory effect on bacteria growth in the absence of S9 fraction in all tested stains (Fig. 14). Since no negative effect was observed on mammalian cells (Fig. 13), the effect seems to be prokariote-specific. Also though the metabolic activation via S9 fraction the antibacterial effect was lost in all strains (Fig. 15). Further tests are required to identify the mechanism and specificity of this inhibition. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 15D).
Fig. 14: Results of the Ames test with protective NC-21P without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenyldiamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 15: Results of the Ames test with protective NC-21P with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
**NC-22P**

NC-22P is photocatalytic water repellent and is based on Nano-TiO\(_2\) and alkyl silane monomers solution in isopropanol. In the *in vitro* cytotoxicity assay no toxic effect was observed for NC-21P in 1:10, 1:100 and 1:1000 dilutions (Fig. 16), compared to the negative control measurements.

![Graph showing fluorescence over time for different dilutions of NC-22P and controls](image)

**Fig. 16: Results of the in vitro cytotoxicity test with protective NC-22P.** NC-22P formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-22P was similar to the negative control, as shown in Fig. 17 and 18, except for the TA 98 strain with metabolic activation (Fig. 18A). Here the number of revertants was doubled in some samples. However, the effect was not concentration-dependent and could be an experimental artefact. For other strains and conditions no mutagenic effects were observed. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 18D).
Fig. 17: Results of the Ames test with protective NC-22P without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylendiamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 18: Results of the Ames test with protective NC-22P with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
NC-32P

NC-32P is an isopropanol-based dispersion with ZnO and AgO NPs. In the *in vitro* cytotoxicity assay no toxic effect on mammalian cells was observed for NC-32P in 1:10, 1:100 and 1:1000 dilutions (Fig. 19), compared to the negative control measurements.

![Graph showing cytotoxicity test results](image)

**Fig. 19:** Results of the *in vitro* cytotoxicity test with protective NC-32P. NC-32P formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-32P was similar to the negative control, except for TA 98 strain, in which the mutagenic effect of the NP formulation was concentration dependent (Fig. 20 and 21). The 1:10 dispersion caused 3.8-fold increase in revertant number (Fig 20A). However, the effect was weakened by the addition of the S9 fraction (Fig. 21A). Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 21D).
Fig. 20: Results of the Ames test with protective NC-32P without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylendiamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 21: Results of the Ames test with consolidant NC-32P with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
NC-36P
NC-36P is a water dispersion of acrylic co-polymer and has a very low amount of volatile organic compounds (VOCs). According to manufacturers instructions it may be considered harmless by inhalation, although the VOCs may be slowly released upon application and drying. It is harmful if swallowed, but it may cause only minor skin irritation or sensitization.

In the *in vitro* cytotoxicity assay no toxic effect was observed for NC-36P in 1:10, 1:100 and 1:1000 dilutions (Fig. 22), compared to the negative control measurements.

![Graph showing cytotoxicity test results](image_url)

**Fig. 22:** Results of the *in vitro* cytotoxicity test with protective NC-36P. NC-36P formulation was used in 1:10, 1:100 and 1:1000 dilutions in water; water and isopropanol were used as negative controls.

In Ames test number of revertants with NC-36P was similar to the negative control, therefore no mutagenic effects were observed in any bacterial strain, as shown in Fig. 23 and 24. Positive and negative controls showed expected number of revertants for each strain. In case of *E. coli* WP2 uvrA strain with S9 metabolic activation the mutagenic effect of 2-Aminoanthracene was weaker than in *S. typhimurium* strains, as expected from the suppliers manual (Xenometrix, Allschwil, Switzerland) (Fig 24D).
Fig. 23: Results of the Ames test with protective NC-36P without S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 4NP – 4-Nitro-o-Phenylenediamine, Na-azide – Sodium azide, NH4-Ac – 9-Aminoacridine, 4-NQO – 4-Nitroquinoline-N-oxide.
Fig. 24: Results of the Ames test with protective NC-36P with S9 fraction. Isoprop – isopropanol, DMSO – dimethyl sulfoxide, 2AA – 2-Aminoanthracene.
5 Conclusions

This study allowed comparative examination of NPs that belong to different classes under the same conditions using the same methods. Though intended to be applied in the same field of stone preservation, these NPs are based on different chemical compounds. Most tested NP formulations, NC-25C, NC-27CP, NC-29C, NC-21P, NC-22P, NC-32P, are based on metal oxides TiO$_2$, ZnO, AgO, ZrO$_2$. Silicate-based (NC-12C) and carbon polymeric NP (NC-36P) treatments are also represented in this study. Generally, no cytotoxic effects in vitro against mammalian cells could be detected. Some formulations (NC-27CP, NC-21P and NC-36P) also had no mutagenic effects in bacterial strains. Others (NC-25C, NC-29C and NC-22P) showed generally good results in mutagenicity assay, with exception for S. typhimurium TA 98 strain. However, the mutagenic effect was low and not concentration-dependent, which suggests an experimental artifact. Two formulations, NC-12C and NC-32P showed a concentration-dependent mutagenic effect on TA 98 strain. Though the revertant numbers were much lower than in the samples with positive control substance, further analysis is needed to clarify this result. Generally, the TA 98 strain was more sensitive to mutagenic effects than other tested strains. Further analysis with alternative strains could determine, whether the observed effect was significant for NPs safety for human health and environment. The protective NC-21P is a photocatalytic water repellent, but it unexpectedly showed an antibacterial effect in all bacterial strains in the absence of the metabolic activation fraction. This effect can be considered an additional positive application feature of this formulation intended to prevent bacterial biofilm growth on stone surface. Though further studies, especially in vivo animal studies, are recommended, the results of this study suggest that health- and environment-related risks of all tested nanomaterials application is low.
6 References


Fruitier-Pölloth, C. (2012) The toxicological mode of action and the safety of synthetic amorphous silica—A nanostructured material Toxicology, 294: 61-79


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Grant Agreement no: 646178


Shi, H., Magaye, R., Castranova, V. and Zhao, J. (2013) Titanium dioxide nanoparticles: a review of current toxicological data. Particle and Fibre Toxicology. 10: 15

