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Effetti genotossici di nanoparticelle in organismi bioindicatori (*Mytilus edulis*) ed in colture cellulari di pesce

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Riassunto

La nanotecnologia è un campo in rapido sviluppo in quanto attrae investimenti significativi da parte di industrie e governi. I nanomateriali (NMs) usati sono tipicamente descritti come nanoparticelle (NPs) o nanotubi (NTs) con dimensioni che possono variare tra 1 e 100 nm.

Le piccoli dimensioni sono una delle cause delle loro peculiari caratteristiche fisico–chimiche diverse da quelle degli altri materiali solidi, e i loro effetti sull'ambiente e sugli organismi viventi sono ancora sconosciuti. E' quindi importante e necessario comprenderne la loro tossicità, il destino e gli effetti ambientali eventualmente provocati, in modo da creare un protocollo sotto controllo legislativo prima che vengano rilasciate in natura. Inoltre, la vasta gamma di nanomateriali esistente richiede la comprensione dei rischi che ciascun composto può comportare.

Nella presente tesi, condotta presso i laboratori CEFAS (Weymouth, UK), sono stati scelti Cadmio (4 nm $\pm$ 1 nm) e Argento (13 nm $\pm$ 1 nm) in forma di nanoparticelle in quanto ben nota la tossicità dei due elementi, e perché ultimamente utilizzati sotto forma di nanoparticelle in diverse applicazioni nel campo medico, nella cura dei tumori, nello sviluppo di strumentazioni, come pannelli fotovoltaici.

Le nanoparticelle usate negli esperimenti di esposizione devono avere la capacità di entrare in soluzione facilmente, dunque nel presente studio, le nanoparticelle utilizzate sono state rivestite da *thiol-terminated methyl polyethylene glycol* in modo da inibire la formazione di agglomerati. Scopo dello studio è stata la valutazione di eventuali effetti tossici a livello del DNA negli emociti di mitilo (*Mytilus edulis*) e in coltura cellulare di trota arcobaleno (RTG), oltre

all'attività enzimatica della lattico deidrogenasi (LDH) e la proliferazione cellulare, solo per RTG.

La linea cellulare utilizzata, sebbene non appartenente ad una specie marina, è da considerarsi, come tutte le linee cellulari in generale, un modello per l'analisi degli effetti genotossici in quanto essendo state geneticamente modificate per poter sopravvivere e moltiplicarsi *in vivo*, si diversificano dall'animale originale.

Il Comet assay, noto anche come *single cell gel electrophoresis* (SCGE), è una tecnica microelettroforetica per la diretta visualizzazione del danno al DNA in singole cellule. Durante l'elettroforesi i frammenti di DNA aventi basso peso molecolare si muovono più rapidamente del DNA integro (testa della cometa). Le comete e quindi l'estensione del danno al DNA, vengono misurate con un sistema di analisi dell'immagine computerizzata, che consente di rilevare la percentuale di DNA presente nella coda e nella testa della cometa.

Per lo studio degli effetti genotossici è stato utilizzato il Comet assay per entrambi gli organismi. Le colture cellulari di trota non sembrano subire effetti significativi dopo una esposizione di 24 e 48 ore alle nanoparticelle di argento e cadmio a cinque diverse concentrazioni (50; 10; 1; 0.1; 0.01 mg/L), così come non si palesano effetti statisticamente significativi sugli emociti di mitilo se non alla dose di 10 mg/L, che, per il cadmio sembrano essere influenzati da effetti citotossici.

Il test dell'LDH (detto anche test di citotossicità) è un saggio colorimetrico per la quantificazione della mortalità e della lisi cellulare, basato sulla misurazione dell'attività appunto della lattico deidrogenasi (LDH) rilasciata nel supernatante dal citosol di una cellula danneggiata. Nel test di proliferazione cellulare (XTT) le

cellule metabolicamente attive (vive) riducono il sale di tetrazonio a formazan; dalla misura allo spettrofotometro di quest'ultimo è quindi possible ricavare la crescita cellulare all'interno del nostro campione.

Abstract

Nanotechnology is a rapidly developing field, attracting significant investments from industry and governments. Nanomaterials (NMs) used in applications are tipically described as nanoparticles (NPs) or nanotubes (NTs).their small size causes physico-chemical properties that differ from those of other solid materials; hence their environmental effects are unknown. To ensure regulatory compliance with relevant legislative regimes it is necessary to understand the toxicity, the fate and environmental effects of nanoparticles prior to release into the environment.

In this study, conduct in CEFAS laboratory (Weymouth, UK), cadmium NPs (4 nm ± 1 nm) and silver NPs (13 nm ± 1 nm) have been chosen as their toxicity is weel known in their solid forms, and because they are lately used as nanoparticles in several applications in medicine, in the development of new technologies and instruments as for example solar panels.

NPs used in experiments must have consistent batch properties, for this reasons they have been capped with *thiol-terminated methyl polyethylene glycol* to reduce agglomerations. The aim of the present study was the valuation of genotoxic effect on *Mytilus edulis* and rainbow trout gonads cells (RTG-2) DNA, more the enzymatic activity of lactic dehydrogenase (LDH) and cellular proliferation only for RTG-2.

RTG-2, also if it's not a marine species cell line, it has to be considered, as all cell lines in general, a model for detection of genotoxic effect as they have been genetically modify to let them survive and multiply *in vivo*, becoming diffeent from the original animal.

Comet assay, known also as *single cell gel electrophoresis* (SCGE), is a microelctrophoretic technique for direct visualization of DNA damage in single cells. During electrophoresis DNA strands with low molecular weight move quiclier than comlite DNA (head of the comet). Comets, and then the extension of the DNA damage, are measured with an image computerizated system, which consent to register the DNA percentage present in the tail and in the head of the comet. For the carectirization of DNA damge, Comet assay has been used for both the organisms.

RTG-2 seems to not suffer significant effects by silver NPs and cadmium NPs at five different doses (50; 10; 1; 0,1; 0,01 mg/L) after an exposure of 24 and 48 hours, while mussels don't suffer any significant effect after an exposure at no one of the doses used (10; 1; 0,1; 0,001 mg/L) after 4 hours.

LDH test (known as Cytotoxicity test as well) is a colorimetric assay for the quantification of cellular mortality and lysis, based on the detection of the lactic dehydrogenase (LDH) released in the supernatant by the citosol of a damaged cell. In the cell proliferation test (XTT) metabolic active cells reduce tetrazolium salt in formazan, from the measure on the spectrophotometer of this reaction product is possible to obtain the cell population growth in our sample.

1_ Introduction

Nanotechnology includes all those fields of science and engineering where phenomena take place in the nanometre scale, and are utilised in the design, characterisation, production and application of materials, structures, devices and systems.

Many structures exist with nanometre dimensions in the natural world, including essential molecules within the human body, components of foods and many technologies have incidentally involved nanostructures for many years but, it has been possible to actively and intentionally modify molecules and structures within this size range only in the last decades.

The various forms of nanotechnology have the potential to make such a very significant impact on society, that it is considered to be the biggest engineering innovation since the Industrial Revolution.

It can be generally assumed that the application of nanotechnology will be very beneficial to individuals and organisations. Many applications involve new materials with radically different properties and functions at the nanoscale. These include materials in the form of very thin films used in catalysis and electronics, two-dimensional nanotubes and nanowires for optical and magnetic systems, and as well nanoparticles used in cosmetics, pharmaceuticals and coating. The industrial fields most readily embracing nanotechnology are the information and communications sector, including many different facets of pharmaceuticals and drug delivery systems, diagnostics and medical technology, where the terms nanomedicine and bionanotechnology are already commonplace. Nanotechnology products may also offer novel challengies for the reduction of environmental pollution.

However, just as phenomena taking place at the nanoscale it may be quite different to those occurring at larger dimensions and may be exploitable for the benefit of mankind, so these newly identified processes and their products may exposed the same humans and the environment in general, to new health risks, possibly involving quite different mechanisms of interference with the physiology of human and wild.

These possibilities may well be focussed on the fate, the dispersion and persistence of free nanoparticles generated in nanotechnology processes and either intentionally or unintentionally released into the environment.

1.1_ Definitions

There are several definitions of nanotechnology and the products of nanotechnology. Nanoparticles (NPs) are defined as materials that have at least one dimension in a range between 1 and 100 nm, from that the used term of nanoscale materials *(Royal society 2004).*

It is within this range of size that materials can possess substantially different properties if compared to the same substances at larger sizes, both because of the substantially increased ratio of surface area to mass, and also because quantum effects begin to play a role at these dimensions, leading to significant changes in several types of physical property.

- Nanoscale*:* having one or more dimensions of the order of 100 nm or less.
- Nanoscience*:* the study of phenomena and manipulation of materials at atomic, molecular and macromolecular scales, where properties differ significantly from those at larger scale.

- Nanotechnology*:* the design, characterisation, production and application of structures, devices and systems by controlling shape and size at the nanoscale.
- Nanomaterial*:* material with one or more external dimensions, or an internal structure, which could exhibit novel characteristics when compared to the same material without nanoscale features.
- Nanoparticle*:* particle with one or more dimensions at the nanoscale.
- Nanocomposite*:* composite in which at least one of the phases has at least one dimension on the nanoscale.
- Nanostructured*:* having a structure at the nanoscale.

1.2_ *Nanoscience and nanotechnology*

What we know about science at nanometre scale is derived from many disciplines, originating in the atomic and molecular concepts in chemistry and physics, and then has been incorporated into molecular life sciences, medicine and engineering. In materials sciences, nanocomposites with nanoscale dispersed phases and nanocrystalline materials in which the very fine grain size affords quite different mechanical properties to conventional microstructures are already in use. In surface science and surface engineering, nanotopographies offer substantially different properties related to adhesion, tribology, optics and electronic behaviour.

Supramolecular chemistry and catalysis have led to novel surface and size dependent chemistry, such as enantioselective catalysis at surfaces. In biological sciences, fundamental understanding of molecular motors and molecular functional entities on the nanometre scale has been responsible for the advances in drug design and targeting.

Nanoscale functionalised entities and devices are in development for analytical and instrumental applications in biology and medicine, including tissue engineering and imaging.

Electronic, electro-optic and optical devices are making biggest impact in the application areas.

The transition from semiconductor (conventional and organic) technology to nanoscale devices has anticipated improved properties and resolution, e.g. fluorescence labelling, scanning probe microscopy and confocal microscopy. Data storage devices use nanotechnology to provide smaller, faster, and lower consumption systems.

In medicine, the understanding and the knowledge of diseases on the nanometre scale is being derived, and drug delivery through functionalised nanostructures may result in improved pharmacokinetic and targeting properties.

1.3_ *Physical and Chemical properties*

The principal parameters of nanoparticles are their shape, size and size distribution, and the morphological (e.g., crystallinity, porosity, and surface roughness) sub-structure of the substance, bulk chemistry of materials, solubility, surface area, state of dispersion, surface chemistry and other physical-chemical properties.

Nanoparticles are present as an aerosol (mostly solid or liquid phase in air), a suspension (mostly solid in liquids) or an emulsion (two liquid phases).

At some point between the Ångstrom level and the micrometre scale, the simple picture of a nanoparticles is a ball or drop. Both physical and chemical properties are derived from atomic and molecular origin in a complex way. For example the electronic and

optical properties and the chemical reactivity of small clusters are completely different from the better known property of each component in the bulk or at extended surfaces.

In this range of dimensions, particle-particle interactions are either dominated by weak Van der Waals forces, stronger polar and electrostatic interactions or covalent interactions. Particle aggregation is determined by the interparticle interaction, depending on the viscosity and polarisability of the fluid.

Particles can be de-agglomerated by applying shear (mixing, sonication, grinding, and turbulence), but unless the conditions are present for a stable dispersion the system will be prone to re-agglomerate rapidly, like in toxicology studies, where particles may agglomerate rapidly when introduced into new environments such as a highly buffered (high ionic strength), or protein rich physiological/biological fluids.

1.4_ *Interactions Nanoparticles-Living Systems and Toxicity*

Nanoparticles may be of the same dimensions as some biological molecules such as proteins and nucleic acids. Many of these biomolecules consist of long macromolecular chains which are folded and shaped by cooperative and weak interaction between side groups, H-bridges and salt bridges.

Functionalized nanoparticles, such as colloidal gold *(Hayatt, 1989),* may intrude into the complex folded structures *(Cheng et al., 1999, Hainfield and Powell, 2000).* Evidence for such interactions is seen from the experience with immunolabelling *(Romano and Romano, 1977)* and related surface functionalisation techniques to target nanoparticles to biomolecules as markers for high resolution Transmission Electron Microscopy (TEM) and optical imaging

systems. Other nanoparticles systems include quantum dots *(Chan and Nie, 1998)* and magnetic nanoparticles *(Josephson et al., 1999).* All nanoparticles, on exposure to tissue and fluids of the body, will immediately absorbed onto their surface some of the macromolecules they encounter at their portal of entry. The specific features of this adsorption process will depend on the surface characteristics of the particles, including surface chemistry and surface energy, and may be modulated by intentional modification of functionalisation of the surfaces *(Schellenberg et al., 2004).*

This is well demonstrated through the use of specific biomolecular linkers that are anchored on the surface of nanoparticles or within vescicles and liposomes *(Nardin, 2000).* In this way the affinity of a nanoparticle can be shaped to fit a particular protein, and thus target a specific biomolecular assembly on a membrane, or within a specific organelle or cell surface.

The specificity of such surface layers is used for analytical purposes *(Elghanian et al., 1997),* for optical labelling of biomolecules in molecular libraries *(Han et al., 2001)* and for drug or gene delivery to cells *(Hood et al., 2002).* In agreement with bulk surface chemistry, metallic nanoparticles are of considerable chemical reactivity while ionic crystal nanoparticles have been observed to accumulate protein layers when exposed to the cytoplasm or in the lymphatic fluid. This protein layer is possibly involved in the interaction of the nanoparticle by the cellular system.

1.4.1_ *Effects of size, shape, surface and bulk composition*

The interaction of nanoparticles with living systems is also affected by the characteristic dimensions. As seen above, NPs of few nm may be contained within biomolecules; an impossible situation for larger particles.

In order to understand and categorized the mechanisms for nanoparticle toxicity we need information about the response of living being to the presence of nanoparticles with varying size, shape, surface and bulk composition, as well as the temporal fate of nanoparticles that are subject to translocation and degradation processes. Very little information about that is available right now, and this implies that there is an urgent need for toxicokinetic data for nanoparticles.

In fact studies focused on the toxicity of nanoparticles have appared only recently and are still rare in literature; data concerning the behaviour and toxicity of particles mainly comes from studies on inhaled nanoparticles *(reviewed by Oberdörster G., 1996, Oberdörster G et al., 2005, Donaldson and Stone, 2003, Borm, 2002, Donaldson et al., 2001a, 2004; Dreher, 2004, Kreyling et al., 2004).*

1.4.2_ *Size*

Size should be the first parameter to be considered when characterizing nanoparticles for toxicity studies. Since many particles system are irregular in shape, particles size is expressed in terms of the equivalent spherical diameter which is defined as the diameter of a spherical particle possensing the same selected property of the (not necessarily spherical) nanoparticles measured

(equivalent spherical volume diameter is defined as the diameter of a sphere with the same volume as the particles measured).

Likewise, the Stokes diameter is the diameter of a sphere of equivalent density that settles at the same rate as the measured one.

For aerosols, the most commonly used size metric is the aerodynamic equivalent diameter, defined as the diameter of a unit density sphere having the same terminal settling speed as the particle in question, whatever its size, shape, and density. It is used in combination with inhalation models to predict where in the respiratory tract such particles may be deposited.

Reduction in size to the nanoscale level results in an enormous increase of surface to volume ratio, so relatively more molecules of the chemical are present on the surface, thus enhancing the intrinsic toxicity *(Donaldson et al., 2004).* This may be one of the reasons why nanoparticles are generally more toxic than larger particles of the same insoluble material when compared on a mass dose base. In the studies of low toxicity particles, TiO_2 induced a more severe lung inflammation and particle lymph node burden compared to $BaSO_4$ dosed at mass burden in milligrams *(Tran et al., 2000).* Surface area was therefore a driver for inflammation for these materials; the differences in severity of the response disappeared when the dose was expressed as surface area. These examples emphasize the importance of particle size, and by implication, the amount of surface area presented to the biological system for particle toxicity.

Anyway, it is difficult to draw firm conclusions regarding size effects from existing literature due to the fact that studies are performed

with different cell lines, tissues, animal models, different types of ultrafine particles, and methods of administration.

1.4.3_ *Chemical Composition*

The chemical composition and the toxicological properties of any chemical elements are of importance for the toxicity of the particles *(Donaldson et al., 2004).* In addition chemicals adsorbed on the surface may affect the reactivity of nanoparticles.

Nanoparticles can have a very complex composition when they are in ambient air, and these components, organics or metals, can interact.

Metallic iron was able to potentiate the effect of carbon black nanoparticles, resulting in enchanced reactivity, including oxidative stress *(Wilson et al., 2002).* It is also possible to see the opposite effects, a diminishing of cytotoxicity, as observed by *Gupta and Gupta (2005)* coating superparamagnetic iron oxide nanoparticles with pullulan in *vitro* cytoxicity. Also for dextran and albumin derivatised iron oxide nanoparticles a reduction was noted by *Berry et al. (2003)*.

1.4.4_ *Shape*

Shape is also an important factor to considerate. Fibre provide a significant example, especially in relation to inhalation, where the physical parameters of thinness and length seem to determine respirability and inflammatory potential. In two recently published *in vivo* studies, single-wall carbon nanotubes (SWCNTs) were demonstrated to induce lung granulomas after intratracheal administration *(Lam 2004, Warheit et al., 2004)*, indicating that these nanotubes cannot be classified as a new form of graphite on

material safety data sheets. On a dose per mass basis the nanotubes were more toxic than quartz particles, well known for their lung toxicity.

1.5_ *Toxicity (ecotoxicity)*

As pointed by *Colvin (2003)* on his discussion on the impact of engineered materials, there is a demonstrated lack of data on the exposure and effects of nanoparticles. In fact just few studies have been carried out with species normally used for ecotoxicological analysis. However, considering that a large number of the above cited human toxicology studies have examinated the uptake and effects of nanoparticles at cellular level, it can be hypothesized that these observations may also hold for other species. A work to support this hypothesis is needed.

From the cellular point of view, different uptake mechanisms are considered to be relevant for NP. The relevance of these different pathways has been shown to depend on the physicochemical properties of the NP (e.g.; chemical composition, size/geometry, surface charge, coating/ligands, aggregation status), the exposed cell type (professional phagocytes versus other cell types), as well as on their microenvironment (e.g.; lung surfactant, opsonins).

The importance of the physicochemical properties of NP in the toxic and functional effects in macrophages (phagocytosis, inflammatory mediator release, calcium release, cytoskeletal function) has been shown by various investigators *(Renwick et al., 2004; Jia et al., 2005).*

Contrasting uptake mechanisms of NP in professional phagocytes versus other cell types in relation to different behaviour in the cell membrane structures (e.g., receptors) are indicated by a number of

studies. For instance, the surface modification of superparamagnetic NP with polyethylene glycol or folic acid has been shown to enhance their uptake by cancer cells but not by macrophages *(Zhang et al., 2002).*

1.5.1_ *Reactive oxygen species*

The generation of reactive oxygen species (ROS) upon exposure of cells to particulate matter is nowadays generally considered a major contributor to NP toxicity *(Donaldson et al., 2001; Nel et al., 2006).* Mechanistically, the enhancement of ROS generation by NP is envisaged to occur at several levels, including: (a) the chemical reactivity both of particles and of impurities found in particle preparations, and (b) the physical interaction of particles with cellular structures involved in the catalysis of biological reduction-oxidation (redox) processes.

NPs induced ROS formation, if exceeding the cellular antioxidative machinery's defensive capacity, may cause oxidative damage to biomolecules. Beyond that ROS are well known signalling modulators at concentrations well below those that cause detrimental oxidation. As a consequence, exposure of cells to NPs may affect, via ROS formation, cellular signalling cascades that control cellular proliferation, inflammatory processes and cell death.

Molecule oxygen, present in biological fluids in high micromolar concetrations, is activated and turned into a 'reactive oxygen species' (ROS) by energy transfer reactions. While singlet oxygen ($^1O_2$), an electronically excited and non-radical form of molecular oxygen, is generated by energy transfer, a radical, superoxyde ($O_2^{\cdot -}$), results from the simple reduction of oxygen.

$$O_2 + e^- \rightarrow O_2^{\cdot -}$$

The monovalent reduction of molecular oxygen may be specifically enzymatically catalyzed (such as NADPH oxidases), or may occur as a by-product of enzymatic reactions, as a side reaction of electron transport along the mitochondrial respiratory chain, or may occur during redox cycling.

Superoxide disproportionation (dismutation), occurring both spontaneously and as catalyzed by manganese- or zinc-dependent superoxide dismutases, yields hydrogen peroxide.

$$2\ O_2^{\cdot -} + 2\ H^+ \rightarrow H_2O_2 + O_2$$

Hydrogen peroxide may be fully reduced to water by peroxidases as glutathione peroxidases, heme peroxidases or a second molecule of H_2O_2 as electron donor, having like result another dismutation reaction.

$$H_2O_2 + XH_2 \rightarrow 2\ H_2O + X$$

$$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$$

$$H_2O_2 + H_2O_2 \rightarrow 2\ H_2O + O_2$$

Partial rather than full reduction of hydrogen peroxide may occur when redox active transition metal ions are present in their reduced forms (Me^{n+}), resulting in the production of hydroxyl radicals (Fenton reaction), most reactive oxidizing species and potent initiators of radical chain reactions with diverse biomolecules.

H_2O_2 (='HO-OH') + e^- –> HO^- + $\cdot OH$ (with Me^{n+} –> $Me^{(n+1)+}$ + e^-)

1.5.2_ *Genotoxicity*

It is the capacity of a substance to modify the DNA structure of a living being.

To maintain the integrity of the DNA molecule before initiating DNA replication, transcription and cell division, cells are provided with several efficient DNA repair mechanisms to prevent such potentially adverse effects *(Lindahl, 1993).*

In this regard, it is nowadays known that DNA damage itself can trigger cell cycle arrest whereby the prolongation of G1 and G2 phases provides additional DNA repair time prior to DNA synthesis and mitosis respectively *(Zhou & Elledge, 2000).*

Damage to genomic DNA, both from endogenous and exogenous sources, may induce three major consequences:

- induction and fixation of mutations;
- induction of DNA cycle arrest;
- activation of signal transduction pathways which promote apoptosis.

Mutation can involve a single gene, a block of genes or even whole chromosomes. Mutations are considered to play a key role in

carcinogenesis (*Ames* 1979; *Devereux et al.* 1999) and in interference with various metabolic processes occurring into the cells such as DNA replication and DNA transcription which may result in cell death *(Andreassen et al., 2006).*

Severe damage to the nuclear DNA is known to trigger the signal transduction pathways which promote cellular apoptosis (programmed cell death).

As already outlined, induction of ROS is considered to represent a major mechanism whereby NP can elicit their toxic effect within the cells. The ability of NP to cause damage to the nuclear DNA is indicated in several studies, and several of these show that ROS are involved herein via oxidative attack to DNA. Such oxidative DNA damage has also been seen in the DNA damaging effects of toxic particle in the micrometre size range such as quartz and asbestos, whereby DNA damage could be reduced or abrogated with radical scavengers and antioxidants *(Xu et al., 1999; Shi et al., 1994; Schins, 2002).* Oxidative DNA damage can lead to base pair mutations, deletions or insertions, all being commonly observed in mutated oncogenes and tumor suppressor genes *(Wiseman & Halliwell, 1996).* Well investigated oxidative lesions include 8-hydroxyguanine (8-OHdG), which is considered to be predominantly caused by hydroxilradicals.

The ROS do not need to interact directly with the DNA to cause deleterious genetic change. The mitotic spindle consisting of tubulin fibres can also be damaged leading to aneuploidy by oxidative deterioration as found following exposure to asbestos fibres *(Barret et al., 1989; Osgood, 1994; Dopp et al., 1997)*. The most potent asbestos fibre; crocidolite, contains the biggest proportion of Fenton catalyst Iron, hence produces the most elevated rate of ROS

production and damage *(Shukla et al., 2003; Poser et al., 2004)*. In addition to the possible effects on the mitotic apparatus, the cytoskeleton which consist of filaments and has extensive roles in cellular trafficking, cell motility and endocytosis has been shown to be inhibited by *in vitro* ultrafine TiO_2 exposure in primary alveolar dog macrophages due to disruption of cytoplasmic calcium levels *(Moller et al., 2002; Moller et al., 2005)*.

1.6_ *Biological effects of NPs*

The literature on the ecotoxicology of NPs is still emerging, however there have been several recent reviews on the ecotoxicity of manufactured NPs and NMs *(Oberdörster et al. 2006; Crane and Hardy 2007; Moore 2006).* Early studies have been observational, and they have tried to document toxic effects, and the concentrations of NPs that produce these effects in different groups of organisms.

The literature on mammalian models has recently been reviewed in the context of the environment and routes of human exposure to manufactured NPs *(Handy and Shaw, 2007).* These mammalian reports have focused on respiratory toxicology and inflammation reactions upon NP exposure *(Oberdörster et al., 1992; Burmudez et al., 2004; Lam et al., 2004)*.

Hardy et al. (2008) pointed out that mammalian lung epithelium has some relevance to ecotoxicology because the lung is representative of a typical mucous epithelial tissue and is not fundamentally different in structure to other epithelia such as the gills or guts of aquatic organisms, or the body surface of earth worms. Fish gills are certainly sensitive to some manufactured NPs (TiO_2 NPs, *Federici et*

al., 2007; single walled carbon nanotubes, SWCNT, Smith et al., 2007).

The effects of TiO_2 NPs, for instance,which is used in sunscreens, showing an induced photo and non-photocatalytic production of reactive oxygen species (ROS) leading to oxidative deterioration of lipids, proteins and nucleic acids *(Gurr et al., 2005; National-Nanotechnology-Initiative, 2006)* have been studied in rainbow trout *(Oncorhynchus mykiss)*.

There are many gaps in our knowledge on the ecotoxicology of NPs; most of the available acute toxicity data are on freshwater species, and mainly species used for regulatory toxicology *(D. magna, Lovern and Klaper, 2006; fathead minnows, Pimephales promelas, Zhu et al., 2006)*.

More studies are needed on marine and terrestrial invertebrate species, as well as other vertebrates including amphibians, reptiles and birds. Anyway most of these studies has been done in laboratory conditions, and toxicity in laboratory solutions may be somewhat different from that in real environmental samples.

Development of methodologies to measure NPs in complex environmental matrices (soil, sediments, natural waters) will be an urgent and essential prerequisite to environmentally realistic studies of NP ecotoxicity.

To date, ecotoxicological studies have not been performed to the level of detail that would enable a mechanistic analysis of absorption, distribution, metabolism and excretion. The first step in the biological uptake of any substance is the absorption of the material onto the exterior surface of the organism *(Hardy and Eddy, 2004)*, and the physical-chemistry aggregation or precipitation of NPs on the exterior surface of organisms. This notion is supported

by experimental observations (aggregation of SWCNT on the gill mucus of rainbow trout *(Smith et al., 2007)*). The next step is the uptake cross the cell membrane and the mechanisms involved remain to be investigate.

It seems unlikely that NPs could be moved across the cell membrane via ion transporters because the particles are much bigger than ions, and presumably would not fit the binding sites.

Moore (2006) argued the case for endocytosis and pointed out that marine bivalves such as *Mytilus edulis* might take up NPs using endocytosis, and demonstrated that polyester NPs were taken up into endosomes and lysosomes of mussels.

This model of uptake may be especially relevant where aggregation of NPs on the surfaces of the organism occur. *Moore (2006)* also raised concerns about NPs acting delivery vehicles for other chemicals *via* endocytosis pathways.

Particles can be identified in tissues using the electron microscope, but the sample preparation takes time, and there is always the risk of introducing aggregations artefacts during processing of the material.

The target organs may also reflect the biology or life style of the organism. For example crustaceans are well known for their ability to sequester toxic metals into granules in the hepatopancreas and other tissues *(Barka et al., 2007)*.

As seen above, it is clear that the exposure to toxicans may induce a chain of events producing several damage on the DNA and on the metabolism of a living being. The identification and the quantification of such events can be used by researchers like markers of the fitness of an organism, of the environment, of a population studied; in a word, as biomarkers.

Overall, the ecotoxicology literature on NPs and NMs is at the beginning, but toxic effects have been identified in a range of fish and invertebrate that raise sufficient concern that NPs, in the environment could have adverse effects on wild life, if present at high enough levels. There are several significant knowledge gaps in the ecotoxicology; for example, information is generally lacking on bacteria, plants, and higher vertebrate species. The ecotoxicity of NPs requires a multi-disciplinary approach, and ecotoxicologists need to understand the main issue in particle chemistry in order to interpret ecotoxicological data correctly.

These include the effects of particle size, shape and surface area, and the interactions of the particles with other material in the water or environmental matrix. Our knowledge of the interactions between NPs and abiotic factors is poor; thus there is still need for a detailed investigation on the effects of salinity, pH, water hardness, presence of colloid materials on NPs ecotoxicity.

1.7_ *Biomarkers*

The term biomarker was used for the first time at the end of 70's to indicate environmental health damage due to an exposure to toxic substances.

The idea to utilize biomarkers in ecotoxicology was introduced in the 70's by *Bayne et al. (1976)* and *Payne (1977)* in marine environment.

The most recent definition of biomarker is *"…that biochemical, cellular, physiologic or behaviour variation that can be measured in a tissue, in a biologic fluid or in an entire organism (and population), which gives the evidence of an exposure and/or the effect of one or more pollutant compounds (and/or radiations)" (Depledge, 1994)*.

By the use of biomarkers it is possible to diagnosticate through the study of early responses, the presence of eventual contaminants in environment, where the bioindicator organism lives.

Thus through the use of biomarkers it is possible to detect the onset of environmental alterations that, if persisting, would lead to the impairment of populations dynamics and communities structure. In this respect, biomarkers also have prognostic values in addition to a diagnostic utility.

It is possible to divide biomarkers in different categories on base of the information they can give us. The two biggest and more common categories are : *general biomarkers* and *specific biomarkers.*

The first one is defined as *"…all those molecular, cellular and physiological responses of an organism which can not be reconduced to a specific class of contaminants but that indicate the general stress state of the organism" (Peakall & Shugart, 1993)*; instead the second one as "*all those molecular and biochemical responses happening in an organism after exposure to a specific class of contaminants" (Peakall & Shugart, 1993).*

Biomarkers based on genotoxic effects are often included in ecotoxicological investigations either through laboratory exposure to ecologically relevant pollutants or in biomonitoring studies. Alterations of the genome can occur at different levels, including molecular damages and/or change in the structure or number of chromosome.

In the present thesis three different biological responses were investigated at cellular level, namely: DNA strand breaks (by the Comet assay), cytotoxicity (by LDH cytotoxicity detection test) and cell proliferation (by a colorimetric assay based on the reduction of tetrazolium salt to formazan).

1.7.1_ *Strand breaks (SB)*

The quantitative determination of strand breaks presence, on the single DNA strand and on the double one as well, is frequently used as biomarker to address the genotoxic effect of environmental contaminants. SB production is actually well correlated with mutagenic and/or carcinogenic characteristics of many environmental contaminants even different mechanism of action *(*Mitchelmore *& Chipman, 1998).* Breaks may be produced by several factors, which can act directly, like solar radiations and X ray, or indirectly, for instance after metabolic activation.

Some substances, like H_2O_2, are able to induce DNA strand breaks in the blue mussel *Mytilus edulis* and in the trout *Oncorhynchus mykiss*.

Strand breaks can also be produced by the action of repair enzymes which cut the DNA. Therefore, it looks clear how SB quantity evaluation is a good way to address the genotoxic effect of several compounds, each one with the capacity to modify the DNA in a different way.

A number of techniques for detecting DNA damage, as opposed to the biological effects (e.g., micronuclei, mutations, structural chromosomal aberrations) that results from DNA damage, have been used to identify substances with genotoxic activity. Until recently, the most frequently used methods involved either the detection of DNA repair synthesis (so-called unscheduled DNA synthesis or UDS) in individual cells, or the detection of DNA SSB (single stand breaks) of ALS (alkali-labile sites) in pooled cell populations using the alkaline elution assay. The UDS technique is based on the replication of DNA during the excision repair of certain

types of DNA lesions, as demonstrated by the incorporation of tritiated thymidine into the DNA repair sites. While providing information at the level of the individual cell, the technique is technically cumbersome, requires the use of radioactivity, and is limited in sensitivity. The alkaline elution assay ignores the critical importance of intercellular differences in DNA damage and requires relatively large numbers of cells. A more useful approach for assessing DNA damage is the single-cell gel (SCG) or “Comet assay”. The term “Comet is used to identify the individual cell DNA migration patterns produced by this assay (Fig. 1).

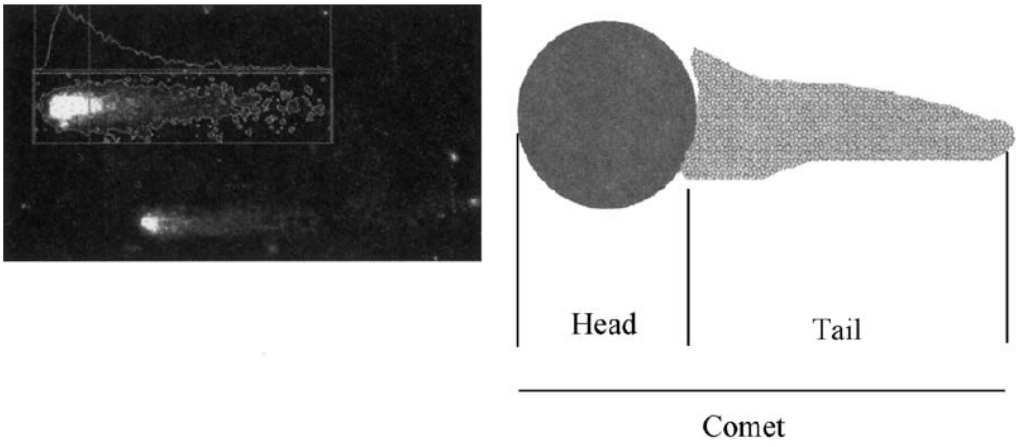


Fig.1_ Typical migration pattern of a nucleus after electrophoresis in the Comet assay.

Östling and Johanson (1984) were the first to develop a microgel electrophoresis technique for detecting DNA damage at the level of a single cell. In their technique, cells embedded in agarose were placed on a microscope slide, the cells were lysed by detergents and high salts, and DNA electrophoresis under neutral conditions. Cells with an increased frequency of DNA double strand breaks (DSB) displayed increased migration of DNA toward the anode. The

migrating DNA was stained with a fluorescent dye (ethidium bromide) and measured using a fluorescence microscope.

The neutral conditions used greatly limited the general utility of the assay. Subsequently, *Sight et al. (1988)* introduced a microgel technique involving electrophoresis under alkaline (pH > 13) conditions for detecting DNA damage in single cells. At this pH, increased DNA migration is associated with increased levels of frank SSB, SSB associated with incomplete excision repair sites, and ALS. Because almost all genotoxic agents more SSB than ALS and DSB, this version of the assay offered greatly increased sensitivity for identifying genotoxic agents (*Tice et al., 1995)*. Since the introduction of the alkaline (pH > 13) Comet assay in 1988, the fields of applications and the number of investigators using this technique have increased almost exponentially. Compared with other genotoxicity assays, the advantages of the technique include:

- Its demonstrated sensitivity for detecting low levels of DNA damage
- The requirement of small numbers of cells/sample
- Flexibility
- Low cost
- The ability to conduct studies using relatively short time period (a few days)

1.7.2_ *LDH (Cytotoxicity Detection Test)*

This test is designated as a precise, fast and simple colorimetric alternative to quantitate cytotoxicity/cytolisis based on the measurement of LDH activity released from damaged cells.

Thus, the Cytotoxicity Detection Test can be used in many different *in vitro* cell systems when damage to the plasma membrane occurs.

Examples are:

- Detection and quantification of cell mediated cytotoxicity induced by cytotoxic T-lymphocytes (CTL), natural killer (NK) cells, lymphokine activated killer (LAK) cells or monocytes
- Determination of mediator-induced cytolisis
- Measurement of antibody-dependent cellular cytotoxicity (ADCC) and complement-mediated cytolysis.
- Determination of the cytotoxic potential of compounds in environmental and medical research and in the food, cosmetic and pharmaceutical industries
- Determination of cell death in bioreactors

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme present in all the cells. It is rapidly released into the cell culture supernatant upon damage of the plasma membrane.

The supernatant is cell-free collected and incubated with a reaction mixture. The LDH activity is determined in an enzymatic test: in the first step NAD^+ is reduced to $NADH/H^+$ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step the catalyst

(diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan (Fig. 2).

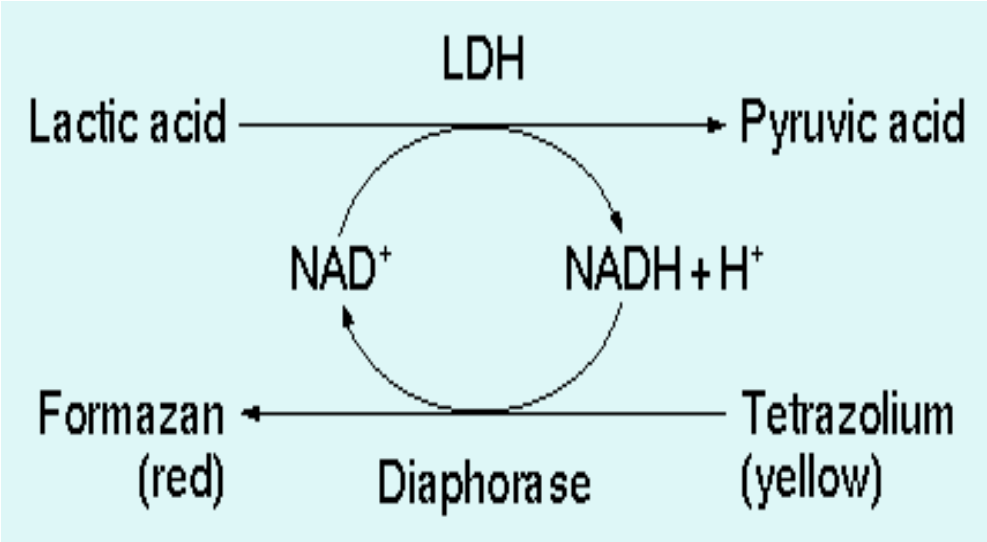


Fig. 2_ Enzymatic reaction occurring in the Cytotoxicity test (LDH) which reduce tetrazolium salt in formazan.

1.7.3_ *Cell Proliferation*

The determination of cellular proliferation, viability and activation are key areas in a wide variety of cell biological approaches. The need for sensitive, quantitative, reliable and automated methods led to the development of standard assay. Cellular proliferation and viability assays are of particular importance for routine applications

The assay is based on the cleavage of the yellow tetrazolium salt XTT (sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) to form an orange formazan dye by metabolic active cells. An increase in number of living cells results in an increase of overall activity of mitochondrial dehydrogenases (on which the cleavage of XTT depends) in the sample. Therefore, this conversion only occurs in viable cells. The formazan dye formed is soluble in aqueous solutions and is directly quantified using a scanning multi-well spectrophotometer.

Cells, grown in a 96 well tissue culture plate, are incubated with the yellow XTT solution for 4-24 hours. After this incubation period, orange formazan solution is formed.

The assay is designed for the spectrophotometric quantification of cell growth and viability without the use of radioactive isotopes.

It is used for the measurement of cell proliferation in response to growth factors, cytokines and nutrients.

It is also used for the measurement of cytotoxicity, like the quantification of tumor necrosis factor or the assessment of cytotoxic or growth inhibiting agents such as inhibitory antibodies.

In contrast to cell viability analysis, cell proliferation assessment is defined as the measurement of actively dividing cells in a sample. Quiescent non-growing healthy cells are not detected by cell proliferation assay.

1.8_ *Mytilus edulis*

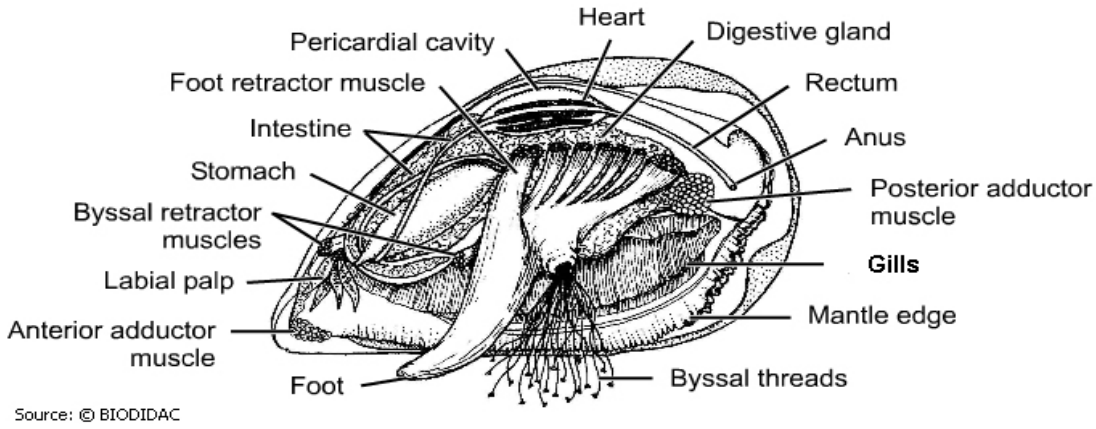


Fig. 3_ Anatomy of blue mussel *Mytilus edulis.*

The blue mussel is characterized by a bluish black shell, about 4 to 5 cm in length, pointed wedge-shaped at one end. The shell has got two halves, both about alike in shape. After a blue mussel shell has been opened, the large closing muscle and four pairs of gills

become visible. The mussel foot is large and muscular. When the mussel is closed the foot is usually the only body part visible from outside. At the foot's end there is the byssus gland producing a hard thread, that renders the mussel capable of attaching itself to the ground. The mussel can move by its byssus thread by cutting it off and building it again. It is even able to defend itself with byssus thread by tying predator snails with byssus threads down.

Nutrition_ The blue mussel is a filtrator (Fig. 4). It whirls sea water into the pallial cavity by cilia, both to breathe, or to filter nutritive particles from the water current. Digestible particles are whirled further into the mouth opening, indigestive particles are coated in mucus and excreted. A medium size mussel pumps about one litre of sea water per hour. Particles are not only sorted out by filtration near the gills, they are also sorted before the mouth opening. Though, mussels can fall dry for a certain time. The particles excreted by a mussel are accumulated around its shell, so it is placed on an ever growing heap of silt. The amount of silt accumulated can become noticeably high especially around mussel beds.

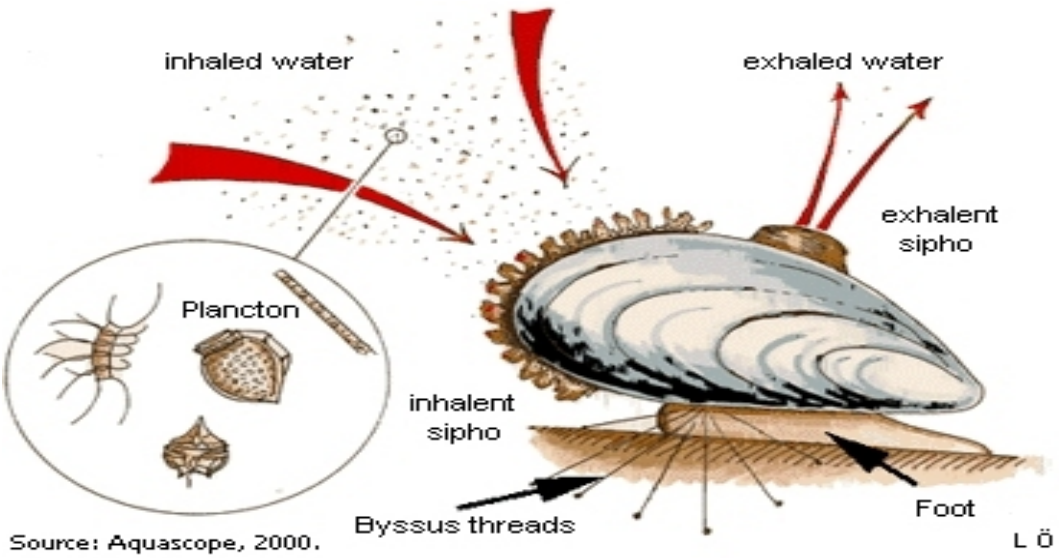
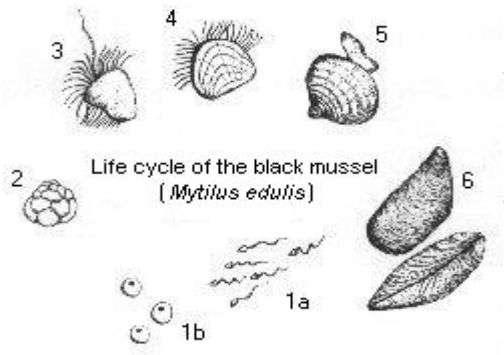


Fig. 4_ Alimentary strategy of blue mussel *Mytilus edulis*.

Reproduction_ Mussels have separate sexes. The female mussel spawns from May to October, depending on the water temperature, the current and other factors. Each spawn contains between 5 and 12 million eggs (1b). Thus mussels are able to withstand decimation by predators through an increase in number.



From the egg fertilized by a sperm cell (1a) the trochophora larva (3) develops. It possesses a terminal ciliar crest and an equatorial ciliar belt. After few days from the trochophora the veliger larva develops (4), recognizable by the sail-shaped cilia-bearing protrusions. After a total of four to six weeks the larvae reach about 0.25 mm size and change into juvenile mussels (5).

After this metamorphosis they cling to filamentary structures, like for example cnidarian colonies (polyps) and sea worms. Some weeks later the young mussels grow until almost doubling their size and begin to drift again. The greater the mussels the less distance they are drifted. Only the largest mussels (6) settle on the tidal zone, the smaller ones settle in deeper regions as they would not survive in the tides. After reaching their final size, mussels attach themselves to the ground with byssus threads. That substrate can be the sea floor, as well as other mussels. There seems to be a tendency of mussels to attach to each other in groups and to form mussel beds that way. This happens especially on soft ground, such as in the Wadden Sea, as blue mussels need to avoid sink into the ground not to suffocate. Other mussels living on silt have developed extended siphos, so they can live underground, but the blue mussel has not. Mussels also attach themselves to poles and rocks.

Distribution_ Common mussels are as common as their name indicates, they are cosmopolites. There are blue and black mussels belonging to the same species all over the American East coast as well as on all Western and Southern European coasts. There is even a species of black mussels in Japanese coastal waters. Black and blue mussels are inhabitants of the litoral zone, of the coastal region. They mostly live where they can remain submerged all time. This zone called sublitoral is where the largest part of blue mussels grow. However mussels continuously accumulating silt are in danger of rising themselves over the water surface. Though, during winter, a certain amount of silt is eroded again. Dangers for the mussel are represented by cold in winter, that may destroy whole mussel beds, especially during ice drift, and many coast-living animals that feed on the mussels, most of all the oystercatcher, but also other birds. Under water, mussels are threatened by starfish, crabs and eider ducks. Additionally the blue mussel belongs to the favourite prey of other molluscs, like sea snails, such as the whelk (*Buccinum undatum*), and other ones which eat mussels by trying to drill through the shell with their radula. Some of them try to outwit the mussel by waiting in front of it until it is forced to open its shell halves to breathe. Then they push their sipho into the gap and begin to eat the mussel, without it being able to defend itself.

Ecological Importance_ By their depositing silt mussels lead to the development of a unique biotope around their colonies. Mussel colonies are often grown over by seaweed, for example the bladderless wrack, (*Fucus mytili*, compare *Mytilus*: the mussel). On mussel polyps, bryozoans and barnacles (*Balanus*) thrive. Some other molluscs feed on the organisms thriving on the mussel shell, such as periwinkles (*Littorina*), and the tiny ancient chitons. By

filtering large amounts of sea water mussels have got an especially high ecological importance - one blue mussel at a prevailing water temperature of 14 °C filters about 1.5 litres of sea water per hour.
By filtering sea water mussels tend to accumulate harmful substances out of the sea water, like heavy metals in endangered areas. Different shellfish poisoning illnesses can occur after consumption of mussels that have accumulated algae poisons. That is also a sign for the increasing sea water pollution, as poisonous algae occur in increasing extent due to the growing eutrophization of sea water. Contamination by bacteria (e.g. salmonellae) is also not free of danger, as especially in coastal areas where mussels are eaten directly from the catch.

1.9_ *RTG-2 cell culture*

Cell culture is the process by which either prokaryotic or eukaryotic cells are grown under controlled conditions. In practice the term “cell culture” has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells.
The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a routine laboratory technique in the 1950s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century.
*Isolation of cells*_ Cells can be isolated from tissue for *ex vivo* culture in several ways. Cells can be easily purified from blood, however only the white cells are capable of growth in culture.
Mononuclear cells can be released from soft tissues by *enzymes* such as collagenase, trypsin or pronase, which break down the extracellualr matrix. Alternatively, pieces of tissue can be placed in

growth media, and the cells that grow out are available for culture. This method is known as *explant culture.*

Cells that are cultured directly from a subject are known as *primary cells.* With the exception of some derived from tumours, most primary cell cultures have limited lifespan. After a certain of population doublings cells undergo the process of senescence and stop dividing, while generally retaining viability.

An established or immortalised cell line has required the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of telomerase gene.

There are numerous well established cell lines representative of particular cell types.

*Maintaining cells in culture_* Cells are grown and maintained with an appropriate temperature and gas mixture in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed.

Aside from temperature and gas mixture, the most commonly varied factor in culture system is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrient components. The growth factors used to supplement media are often derived from animal blood, such as calf serum. These blood-derived ingredients pose the potential for contamination of derived pharmaceutical products with viruses and prions. Current practice is to minimize or eliminate the use of these ingredients where possible.

Some cells naturally live without attaching to a surface, such as cells that exist in the bloodstream. Others require a surface, such a s most cells derived from solid tissues. Cell grown unattached to a

surface are referred to as *suspension cultures.* Other *adherent cultures* cells can be grown on tissue culture plastic, which may be coated with extracellular matrix components (e.g. collagen of fibronectin) to increase adhesion properties and provide other signals needed for growth.

*Manipulation of cultured cells_* As cells generally continue to divide in culture, they grow until to fill the available area or volume. This can generate several issue:

1. Nutrient depletion in the growth media
2. Accumulation of apoptotic/necrotic (dead) cells
3. Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing known as contact inhibition
4. Cell-to-cell contact can stimulate promiscuous and unwanted cellular differentiation

These issues may be dealt with using tissue culture methods that rely on *sterile technique.* These methods aim to avoid contamination with bacteria or yeast that will compete with mammalian and cell death. Manipulations are typically carried out in a *biosafety hood* or laminar flow cabinet to exclude contaminating micro-organisms.

*Media changes_* The purpose of media changes is to replenish nutrients and avoid the build up of potentially harmful metabolic byproducts and dead cells, in the case of adherent cultures, the media can be removed directly by aspiration and replaced (with suspension cultures, cells can be separated from media by centrifugation and resuspended in fresh media).

*Passaging cells_* Passaging or splitting cells involves transferring a small number of cells into a new vessel. Cells can be cultured for a longer time if they are split regularly, as it avoids the senescence

associated with prolonged high cell density. Suspension cultures are easily passaged with a small amount of culture containing a few cells diluted in a larger volume of fresh media, while for adherent cultures, cells first need to be detached; this was historically done with a mixture of trypsin-EDTA, however other enzymes mixes are now available for this purpose.

2_ *Aim of study*

The aim of the present study was the evaluation of the potential genotoxic effects induced by nanoparticles on a marine sentinel species and cultured fish cells.

In particular, a cell culture from gonads of the teleost fish rainbow trout (*Oncorhynchus mykiss*) and haemocytes of blue mussel (*Mytilus edulis*) have been exposed to Ag_2S nanoparticles and CdS nanoparticles at different concentrations. As nanoparticles have the tendency to clump each other we used a capping agent (thiol-terminated methyl polyethylene glycol) normally used in this kind of studies.

DNA integrity was evaluated by the Single Cell Gel Electrophoresis (Comet Assay). Moreover, citotoxicity (by the use of LDH Cytotoxicity test) and Cell proliferation (by XTT assay) were also investigated to have a more exhaustive picture of nanoparticles toxicity.

3_ *Materials and Methods*

3.2_ *The nanoparticles*

CdS (4 ± 1 nm) and Ag_2S (13 ± 7 nm) NPs were sonicated and capped with an agent to reduce aggregation (thiol-terminated methyl plyrthylene glycol, Capping Agent).

Before exposure they were diluted in five different concentrations in L.15 10%FBS (50 mg/l, 10 mg/l, 1 mg/l, 0,1 mg/l, 0,01 mg/l) to test the toxicity in RTG-2.

3.3_ *The exposure*

Mussels, collected on Weymouth coast, were bled from the adductor mussel of 10 animals for experiment with a siring.

During every experiment the haemolymph was collected in Eppendorf tubes (two tubes for each dose) and exposed to four different concentrations of each substance for 4 hours at 15°C. For the mussels NPs and Capping Agent were diluted in HBBS to have a concentration of 100 mg/l (77 µl of CdS in 1 ml of HBBS, 111 µl of Ag_2S in 1 ml of HBBS, 10 µl of Cap. Agent in 1 ml of HBBS). Lately, for the exposure, 100 µl of each substance in 900 µl of haemolymph were used in order to have 10 mg/l, 1 mg/l, 0,1 mg/l, 0,01 mg/l.

For the mussels we run two experiments, divided into two replicas, for each concentration.

We used RTG-2 cells derived from gonads of rainbow trout *Oncorhynchus mykiss* generally used in toxicology studies. Cells were maintained at optimal growth conditions for the particular cell line in 75-cm^2 flasks under 5% CO_2 at 22°C, in Leibovitz medium (L.15) 10% FBS without antibiotics. Cells were passaged when the monolayer reached approximately 80-90% confluence. For

experimentation, cells were washed twice with Dulbecco's PBS, trypsinazed (500-700 µl of trypsin for each flask), counted with Tripan Blue in a counter slide, and diluted to reach the wanted concentration (1 X 10^5/ml) in L.15 10% FBS upon nominated. Reached the concentration useful for the experiment cells were transferred into 6-well plates (2 ml of volume for well) for Comet Assay and in 96-wells plates for Cytotoxicity test and Cell Proliferation test (200 µl for well). After plating, cells were allowed to attach for 24 hours before transfection.

For RTG-2, cells were transferred from 75 cm^2 flasks, after trypsinisation, into 6-well plates with L.15 medium (10%FBS) at 1 x 10^{-5} cells/ml (2 ml for each well) and seeded for 24 hours prior to test exposure at 22°C. After 24 hours, cells were washed twice with PBS and exposed to five different concentrations (50; 10; 1; 0,1; 0,01 mg/L) for each substance (CdS NPs, Ag_2S NPs and Capping Agent) for 24 and 48 hours in L.15 (1% FBS) at 15°C. After the exposure cells were washed twice with PBS and trypsinased (around 500 µl of 0,25% trypsine in PBS). To stop the trypsine activity 1000 µl of L15 10% FBS were used. 1 ml of supernatant was collected in Eppendorf tubes marked with the corresponding concentration. Also in this case we run two definitive experiments divided into two replicas for the Comet assay, the LDH test and Cell proliferation both after 24 hours and 48 hours.

3.4_ *Comet assay (single cell gel electrophoresis)*

Eppendorf tubes were spin down at 3000 rpm for 2 minutes. Taked off the supernatant, pellets were resuspended in 170 µl of LMP (low melting point agarose) 0.5%.

Two drops of 85µl, were placed on each 1% NMP (normal melting point agarose) agarose-coated slides. A coverslip was placed on top of each gel and the slides chilled to allow coverslip removal. After few minutes at 4°C, the slides without coverslip were placed in chilled lysing solution (2,5 M NaCl, 100 mM EDTA, 10 mM Tris-base, 8g NaOH and 1 litre DH_2O; 1% Triton X-100 and 9% DMSO added prior to use) for 1 hour and immersed in electrophoresis buffer (300 mM NaOH, 1 mM EDTA in 1 litre DH_2O; pH 13) for 20 minutes at 4°C. electrophoresis lasted 20 minutes (25 V and 300 mA) before slides were washed in neutralisation buffer (0.4 M Tris pH 7,5).

Air-dried slides were stained with ethidium bromide (2 µg/ml in DH_2O) and scored using Comet Assay IV. We have scored 50 cells for each half slide (every half slide is one replica of the experiment).

3.5_ *LDH*

The Cytotoxicity detection kit (Roche applied science) Cat. Nr. 11 644 793 001 was used for the detection of the cytotoxic effect of NPs.

100 microliters of L.15 media from each well from the 96-well plates after the exposure of 24 and 48 h was taken and split into 96-wells plates (100 µl for well). To this volume, 100 µl of reagent were added and the plate was incubated for 30 min in room temperature, protected from light.

The reaction mixture for 100/200 µl wells was obtained by mixing 0,25 ml catalyst (bottle 1) with 11,25 ml Dye solution (bottle 2). Both solutions have been kept in +4/+8°C.

The absorbance was measured at 490 or 492 nm by a Wallac 1420 VICTOR^2™ plate reader spectrophotometer.

Controls consisted in:

- Cell free control. Only 100 µl of L.15
- Minimum leakage control. 100 µl of cells in L.15 1% FBS
- Maximum leakage (positive) control. 100 µl of cells in L.15 1% FBS with 1% of Triton X

3.6_ *Cell Proliferation*

Cells were plated in a sterile 96 well-tissue culture plate (1 X 10^5 cells for well) in 200 µl L.15 medium.

Before the exposure growth medium was removed and cells were washed twice with PBS.

To each well 200 µl of free serum L.15 were added, with different concentration of NPs and Cap Agent. Plates were lately incubated for 24 and 48 h. After the exposure 100 µl of medium were taken off from each well and 100 µl XTT labeling mixture were added and plates were incubates for 4 hours before the measuring of the absorbance at 450 - 500 nm by a Wallac 1420 VICTOR^{2}™ plate reader spectrophotometer. The XTT labeling mixture was obtained by mixing 5 ml of XTT labeling reagent + 0,1 ml electron coupling reagent, immediately before use.

4_ *Results and discussion*

Pilot experiments have been done with the purpose to test several doses of NPs to find the right ones to be used in this study. The main problem working with nanoparticles is their tendency to clamp each others. In order to reduce NPs aggregation, sonication and capping agents are usually employed; however these procedures are not able to completely prevent NPs aggregation.

To validate our experiments we used MMS (methyl methanesulphonate) as positive control for the Comet assay. MMS is a potent DNA alkylating agent able to induce DNA damage clearly visible with fluorescence microscope, after electrophoresis. Triton X 1% was used as positive control in the cytotoxicity test, being able to induce an 100% cellular mortality through the destruction of cell membranes.

Experimental results showed that Ag_2S and CdS NPs have a toxic effect on mussel haemocytes and RTG2 cell cultures.

Mussel haemocytes

Four hours exposure to all the compounds used resulted to be significantly effective at the top dose of 10 mg/L, while any evidence of a significant effect at the other doses was found (Figs. 5; 6; 7).

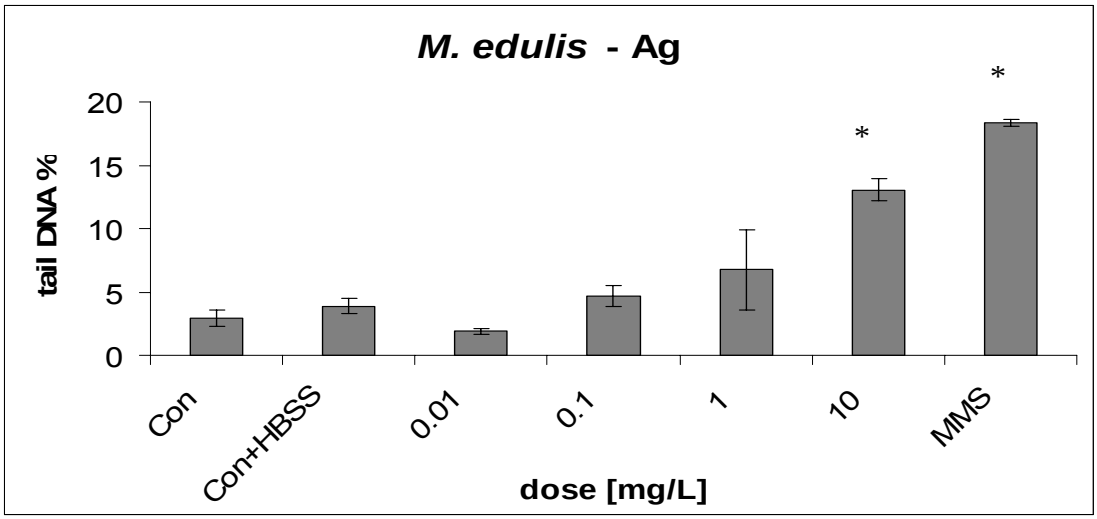


Fig. 5_ DNA migration after 4-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for *Mytilus* Comet Ag

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	18,27	< 0,0001
B: colt	5,39	0,0309

Multiple range test for *Mytilus* Ag

Dose	Mean	Homogeneous Groups
0,01	1,8875	X
Con	2,96	XX
Con+HBSS	3,8825	XX
0,1	4,66	XX
1	6,75	X
10	13,0475	X
MMS	18,3425	X

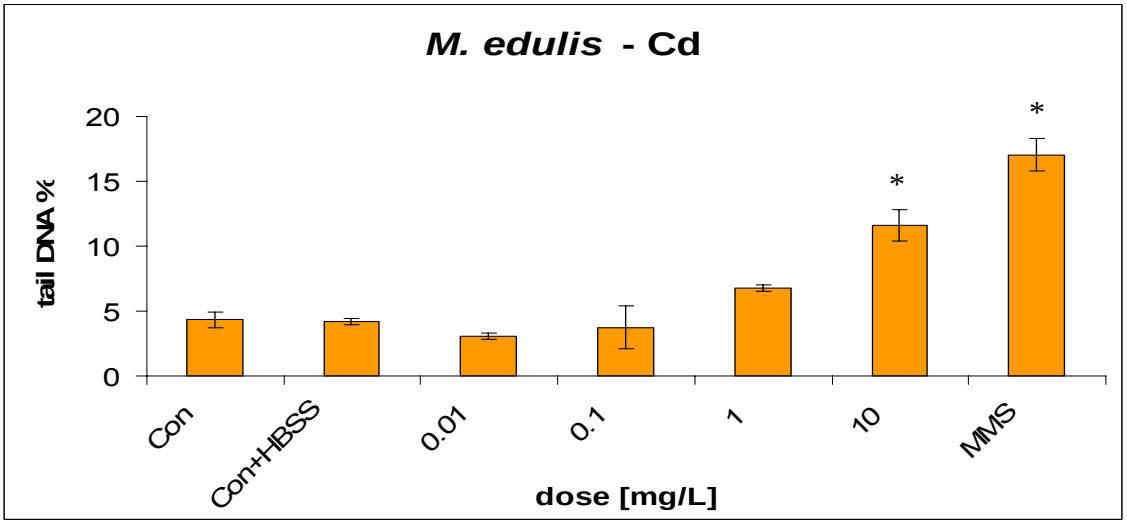


Fig. 6_ DNA migration after 4-hour exposure to CdS NPs and MMS as positive control. * indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for *Mytilus* Comet Cd

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	27,49	< 0,0001
B: colt	1,88	0,1855

Multiple range test for *Mytilus* Cd

Dose	Mean	Homogeneous Groups
0,01	3,0975	X
0,1	3,7325	X
Con+HBSS	4,2025	XX
Con	4,3425	XX
1	6,765	X
10	11,6025	X
MMS	17,0425	X

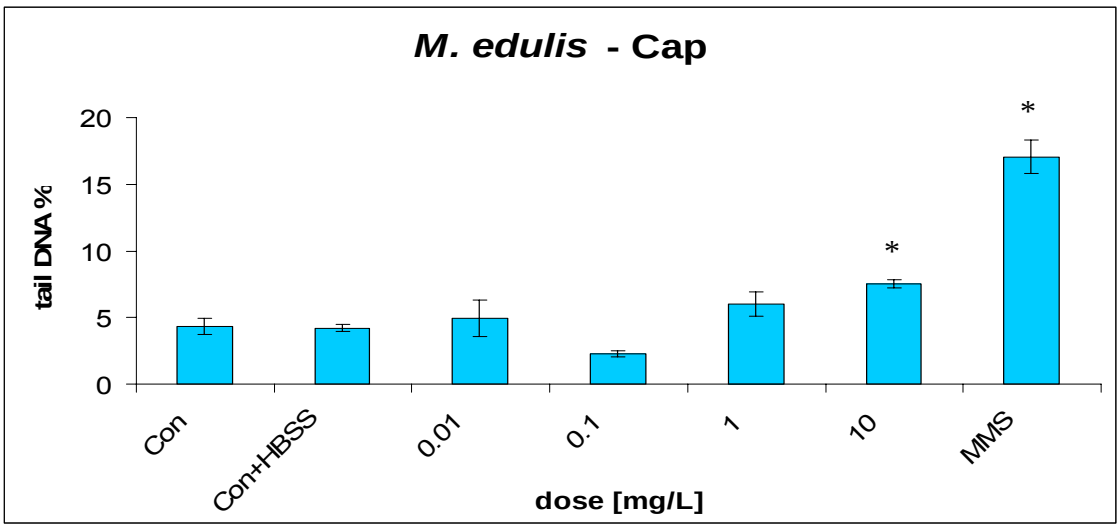


Fig. 7_ DNA migration after 4-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for *Mytilus* Comet Cap

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	27,12	< 0,0001
B: colt	0,10	0,7571

Multiple range test for *Mytilus* Cap

Dose	Mean	Homogeneous Groups
0,1	2,275	X
Con+HBSS	4,2025	XX
Con	4,3425	XX
0,01	4,94875	XXX
1	5,99	XX
10	7,505	X
MMS	17,0425	X

The lack of toxicity at low concentrations might be related to the short exposure period to the substances, likely not sufficient for NPs to induce a significant damage. Similar findings were reported by *Vevers et al. (2007*) after exposure of fish cell cultures to TiO_2, CdS and Ag_2S nanoparticles for 4 hours; no significant toxic effect was detected on DNA if not combined with UV radiation.

Unfortunately, it has not been possible to use an exposure period longer than four hours for the low capacity of mussel haemocytes to survive at once they were taken off from the animal.

Fish cell culture

Cell cultures are very profitable for experiments that need long exposure. After an exposure of 24 hours, Ag_2S nanoparticles induced a significant genotoxic effect only at the top dose of 50 mg/l in L.15 medium, while at the other concentrations they did not show any significant effects on DNA (Fig. 8).

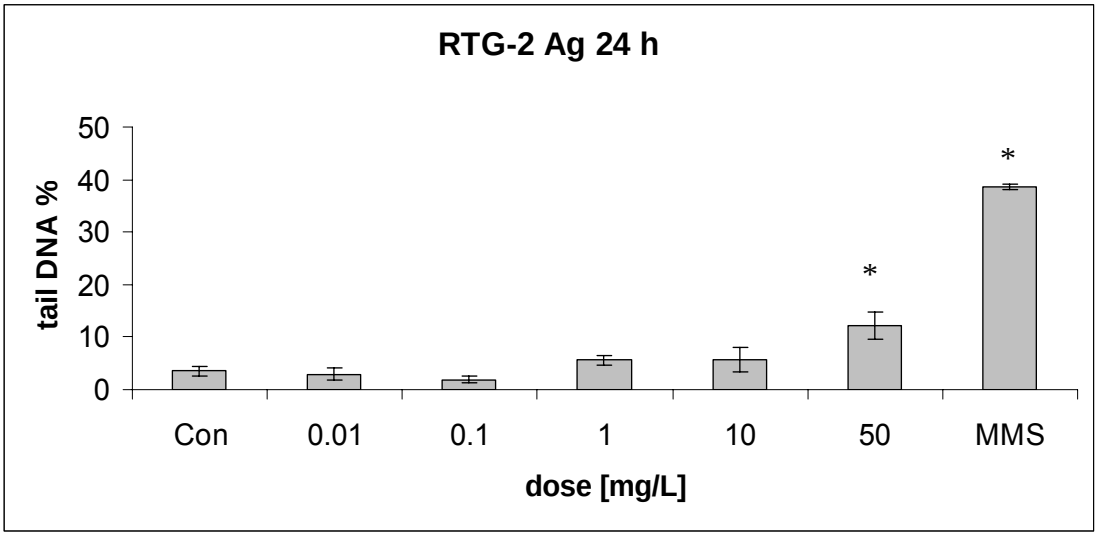


Fig. 8_ DNA migration after 24-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Ag 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	98,89	< 0,0001
B: colt	2,78	0,1110

Multiple range test for RTG-2 Comet Ag 24h

Dose	Mean	Homogeneous Groups
0,1	1,9325	X
0,01	2,9625	X
Con	3,58	X
1	5,615	X
10	5,7225	X
50	12,06	X
MMS	38,635	X

We found the same feature after 48 hours exposure with a significant DNA damage only in cell culture treated with 50 mg/L (Fig. 9).

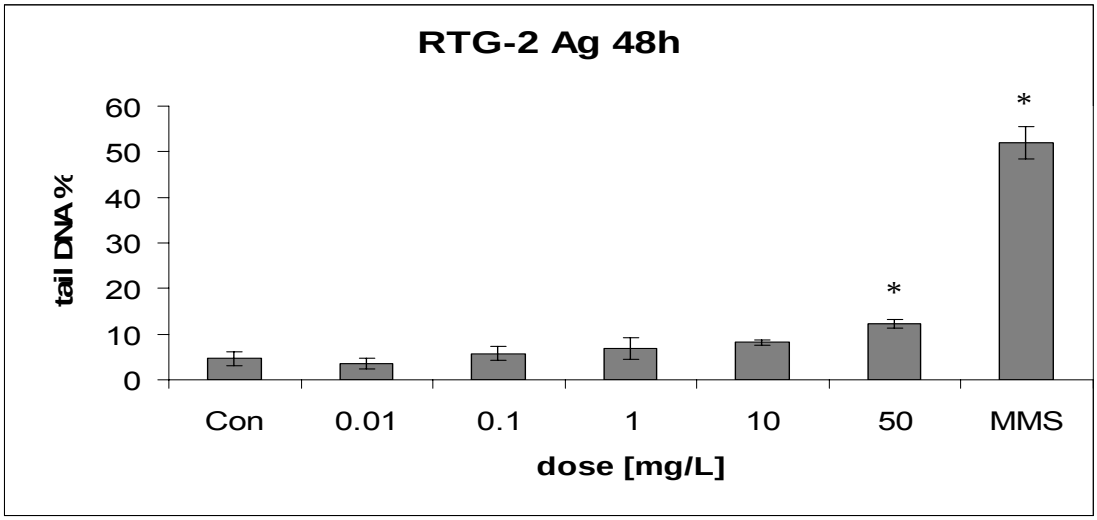


Fig. 9_ DNA migration after 48-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Ag 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	199,31	< 0,0001
B: colt	0,30	0,5880

Multiple range test for RTG-2 Comet Ag 48h

Dose	Mean	Homogeneous Groups
0,01	3,5275	X
Con	4,6675	XX
0,1	5,78	XX
1	6,8575	XX
10	8,18	X
50	12,2775	X
MMS	52,03	X

Instead, using CdS nanoparticles, we have registered a significant genotoxicity at all the concentrations tested after 24 hours exposure, with the highest effect observed at 1 mg/L. After 48 hours of exposure only the 10 mg/L dose produced a statistically significant effect on DNA migration, as shown in Figs. 10/11.

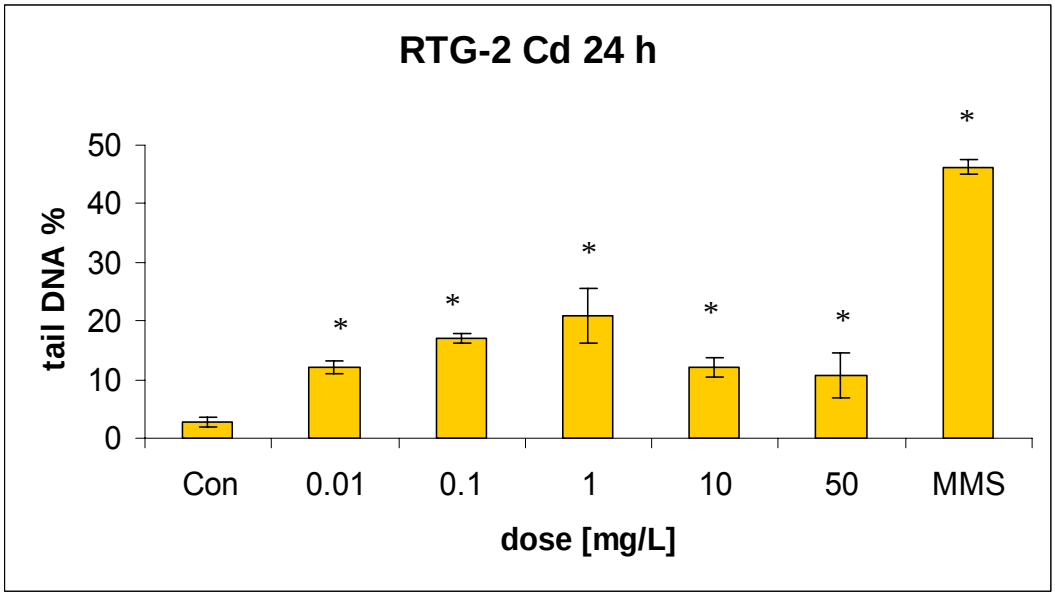


Fig. 10_ DNA migration after 24-hour exposure to CdS NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cd 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	49,51	< 0,0001
B: colt	O,02	0,8926

Multiple range test for RTG-2 Comet Cd 24h

Dose	Mean	Homogeneous Groups
Con	2,82	X
50	10,6975	X
0,01	12,12	XX
10	12,165	XX
0,1	16,9925	XX
1	20,9125	X
MMS	46,2875	X

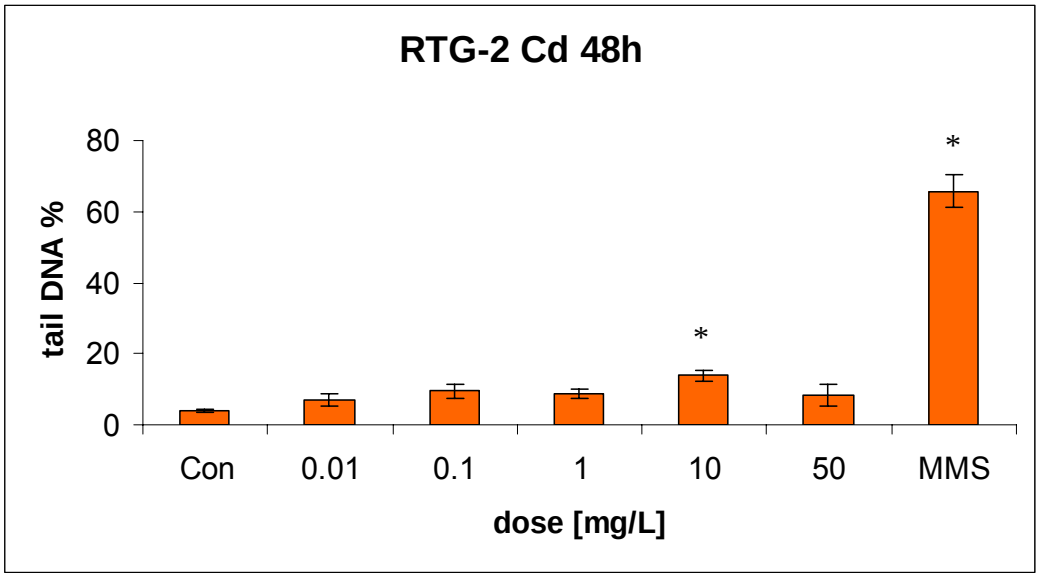


Fig. 11_ DNA migration after 48-hour exposure to CdS NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cd 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	122,46	< 0,0001
B: colt	0,46	0,5072

Multiple range test for RTG-2 Comet Cd 48h

Dose	Mean	Homogeneous Groups
Con	3,945	X
0,01	6,9575	X
50	8,2475	XX
1	8,8275	XX
0,1	9,445	XX
10	13,8275	X
MMS	65,775	X

The dose-effect relationship displayed by CdS NPs after 24 h exposure suggests a higher bioavailability of nanoparticles in the 0.01 – 1 mg/l range of doses, in comparison with the highest concentrations (10 and 50 mg/l). The lowering of the observed effects at higher concentrations is likely related to the clumping of NPs, which prevent penetration through cell membranes. Such a phenomenon (NPs aggregation) was well visible under the optic

microscope in sample exposed to 50 mg/L. After 48 hours exposure 10 mg/L was the only dose inducing a significant DNA damage on RTG-2 cells. DNA repair events can be responsible for leading back the DNA damage induced by 0.01-1 mg/l CdS NPs to control levels. Differently to what we have seen with mussels, capping agent did not induce any significant effect on RTG-2 DNA both after 24 hours and 48 hours exposure (Fig. 12; 13).

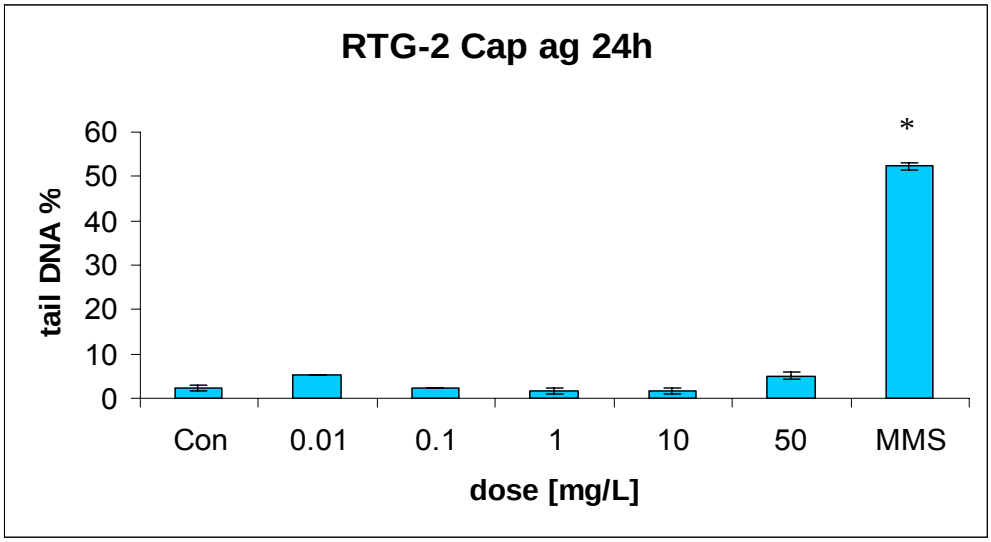


Fig. 12_ DNA migration after 24-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	78,19	< 0,0001
B: colt	0,77	0,3914

Multiple range test for RTG-2 Comet Cap 24h

Dose	Mean	Homogeneous Groups
10	1,5975	X
1	1,65	X
Con	2,355	X
0,1	2,36	X
50	5,095	X
0,01	5,2725	X
MMS	52,3	X

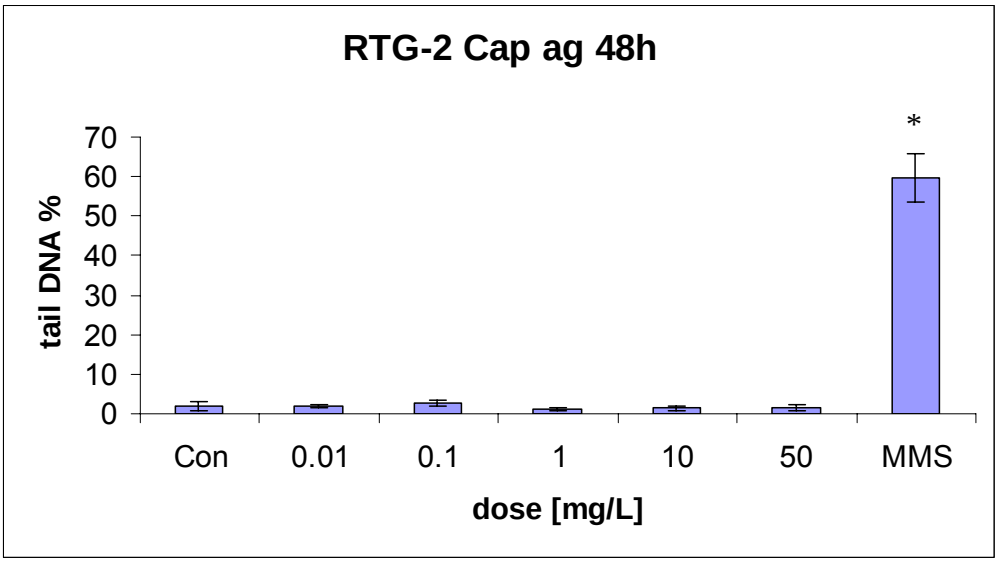


Fig. 13_ DNA migration after 48-hour exposure to Capping Agent (thiol-terminated methyl polyethylene glycol) and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	50,77	< 0,0001
B: colt	2,29	0,1461

Multiple range test for RTG-2 Comet Cap 48h

Dose	Mean	Homogeneous Groups
1	1,1675	X
10	1,4425	X
50	1,565	X
0,01	1,92	X
Con	1,9975	X
0,1	2,7775	X
MMS	59,72	X

Results obtained from cytotoxicity test showed that the exposure of trout cells to Ag_2S NPs induced a significant toxicity only at the top dose of 50 mg/L after 24 hours (Fig. 14).

After an exposure of 48 hours, we also registered a significant toxic effect on cells at 10 mg/L and 1 mg/L doses (Fig. 15). Anyway, Ag_2S NPs caused only a slight decrease in cell viability, never reaching percentage of cytotoxicity close to 100% (Fig. 16; 17).

Cadmium nanoparticles resulted to be more toxic after 24 hours exposure in comparison with what we have seen with Ag_2S (Fig. 18) displaying significant effects at all the doses investigated. Instead, after 48 hours exposure, only three doses: 1, 10 and 50 mg/l exhibited significant cytotoxicity. Cadmium NPs induced a 100% cytotoxicity at 10 mg/L and 50 mg/L, with levels of LDH higher than Triton X 1%, used as positive control.

In spite of the lack of genotoxicity associated with the capping agent in RTG2 cell line, a remarkable effect on cytotoxicity was observed. As shown in Fig. 22 - 25 the Capping agent was cytotoxic at 1 mg/L, 10 mg/L and 50 mg/L after 24 and 48 hours exposure. In both, experiments the peak of LDH leakage was observed at 10 and 50 mg/L doses, corresoponding to a cell mortality of about 100%.

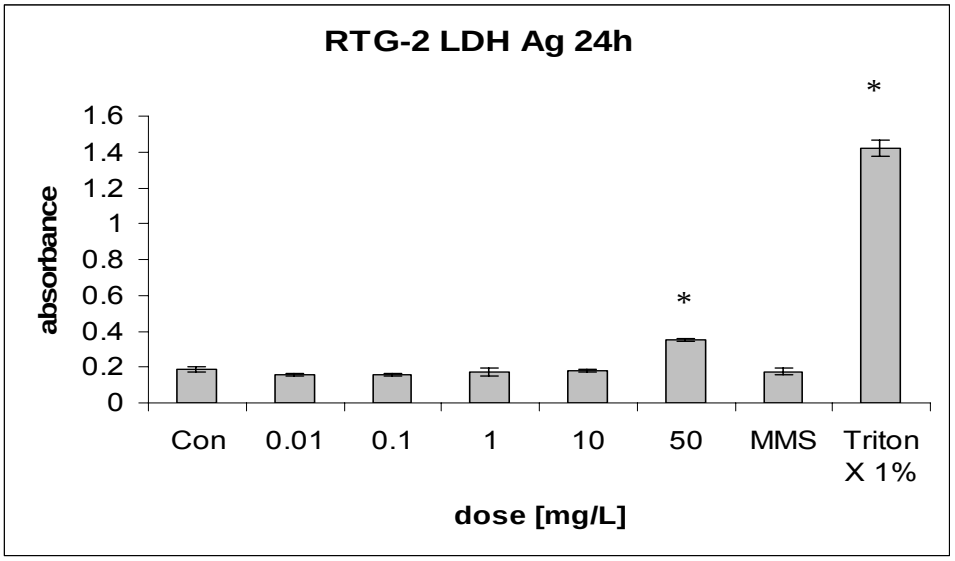


Fig.14_ LDH levels after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Ag 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	568,03	< 0,0001
B: colt	1,03	0,321

Multiple range test for RTG-2 LDH Ag 24h

Dose	Mean	Homogeneous Groups
0,1	0,16	X
0,01	0,16	X
1	0,1725	X
MMS	0,175	X
10	0,18	X
Con	0,185	X
50	0,35	X
Triton X 1%	1,42	X

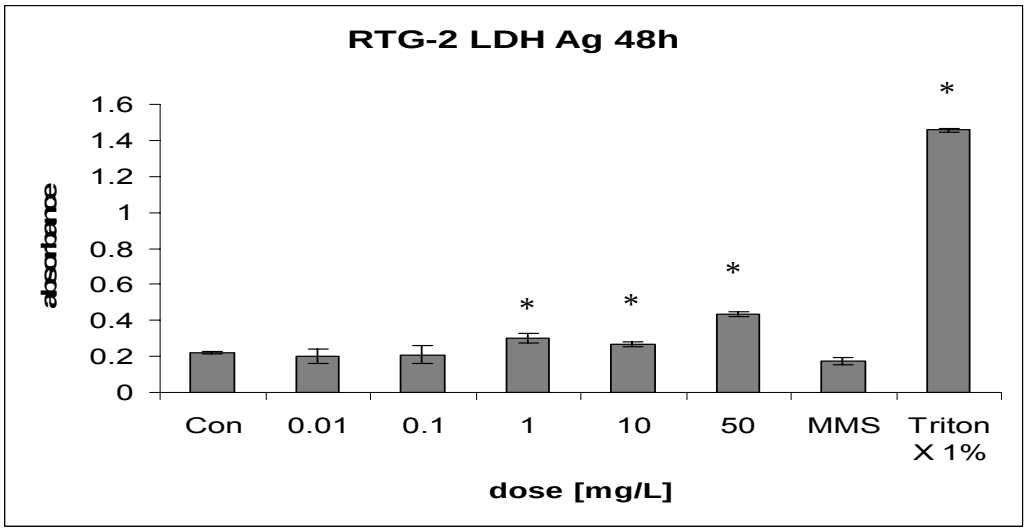


Fig. 15_ LDH levels after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Ag 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	878,68	< 0,0001
B: colt	1,06	0,313

Multiple range test for RTG-2 LDH Ag 48h

Dose	Mean	Homogeneous Groups
MMS	0,175	X
0,01	0,2	XX
0,1	0,21	XX
Con	0,22	X
10	0,2675	X
1	0,3025	X
50	0,435	X
Triton X 1%	1,4575	X

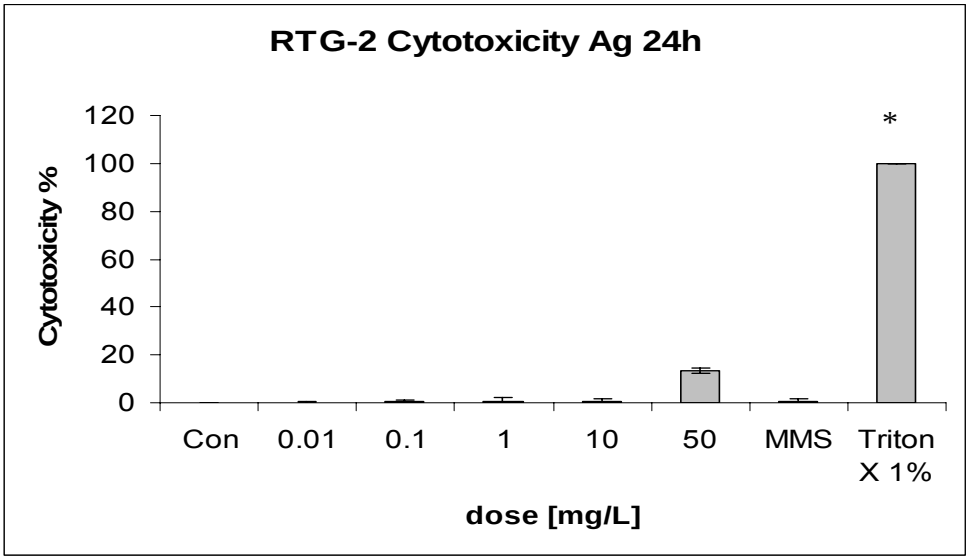


Fig. 16_ Cytotoxicity (%) after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

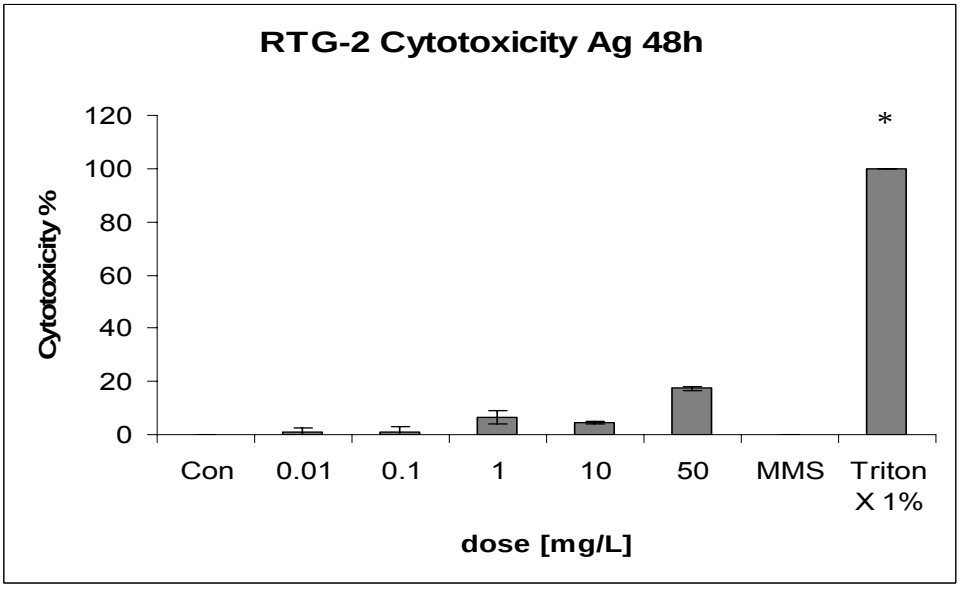


Fig. 17_ Cytotoxicity (%) after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

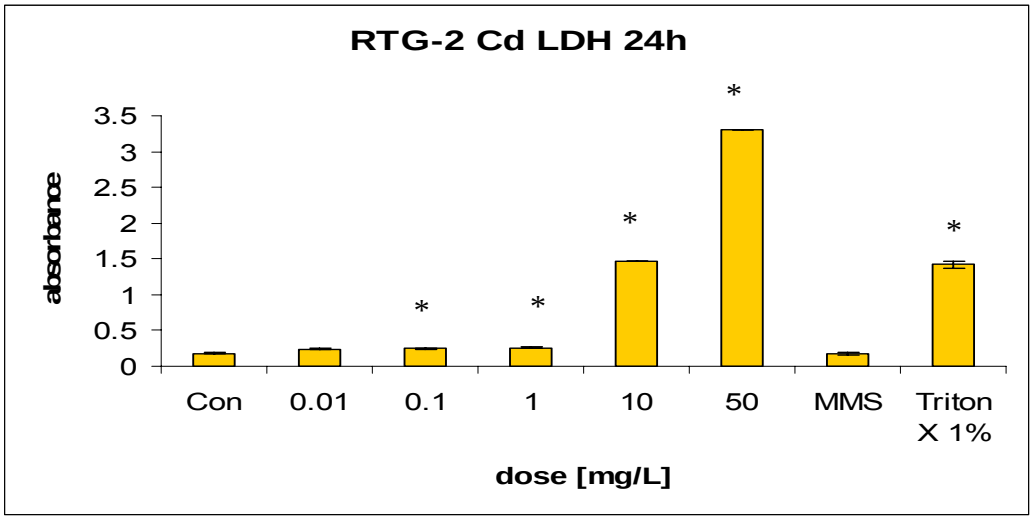


Fig. 18_ LDH levels after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cd 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	2962,81	< 0,0001
B: colt	1,47	0,238

Multiple range test for RTG-2 LDH Cd 24h

Dose	Mean	Homogeneous Groups
MMS	0,175	X
Con	0,185	XX
0,01	0,24	XX
0,1	0,2475	X
1	0,2625	X
Triton X 1%	1,42	X
10	1,4675	X
50	3,3025	X

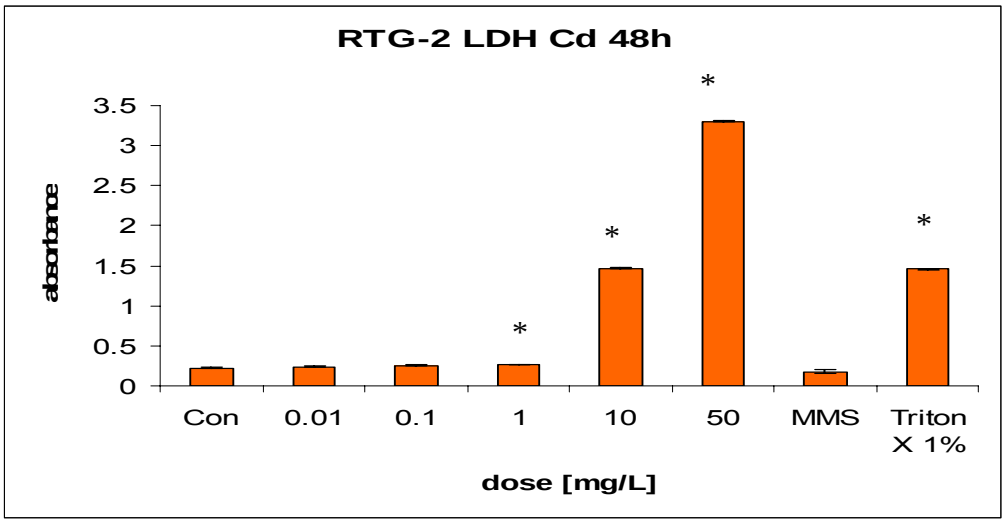


Fig. 19_ LDH levels after 48-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cd 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	5953,88	< 0,0001
B: colt	0,02	0,903

Multiple range test for RTG-2 LDH Cd 48h

Dose	Mean	Homogeneous Groups
MMS	0,175	X
Con	0,22	X
0,01	0,24	XX
0,1	0,255	XX
1	0,2625	X
Triton X 1%	1,4575	X
10	1,4675	X
50	3,3025	X

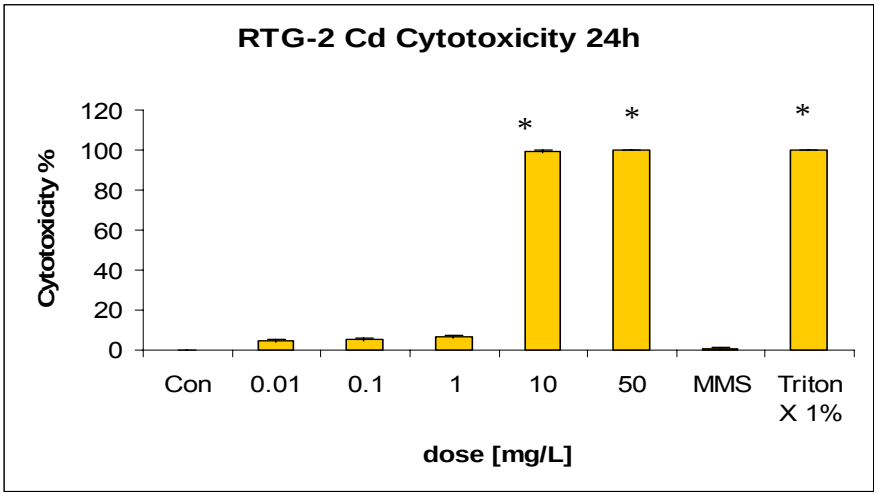


Fig. 20_ Cytotoxicity (%) after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

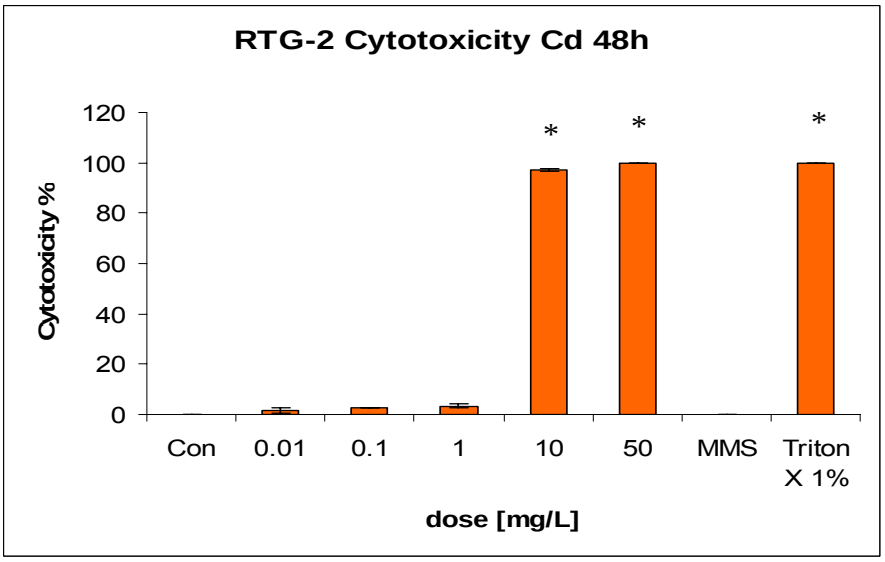


Fig. 21_ Cytotoxicity (%) after 48-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

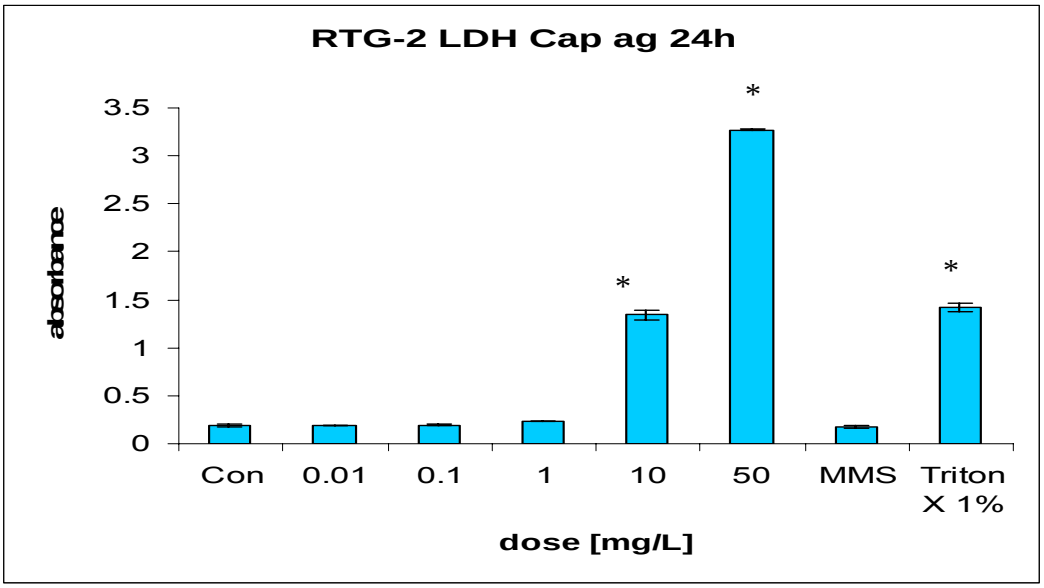


Fig. 22_ LDH levels after 24-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	2376,35	< 0,0001
B: colt	1,65	0,211

Multiple range test for RTG-2 LDH Cap 24h

Dose	Mean	Homogeneous Groups
MMS	0,175	X
Con	0,185	X
0,01	0,1875	X
0,1	0,195	X
1	0,235	X
10	1,3425	X
Triton X 1%	1,42	X
50	3,2725	X

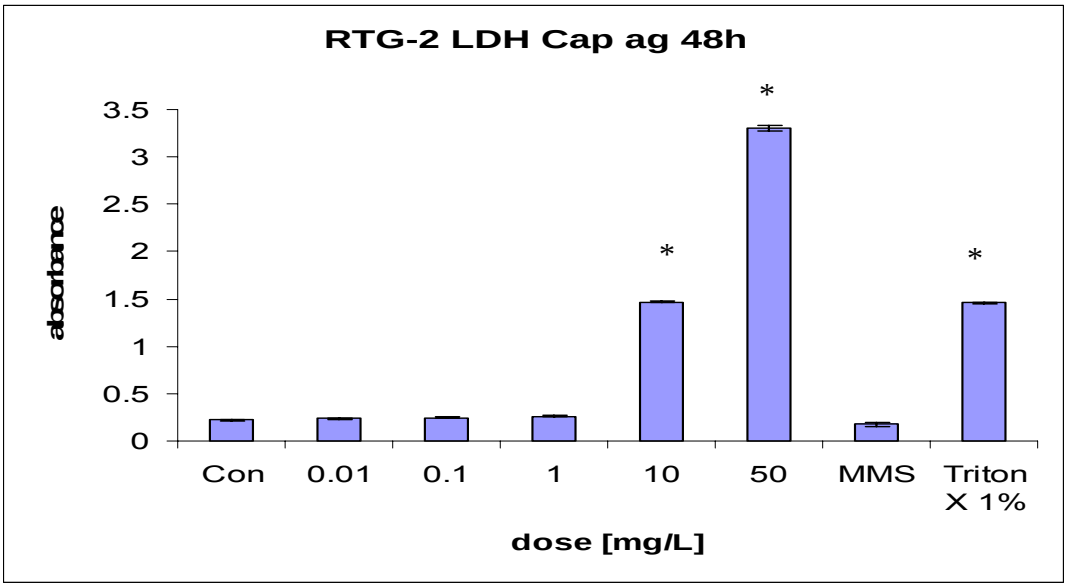


Fig. 23_ LDH levels using a 48-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	6070,41	< 0,0001
B: colt	0,55	0,465

Multiple range test for RTG-2 LDH Cap 24h

Dose	Mean	Homogeneous Groups
MMS	0,175	X
Con	0,22	X
0,01	0,24	X
0,1	0,2475	X
1	0,26	X
Triton X 1%	1,4575	X
10	1,4675	X
50	3,3025	X

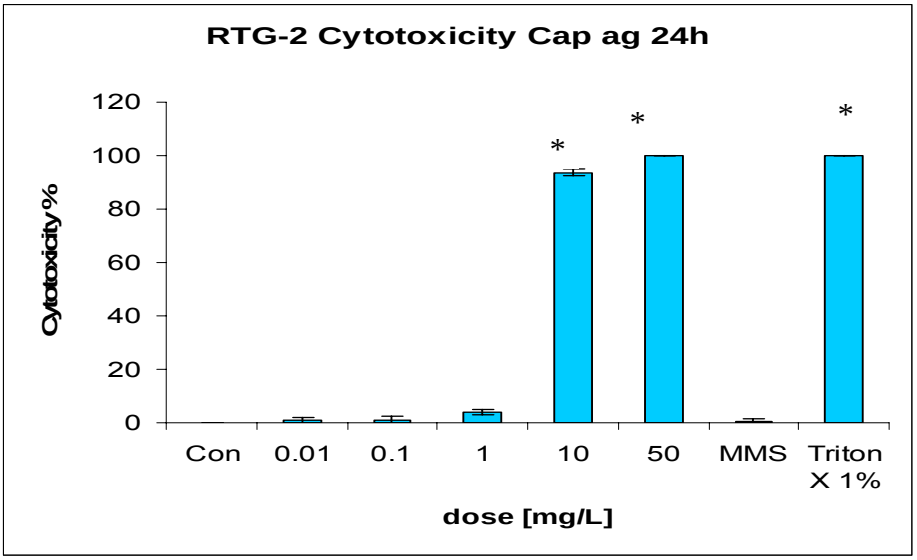


Fig. 24_ Cytotoxicity (%) induced using a 24-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

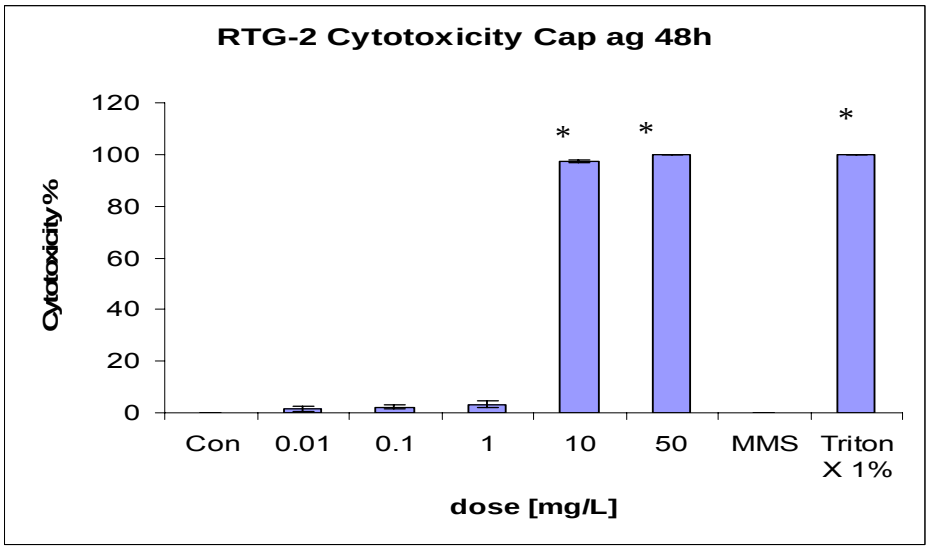


Fig. 25_ Cytotoxicity (%) after 48-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

Cell proliferative effect of NPs was also investigated.

A statistically significant positive effect on cell population was observed at at 0.01 mg/L, 10 mg/L and 50 mg/L of silver nanoparticles after 24 hours exposure and at the highest one after 48 hours. Cadmium NPs induced significant increases of cell proliferation at all the concentrations used after 48 hours, no significant responses were registered after 24 hours. Capping agent induced cell proliferation at 10 mg/L and 50 mg/L after 24 hours and at the three highest doses after 48 hours.

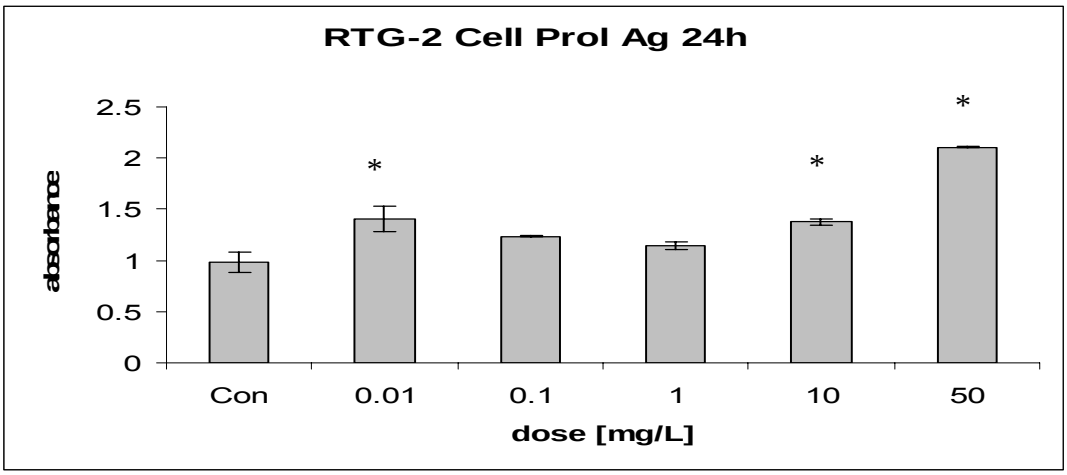


Fig. 26_ Cell proliferation after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control. * indicates a statistically significant decrease respect to control ($p< 0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Ag 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	18,57	< 0,0001
B: colt	0,00	0,955

Multiple range test for RTG-2 Cell prol Ag 24h

Dose	Mean	Homogeneous Groups
Con	0,98	X
1	1,145	XX
0,1	1,235	XX
10	1,375	X
0,01	1,405	X
50	2,1075	X

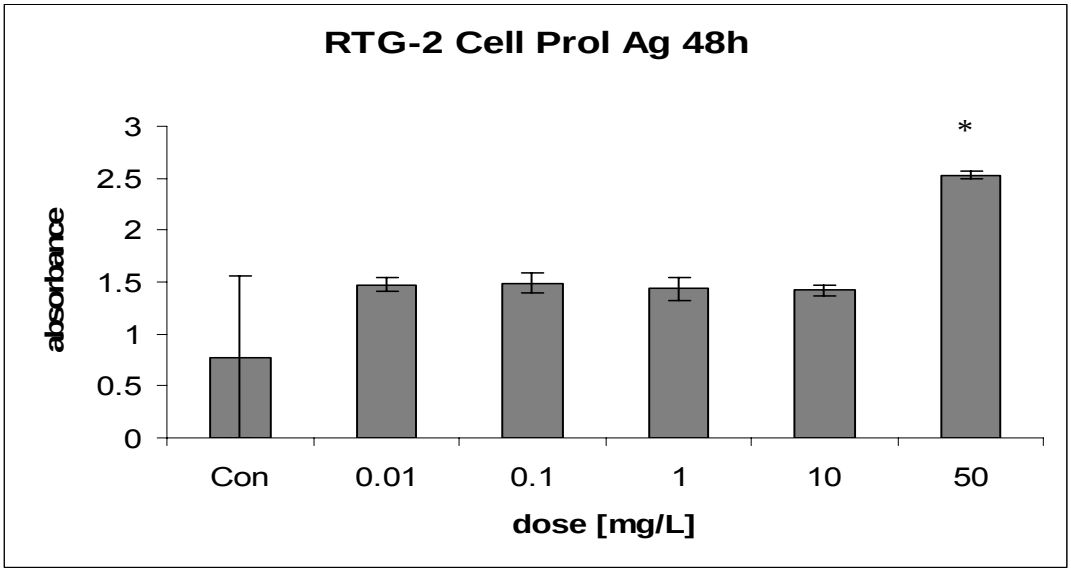


Fig. 27_ Cell proliferation after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control. * indicates a statistically significant decrease respect to control ($p< 0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Ag 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	23,02	< 0,0001
B: colt	1,50	0,238

Multiple range test for RTG-2 Cell prol Ag 48h

Dose	Mean	Homogeneous Groups
Con	1,33	X
10	1,42	X
1	1,435	X
0,01	1,475	X
0,1	1,4925	X
50	2,53	X

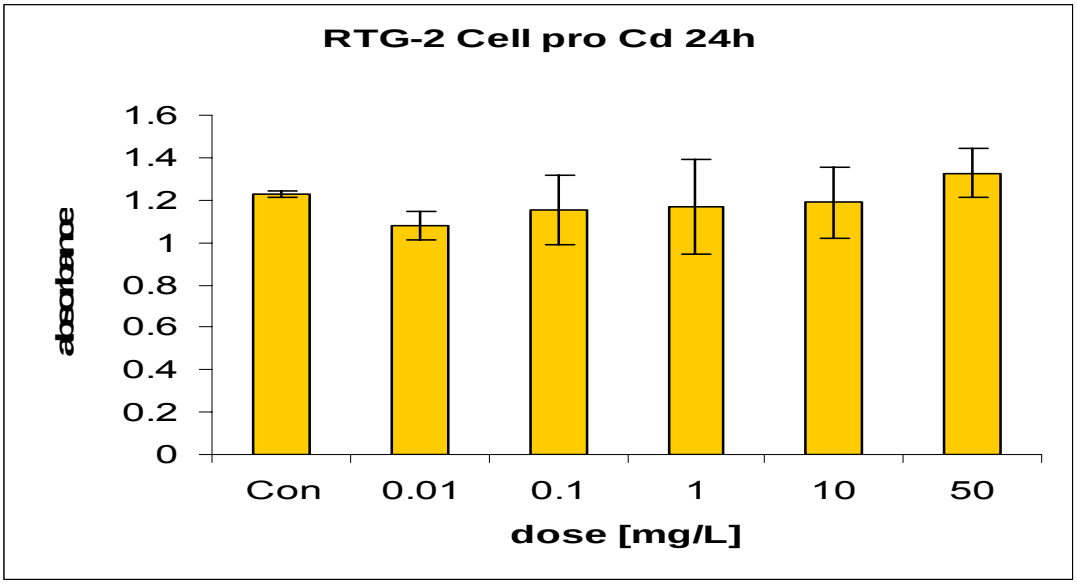


Fig. 28_ Cell proliferation after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p < 0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cd 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	1,50	0,242
B: colt	1,20	0,289

Multiple range test for RTG-2 Cell prol Cd 24h

Dose	Mean	Homogeneous Groups
0,01	1,0825	X
0,1	1,155	XX
1	1,1675	XX
10	1,1875	XX
Con	1,23	XX
50	1,3275	X

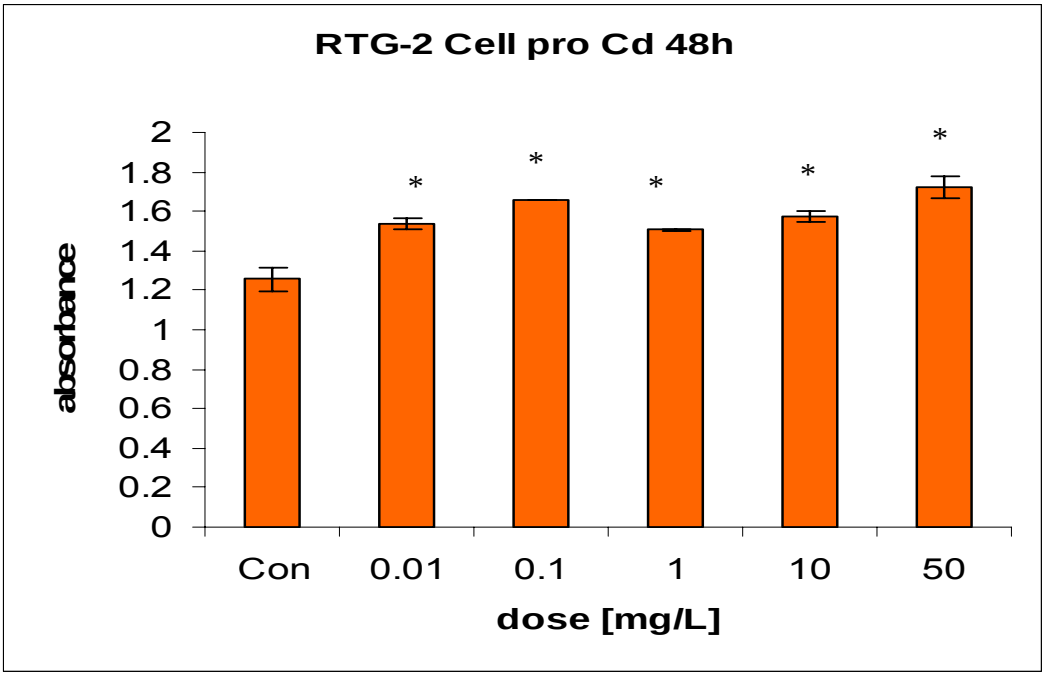


Fig. 29_ Cell proliferation after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p< 0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cd 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	15,36	< 0,0001
B: colt	0,20	0,660

Multiple range test for RTG-2 Cell prol Cd 48h

Dose	Mean	Homogeneous Groups
Con	1,255	X
1	1,505	X
0,01	1,535	X
10	1,5725	XX
0,1	1,6575	XX
50	1,72	X

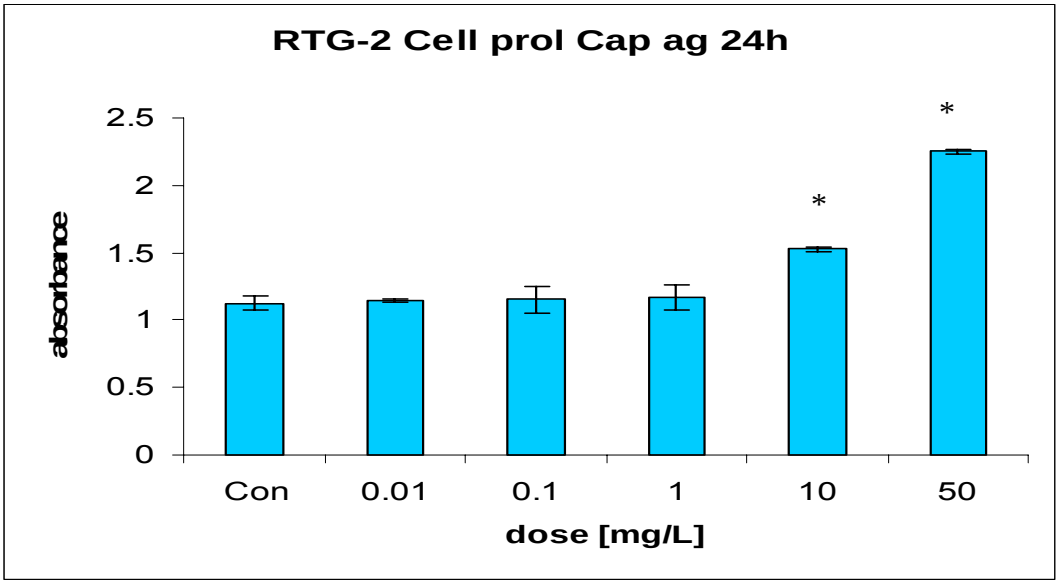


Fig. 30_ Cell proliferation after 24-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	60,31	< 0,0001
B: colt	0,82	0,378

Multiple range test for RTG-2 Cell prol Cap 24h

Dose	Mean	Homogeneous Groups
Con	1,125	X
0,01	1,145	X
0,1	1,1525	X
1	1,1675	X
10	1,525	X
50	2,2525	X

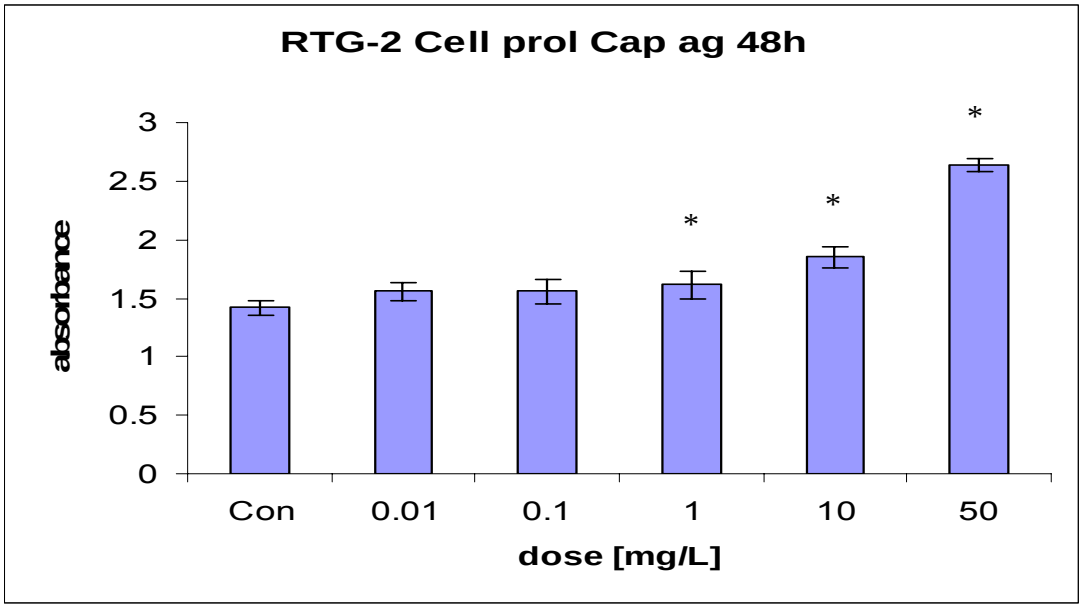


Fig. 31_ Cell proliferation after 48-hour exposure to Capping agent (thiol-terminated methyl polyethylene glycol) and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	59,92	<0,0001
B: colt	1,95	0,180

Multiple range test for RTG-2 Cell prol Cap 48h

Dose	Mean	Homogeneous Groups
Con	1,4175	X
0,1	1,5575	XX
0,01	1,5575	XX
1	1,6125	X
10	1,85	X
50	2,6375	X

Looking at the genotoxic (in mussels) and cytotoxic properties of Capping agent it seemed proper to subtract genotoxicity and cytotoxicity values of Capping agent from ones induced by NPs. After the subtraction both silver and cadmium nanoparticles lost their genotoxic effects in mussels at all the concentrations used (see Fig. 32; 33).

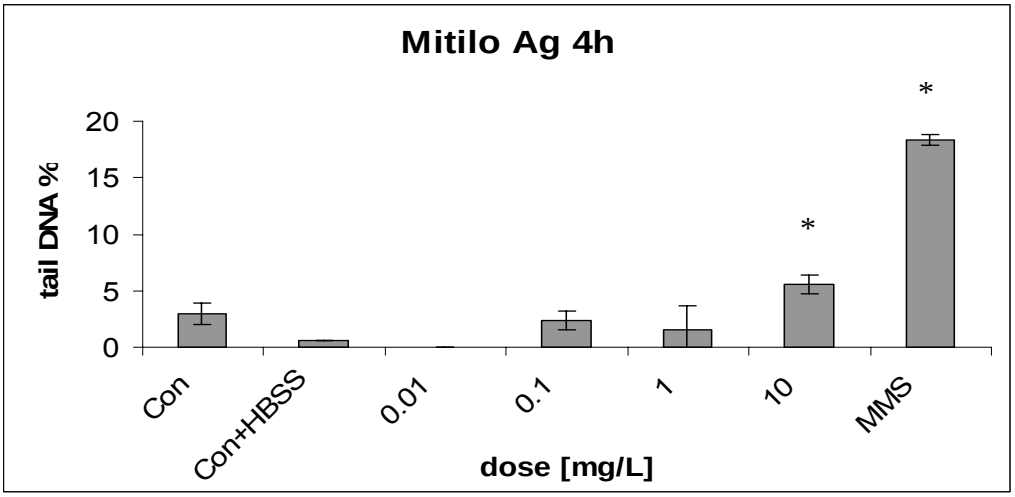


Fig. 32_ DNA migration after 4-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for *Mytilus* Comet Ag - Cap

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	21,34	< 0,0001
B: colt	4,47	0,0472

Multiple range test for *Mytilus* Ag -Cap

Dose	Mean	Homogeneous Groups
0,01	0,0	X
Con+HBSS	0,5725	X
1	1,505	XX
0,1	2,385	XX
Con	2,96	XX
10	5,5425	X
MMS	18,3425	X

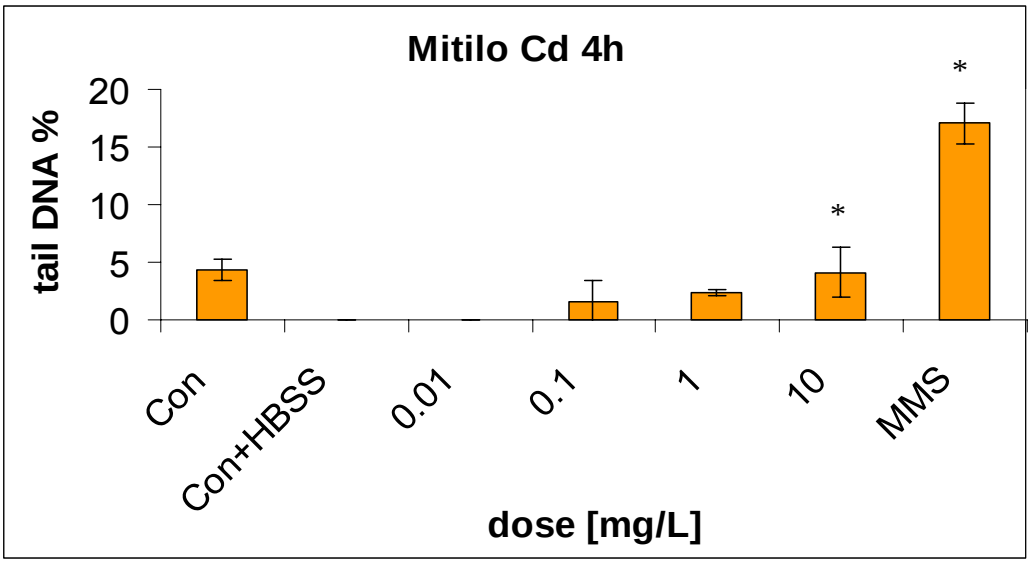


Fig. 33_ DNA migration after 4-hour exposure to CdS NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for *Mytilus* Comet Cd - Cap

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	39,34	< 0,0001
B: colt	1,10	0,3075

Multiple range test for *Mytilus* Cd -Cap

Dose	Mean	Homogeneous Groups
Con+HBSS	0,0	X
0,01	0,0	X
0,1	1,545	XX
1	2,35	XXX
10	4,0975	XX
Con	4,3425	X
MMS	17,0425	X

Comet data from RTG-2 showed some significant changes; genotoxic effects induced by silver nanoparticles on trout DNA were erased after 24 hours at any concentration, while only 50 mg/L was found to be effective after 48 hours (Fig. 34; 35).

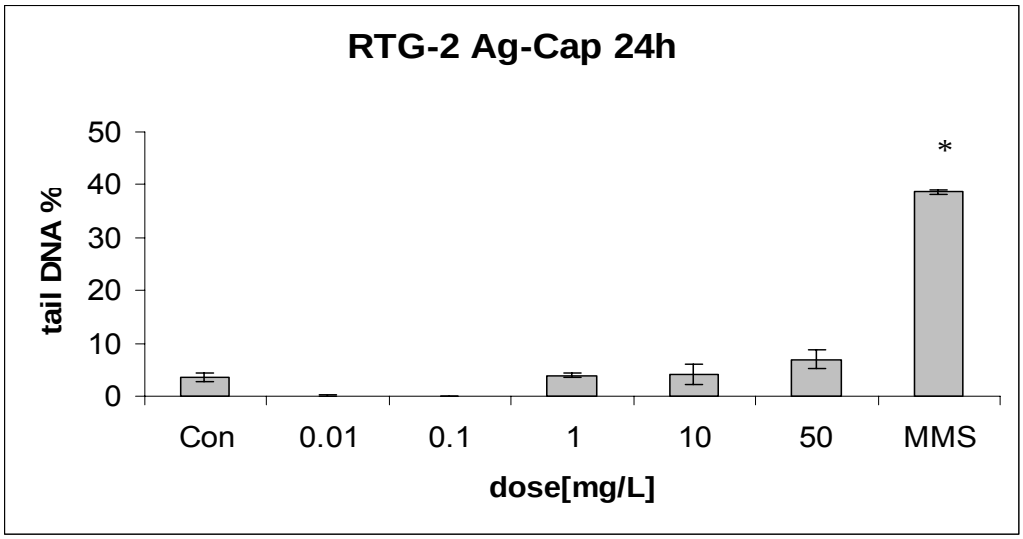


Fig. 34_ DNA migration after 24-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Ag-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	103,43	< 0,0001
B: colt	0,97	0,3354

Multiple range test for RTG-2 Comet Ag-Cap 24h

Dose	Mean	Homogeneous Groups
0,1	0,0	X
0,01	0,095	XX
Con	3,58	XXX
1	3,965	XX
10	4,125	X
50	6,965	X
MMS	38,635	X

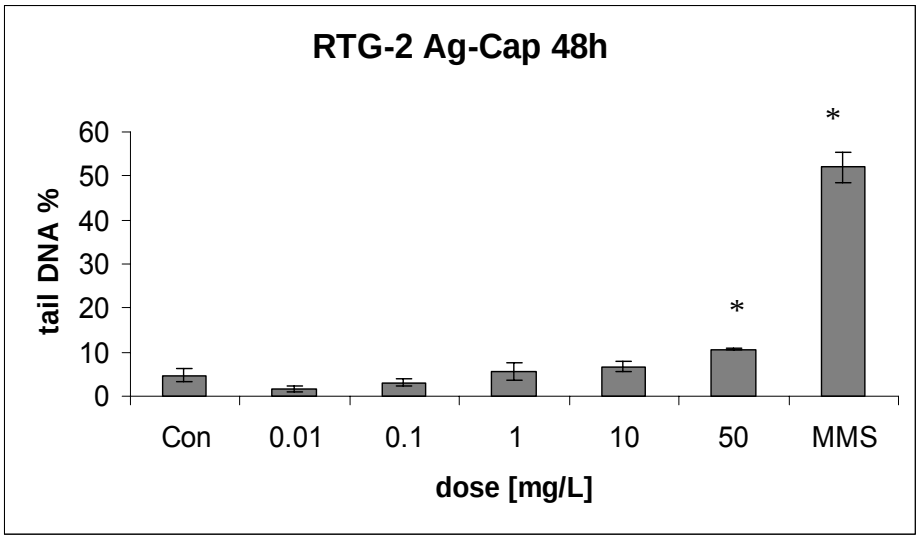


Fig. 35_ DNA migration after 48-hour exposure to Ag_2S NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Ag-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	209,53	< 0,0001
B: colt	0,16	0,6925

Multiple range test for RTG-2 Comet Ag-Cap 48h

Dose	Mean	Homogeneous Groups
0,01	1,6075	X
0,1	3,0025	XX
Con	4,6675	XXX
1	5,69	XX
10	6,7375	X
50	10,7125	X
MMS	52,0375	X

On the contrary, cadmium nanoparticles hold their significant toxicity at the majority of the concentrations (0,1 mg/L, 1 mg/l and 10 mg/L) after 24 hours of exposure, while they maintained their genotoxicity at 10 mg/L after 48 hours.

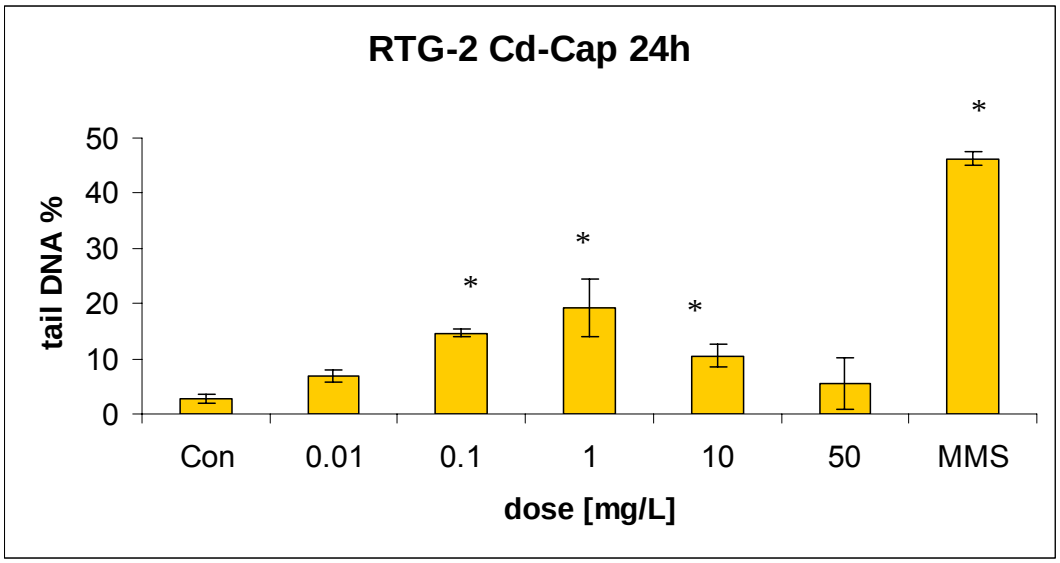


Fig. 36_ DNA migration after 24-hour exposure to CdS NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cd-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	44,48	< 0,0001
B: colt	0,81	0,3797

Multiple range test for RTG-2 Comet Cd-Cap 24h

Dose	Mean	Homogeneous Groups
Con	2,82	X
50	5,6025	XX
0,01	6,8475	XX
10	10,5675	XX
0,1	14,6325	XX
1	19,2625	X
MMS	46,2875	X

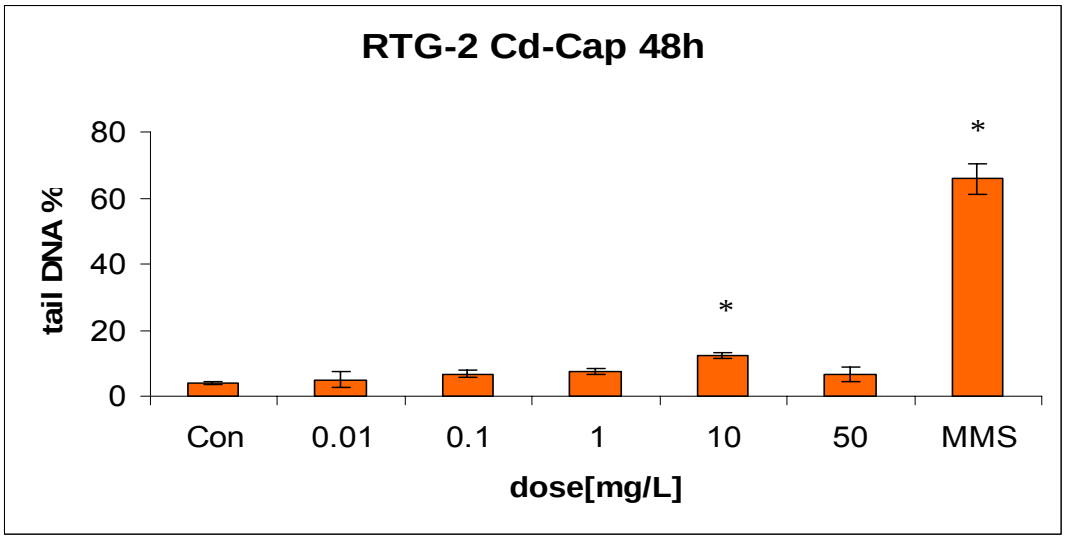


Fig. 37_ DNA migration after 48-hour exposure to CdS NPs and MMS as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. 100 cells scored per replicate treatment (N = 4). Mean percentage of tail DNA is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Comet Cd-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	137,04	< 0,0001
B: colt	0,32	0,5761

Multiple range test for RTG-2 Comet Cd-Cap 48h

Dose	Mean	Homogeneous Groups
Con	3,945	X
0,01	5,0375	X
0,1	6,6675	X
50	6,6825	X
1	7,66	XX
10	12,385	X
MMS	65,595	X

As we can see in the Figs above the dose-effect relationship induced by cadmium nanoparticles after 24 hours exposure was confirmed, as well as the DNA repair events observed at 48 hours exposure.

In the LDH test significant changes are observable after subtracting Capping agent values: no significant effect induced by silver and

cadmium nanoparticles were obtained after both 24 hours and after 48 hours exposure.

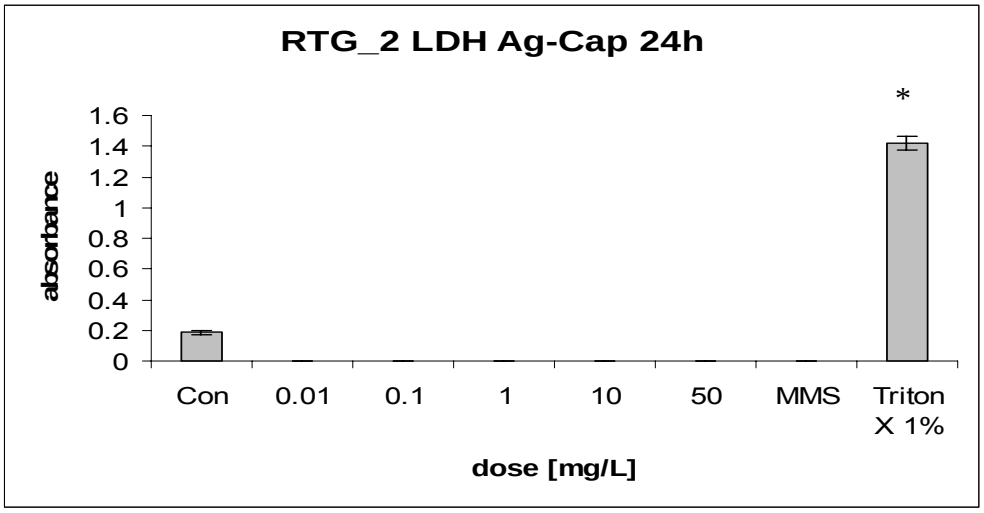


Fig. 38_ LDH levels after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control. * indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Ag-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	826,52	< 0,0001
B: colt	0,85	0,3669

Multiple range test for RTG-2 LDH Ag-Cap 24h

Dose	Mean	Homogeneous Groups
10	0,0	X
0,01	0,0	X
0,1	0,0	X
MMS	0,0	X
50	0,0	X
1	0,0	X
Con	0,185	X
Trion X 1%	1,42	X

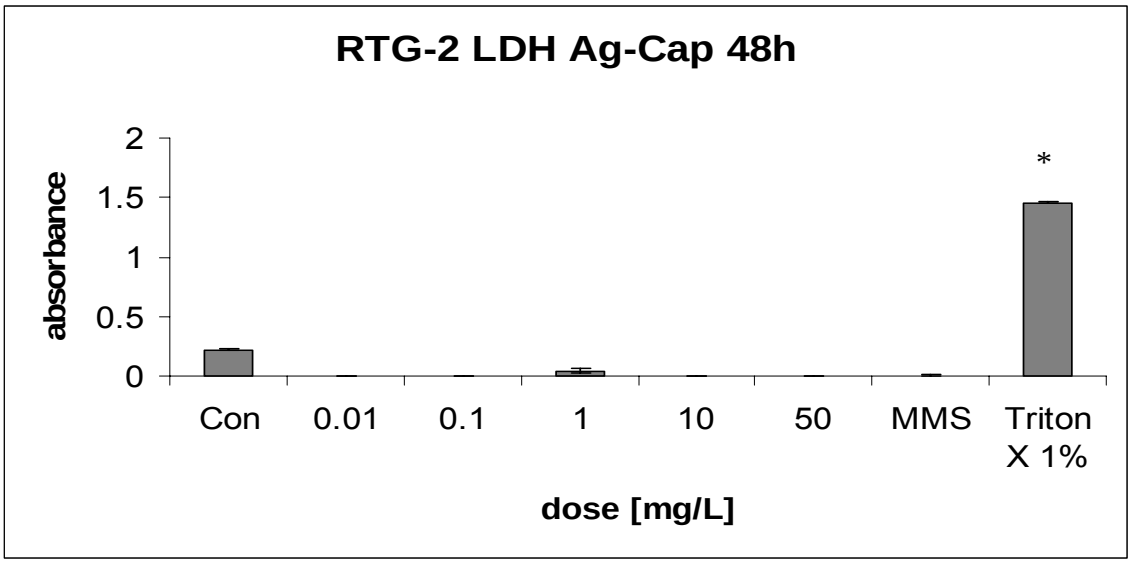


Fig. 39_ LDH levels after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Ag-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	2984,71	< 0,0001
B: colt	0,91	0,3512

Multiple range test for RTG-2 LDH Ag-Cap 48h

Dose	Mean	Homogeneous Groups
10	0,0	X
0,1	0,0	X
0,01	0,0	X
50	0,0	X
MMS	0,005	X
1	0,0425	X
Con	0,22	X
Triton X 1%	1,4575	X

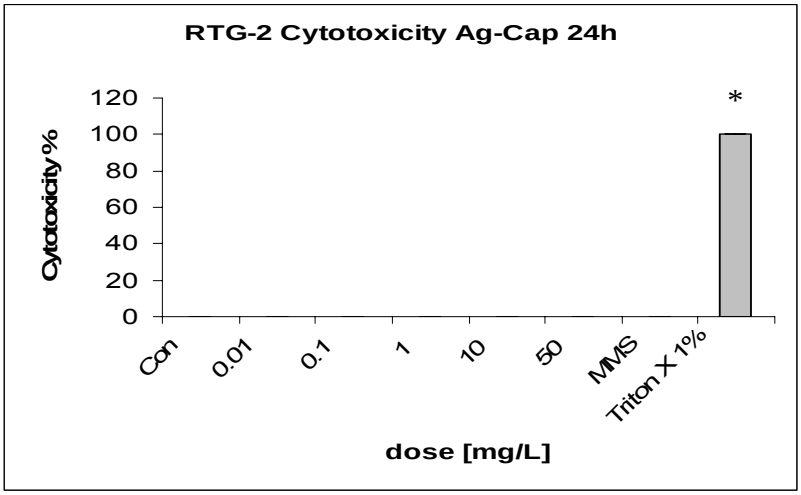


Fig. 40_ Cytotoxicity (%) induced after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

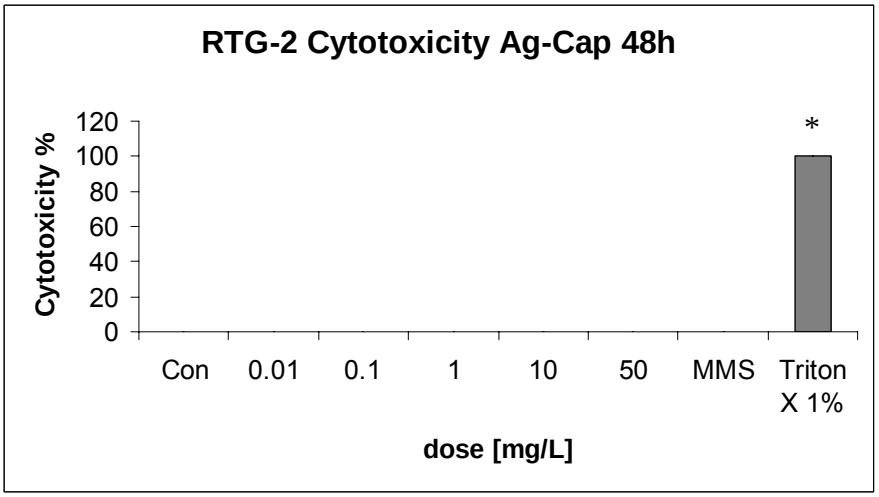


Fig. 41_ Cytotoxicity % after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

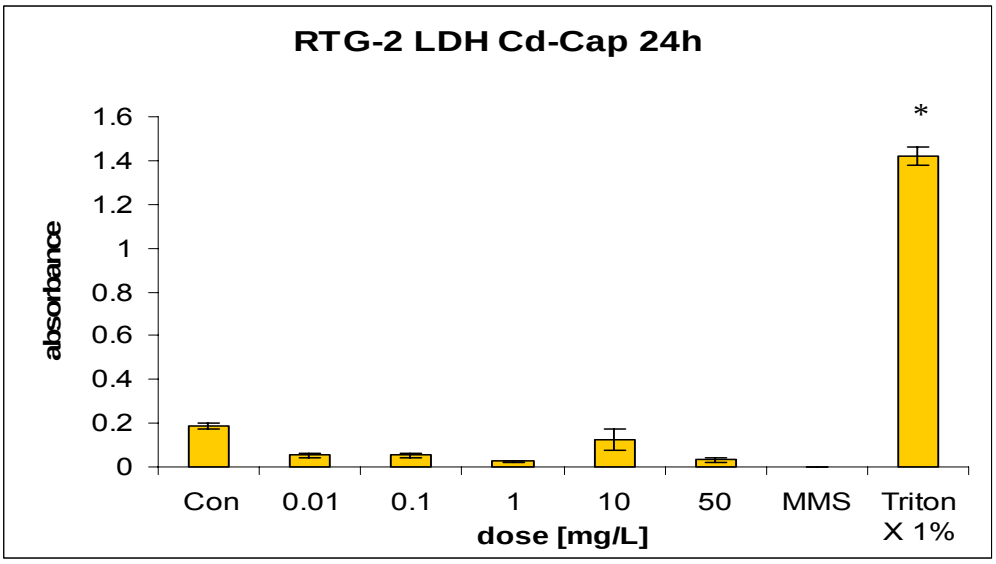


Fig. 42_ LDH levels after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cd-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	408,38	< 0,0001
B: colt	0,27	0,6087

Multiple range test for RTG-2 LDH Cd-Cap 24h

Dose	Mean	Homogeneous Groups
MMS	0,0	X
1	0,0275	X
50	0,0325	X
0,1	0,0525	X
0,01	0,0526	X
10	0,125	X
Con	0,185	X
Triton X 1%	1,42	X

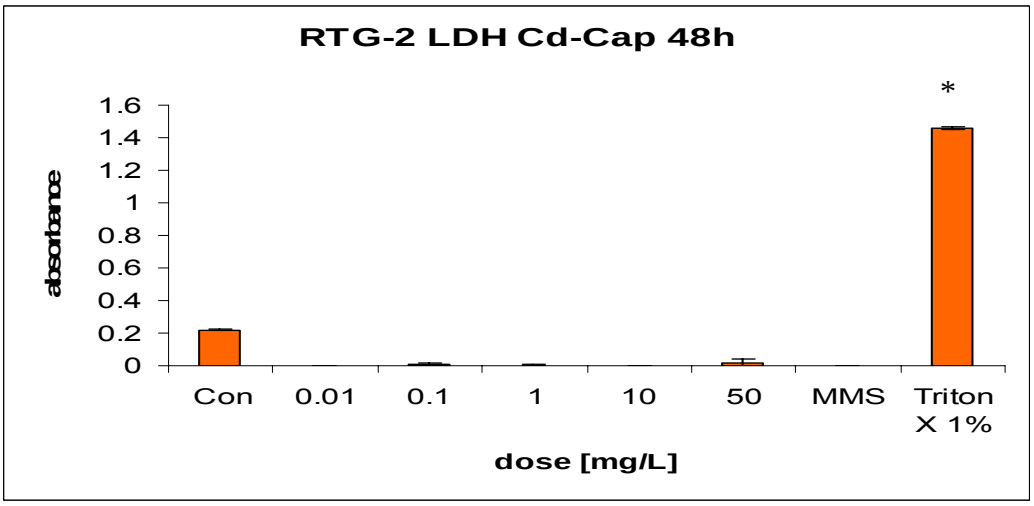


Fig. 43_ LDH levels after 48-hour exposure to CdS NPs and Triton X 1% as positive control. * indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of LDH is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 LDH Cd-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	2506,94	< 0,0001
B: colt	2,45	0,1308

Multiple range test for RTG-2 LDH Cd-Cap 48h

Dose	Mean	Homogeneous Groups
10	0,0	X
MMS	0,0	X
0,01	0,0	X
1	0,0025	X
0,1	0,0075	X
50	0,0175	X
Con	0,22	X
Triton X 1%	1,4575	X

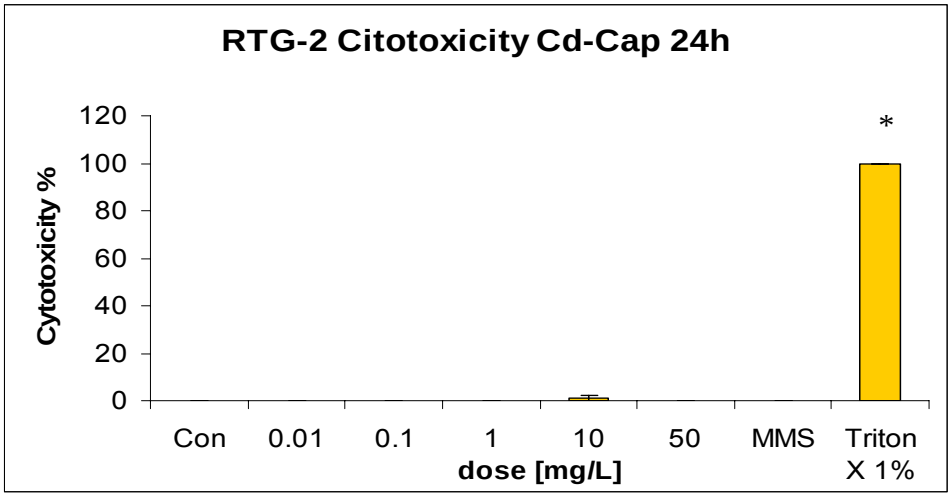


Fig. 44_ Cytotoxicity % after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

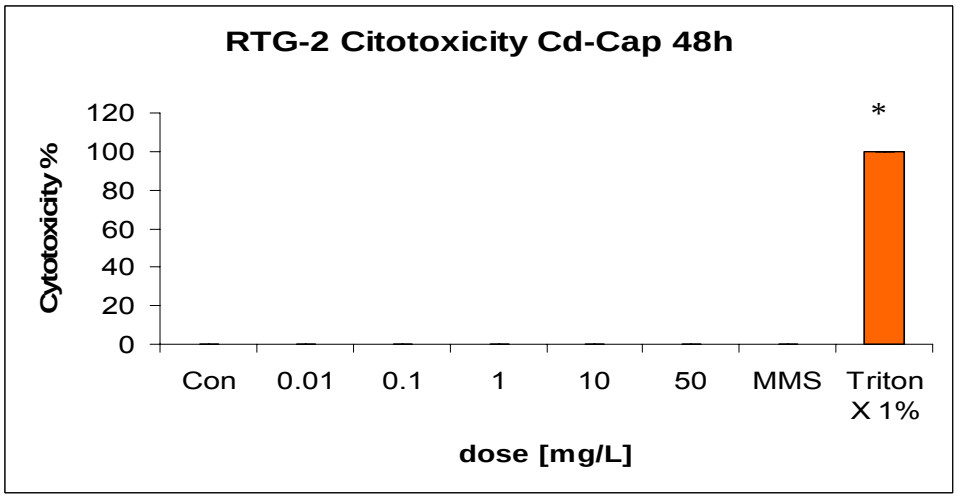


Fig. 45_ Cytotoxicity % after 48-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicate statistically significant ($p<0.05$) increase over the controls. Mean percentage of Cytotoxicity is shown with the upper and lower quartiles as error bars.

After we subtracted the effects of Capping agent, values from the Cell proliferation assay show to correspond to what we have seen in the LDH test and in what there is in literature; in fact now high doses of nanoparticles correspond to a significant decrease of cell population.

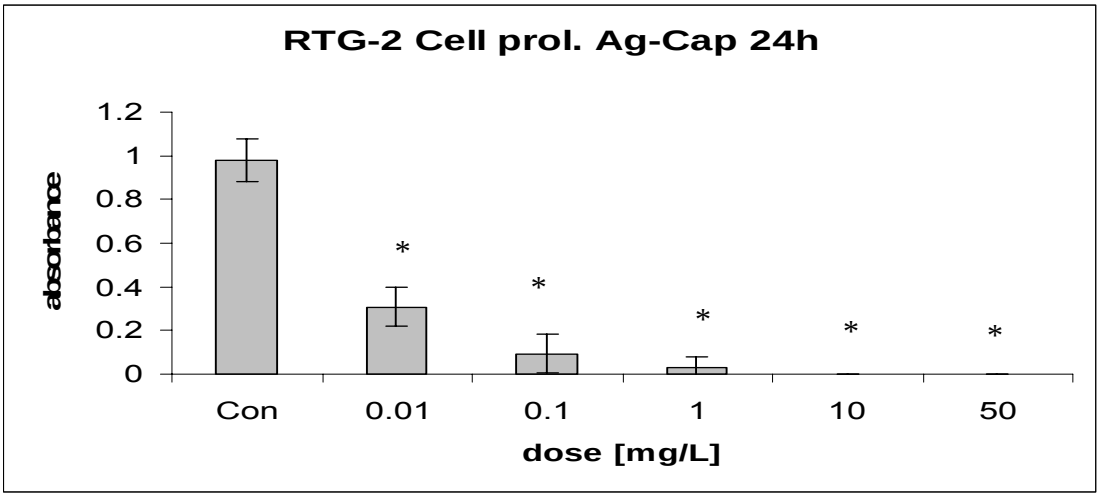


Fig. 46_ Cell proliferation after 24-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Ag-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	19,42	< 0,0001
B: colt	0,28	0,6034

Multiple range test for RTG-2 Cell prol Ag-Cap 24h

Dose	Mean	Homogeneous Groups
50	0,0	X
10	0,0	X
1	0,0325	X
0,1	0,0975	XX
0,01	0,3075	X
Con	0,98	X

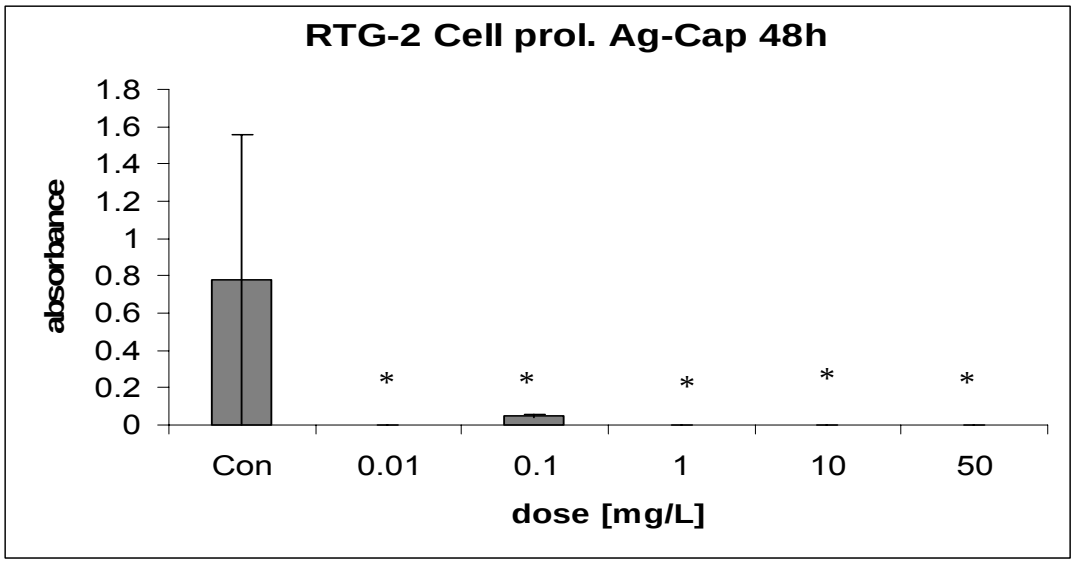


Fig. 47_ Cell proliferation after 48-hour exposure to Ag_2S NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Ag-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	1707,14	< 0,0001
B: colt	0,02	0,8776

Multiple range test for RTG-2 Cell prol Ag-Cap 48h

Dose	Mean	Homogeneous Groups
10	0,0	X
50	0,0	X
0,01	0,0	X
1	0,0	X
0,1	0,05	X
Con	1,33	X

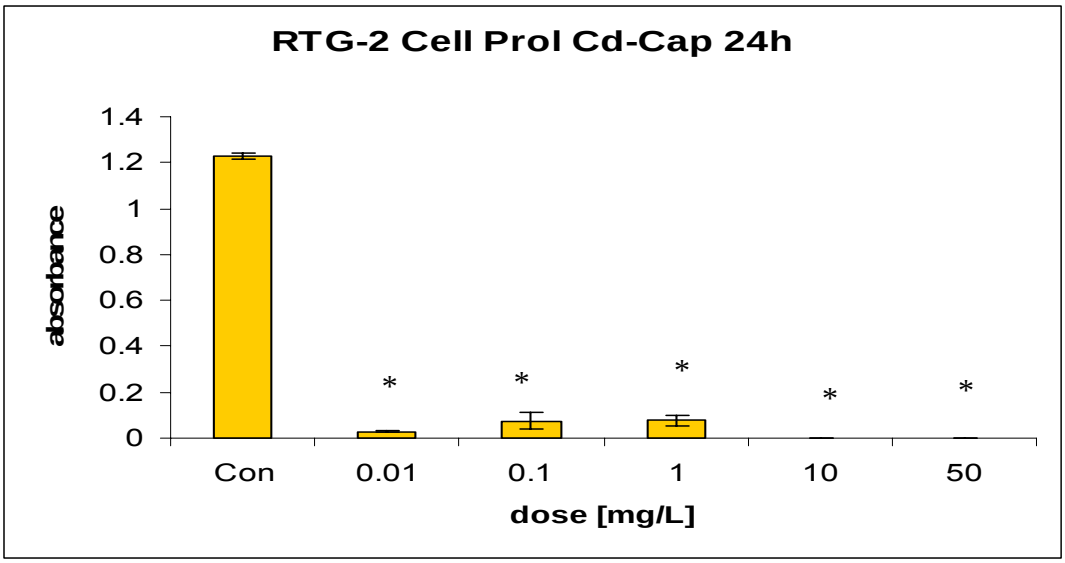


Fig. 48_ Cell proliferation after 24-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cd-Cap 24h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	524,07	< 0,0001
B: colt	3,66	0,0728

Multiple range test for RTG-2 Cell prol Cd-Cap 24h

Dose	Mean	Homogeneous Groups
10	0,0	X
50	0,0	X
0,01	0,0275	XX
0,1	0,075	X
1	0,0775	X
Con	1,23	X

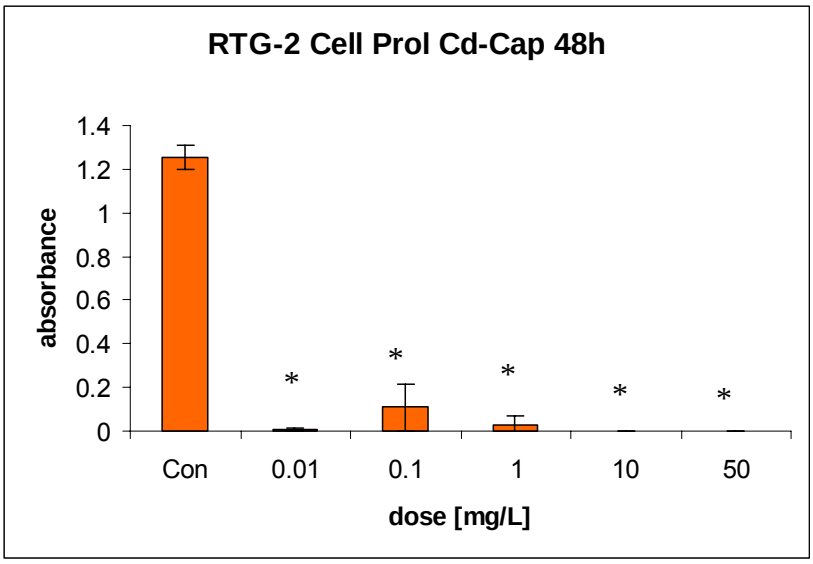


Fig. 49_ Cell proliferation after 48-hour exposure to CdS NPs and Triton X 1% as positive control.
* indicates a statistically significant decrease respect to control ($p<0.05$). Mean percentage of cell proliferation is shown with the upper and lower quartiles as error bars.

Analysis of Variance for RTG-2 Cell prol Cd-Cap 48h

Source	F-Ratio	p-Value
MAIN EFFECTS		
A: dose	169,98	< 0,0001
B: colt	1,82	0,1948

Multiple range test for RTG-2 Cell prol Ag-Cap 48h

Dose	Mean	Homogeneous Groups
50	0,0	X
10	0,0	X
0,01	0,005	X
1	0,025	X
0,1	0,1075	X
Con	1,255	X

Our results are in line with other studies on the biological effects of nanoparticles. *Reeves et al. (2007),* for istance, affirms that $TiCO_2$ nanoparticles induce a genotoxic effect on DNA of goldfish skin cells (GFSk-S1) at all the concentrations used. In that study it was suggested that 8-hydroxyguanine (8-OH-G) was probably the most likely product of TiO_2-induced oxidative stress.

This product is important because is both relatively easily formed and is mutagenic and carcinogenic. Similar results were also found recet studies on mammalian cells; these researches confirmed the genotoxic potential of TiO_2 nanoparticles at concentrations similar to those studied by *Reevers et al (2007*). *Gurr et al.* observed increased levels of oxidative DNA damage when BEAS-2B cells, a human bronchial epithelial cell line, were treated with 10 μg/ml anatase (10 nm) TiO_2 particles for 1 hour. *Vevers et al.* (*2007*) instead found TiO_2 to be non effective as genotoxicant, at least in the absence of UV-illumination on RTG-2 cells after an exposure of 4 hours. They observed a reduction in lysosomal integrity at the highest dose (50 mg/L) after 24 hours of exposure but this was not correlated to any detectable DNA damage. Moreover they reported that even if NPs are not passing into the cytoplasm to cause oxidative damage, there is still a potential to reduce cell functionality due to lipid peroxidation products that permeate throughout the cell causing a cascade of oxidative deterioration promoted by the presence of transition metal ions, which can substain the Haber-Weiss and Fenton reactions (main sources of the very reactive hydroxil radical). As silver and cadmium nanoparticles have similar properties to TiO_2 it possible that the same process occurs when cells are expose to these two nanoparticles. Unfortunately, literature about silver and cadmium NPs is very limited, as nanotechnology is

a new field and the thier potential as emerging pollutant is still scarcely investigated

Cadmium is well known to be mutagenic, teratogenic and carcinogenic. In earlier studies with cultured mammalian cells, cadmium has been shown to induce lipid peroxidation, DNA single-strand breaks and chromosomal aberrations in its bulk form. Both the interaction with DNA repair processes and the induction of oxidative DNA damage may account for cadmium genotoxicity.

Many studies have highlighted the potential role of active oxygen species and free radicals as mediators in the induction of genotoxicity by cadmium. The fact that reactive oxygen species induce apoptosis (programmed cell death) suggest that oxidative stress may be also involved in cadmium-induced apoptosis (*Risso-de Faveri C., 2001)*

Concerns with silver toxicity are commonly limited to invertebrates and plants, because silver is a relatively rare element and not very toxic to humans or other vertebrates. Although found in trace levels in human tissues, it has no known physiologic function (*ATSDR, 1990*) and its presence in tissues is considered to be a contaminant (*RAIS, 2005*). Exceptionally high levels of silver in humans (> 1 g body burden) cause argyria, "*a gray or blue-gray, permanent discoloration of the skin and mucous membranes that is not a toxic effect per se, but is considered cosmetically disfiguring*" (*RAIS, 2005*). Silver has also been reported to cause mild allergic reactions from dermal exposures, respiratory tract irritation, impaired night vision, and abdominal pain from chronic inhalation. In contrast, silver is highly toxic to micro-organisms and invertebrates. More than three decades ago, *Bryan* (*1971*) ranked it second only to mercury in its toxicity to marine invertebrates. Later, the same Author ranked Ag

as one of the three most toxic elements (the others being copper and mercury) to marine invertebrates in estuarine and marine waters (*Bryan, 1984)*. The relatively high toxicity of silver was subsequently corroborated by *Eisler* (*1996*), who reported that ionic silver (Ag^+) "*is one of the most toxic metals known to aquatic organism in laboratory testing"*, and more recently by *Ratte* (*1999*), who stated that ionic silver *"is one of the most toxic forms of a heavy metal, surpassed only by mercury and thus has been assigned to the highest toxicity class, togheter with cadmium, chromium (VI), copper and mercury." (Flegal A.R., 2007).*

This thesis is a pilot work to collect first informations for the evaluation of the potential risk of NPs in acqautic organisms. The use of RTG-2 cells and *M. edulis* haemocytes enables a degree of extrapolation to *in vivo* exposures in living organisms, although care is needed. For example, the concentration of NPs found to be innocuous after 48 hours in this study would be lethal in an aquatic 14-days exposure in trout (*Oncorhynchus mykiss*), primarly due to oxidative gill damage (*Vevers et al., 2007*).

A chronic *in vivo* exposure experiment where more environmental realistic concentrations of NPs are used and bioaccumulation measured after longer period would be very useful (*Vevers et al., 2007*). A lower concentration of NPs would limit the potential to agglomerate and would therefore increase the chance of passage into cytoplasm, as it has been seen in this study. It would be advantageous to analyze the phagocytotic capacity of cells using fluorescent albumin coated beads and time lapse fluorescent microscopy. This would provide information of intracellular transport.

Results shown in this study with silver and cadmium NPs indicate that extremely high exposure doses are required to generate

genotoxic and cytotoxic effects *in vitro*, on the contrary of what is known about cadmium and silver in their bulk forms.

Future investigations may focus on other subtle cellular changes with more environmentally relevant levels of NPs. As the growth of nanotechnologies broadens to include novel materials and uses, the risk of toxicological potential in the aquatic environment needs to be fully characterized before an asbestos-type episode arises (*Donaldson et al., 2004*).

5_ *Conclusions*

- Silver nanoparticles were found to induce a genotoxic effect in mussel haemocytes after 4 hours exposure only at the dose of 10 mg/l , while no effect was detected in trout cell cultures after 24 hours exposure while an increase of DNA damage was detected after treating cells with the highest dose of 50 mg/l after 48 hours. Neither LDH test, nor the cytotoxicity test revealed any cytotoxic effect both at 24 and at 48 hours .

- Cadmium nanoparticles were found to induce a genotoxic effect in mussel haemocytes after 4 hours exposure only at the dose of 10 mg/l as well. A dose-effect relationship was found after exposing RTG-2 cells for 24hours, while DNA repair events are likely responsible for the lack of DNA damage observable after 48 hours, except for the dose of 10 mg/l.
Also in this case neither LDH test, nor the cytotoxicity test revealed any cytotoxic effect both at 24 and at 48 hours.

- The Capping agent, used in order to avoid the aggregation of nanoparticles, was found to possess a weak cell proliferation capacity at the highest doses. For this reason, after the subtraction of the effects of Capping agent, values from the Cell proliferation assay showed that high doses of silver and cadmium nanoparticles corresponded to a decrease of cell population.

Taken together, the results obtained underline the profitability of fish cell cultures to evaluate the possible toxicity induced by nanoparticles, as vehicle of environmental toxicants.

Future perspectives concern the use of a wider spectrum of NPs and a deeper evaluation of Cadmium NPs genotoxicity, also including oxidative stress parameters and DNA repair inhibitors to better investigate their mechanism of action.

The choice of an agent able to prevent the NPs aggregation also appears to be crucial. In the present work the thiol-terminated methyl polyethylene glycol showed to possess a genotoxic effect in mussel cells and a cell proliferation capacity in RTG-2 cells. Thus, further investigation needs to be done to select an ideal Capping agent able to solve the problem of NPs aggregation but lacking of any toxic aqctivity, in order to obtain a better interpretation of results.

6_ *References*

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