Baseline

Neutral Red cytotoxicity assays for assessing *in vivo* carbon nanotube ecotoxicity in mussels – comparing microscope and microplate methods

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Highlights

- Mussels *Mytillus sp* were exposed *in vivo* to 50, 250 and 500 μgL^{-1} single (SWCNTs) and multi-walled carbon nanotubes (MWCNTs).
- SWCNTs and MWCNTs caused concentration dependent decreases in neutral red retention time (NRR).
- A concentration dependent decrease in optical density was not observed using the microplate method (NRU).
- We conclude that the NRU method is not sensitive enough to assess carbon nanotube ecotoxicity *in vivo* under environmentally relevant conditions.

Abstract

The purpose of the present study was to compare two neutral red retention methods, the more established but very labour-intensive microscope method (NRR) against the more recently developed microplate method (NRU). The intention was to explore whether the sample volume throughput could be increased and potential operator bias avoided. Mussels *Mytilus sp* were exposed *in vivo* to 50, 250 and 500 μgL^{-1} single (SWCNTs) or multi-walled carbon nanotubes (MWCNTs). Using the NRR method, SWCNTs and MWCNTs caused concentration dependent decreases in neutral red retention time. However, a concentration dependent decrease in optical density was not observed using the NRU method. We conclude that the NRU method is not sensitive enough to assess carbon nanotube ecotoxicity *in vivo* in environmentally relevant media, and recommend using the NRR method.

Nanomaterials (NMs) are defined as having at least one dimension between 1 and 100nm (EC, 2011). Nanomaterials have been present in the environment for thousands of years, either naturally or from incidental human activity, in the form of volcanic ash, sea salt, viruses and soot from the partial combustion of fossil fuels (Nowack and Bucheli, 2007). Over the last 50 years the development of engineered nanomaterials (ENMs) has increased greatly giving rise to a multitude of manifestations, including nanoparticles, nanocomposites, nanostructured materials/coatings and nanotubes, each displaying different and notable properties (Tedesco and Sheehan, 2010), which has led to their widespread use in a range of everyday products such as lotions, cosmetics, medicines and paint (Kahru and Dubourguier, 2010). The latest available Woodrow Wilson Institute Nanotechnology Consumer Products Inventory (2013) reported 1,628 consumer products that have been introduced to the market since 2005, up 24% since the last update in 2010[1]. Increased production and use has led to increased incidences of engineered nanomaterials being released in to the environment during the project life cycle (Nowack and Gottschalk, 2011; Tsai, 2015; Wigger et al., 2015), and raised particular concern about the associated risk and the suitability of existing environmental risk assessment (Sørensen et al., 2015). However, the variability of their physical and chemical properties make nanomaterials very different in terms of behaviour from corresponding bulk materials, and these cannot necessarily be used to aid in predicting toxicity (Grieger *et al*., 2015; Stone *et al.*, 2014), and therefore require characterisation as far as possible under exposure conditions (Brenner *et al.,* 2014). Owing to their low mass and remarkable strength, carbon nanotubes (CNTs) are finding use in a wide variety of industries, including aerospace, maritime, renewable energy and medicine (Cheung *et al.,* 1998). Carbon nanotubes are high-aspect ratio hollow tubes, made from graphene sheets and can be found in two main engineered forms, single walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) (Kohler *et al.,* 2002). SWCNTs measure between 0.5-1.5nm in diameter and can be several μm in length while MWCNTs can be over 100nm in diameter due to the layering of different sized CNTs with a distance of approximately 0.34nm between each layer. Their increased use has therefore intensified the need to investigate the fate and impacts on the receiving environment. However, there are substantial gaps in our knowledge (Stone *et al.,* 2014) making meaningful environmental impact assessments, on which management decisions are based, very difficult. Whilst understanding the drivers of CNT behaviour, such as size, surface area, shape and aspect ratio, charge and functionalization has

[1] http://www.wilsoncenter.org/article/inventory-finds-increase-consumer-products-containing-nanoscale-materials

improved predictions of environmental fate, and bioavailability, the detection and identification of engineered nanomaterials in complex environmental matrices, as well as environmentally relevant hazard identification, remains challenging.

Mytilus sp is regarded as a good biomarker species in response to contamination and stress (Shepard and Bradley, 2000). They are active filter feeders efficiently obtaining food particles from the water column, including adsorbed contaminants and other particulate pollutants such as CNTs (Al-Shaeri *et al.,* 2013; Brenner *et al.,* 2014). Once removed from suspension by the gills, *Mytilus sp.* have been shown to selectively exclude the majority of SWCNTs from filtered material and excrete them before ingestion as pseudofaeces. Nevertheless, a toxic response in the gill epithelia consistent with oxidative stress and subsequent DNA damage was observed and attributed mainly to metal co-contaminants (Al-Shaeri *et al.,* 2013). More recently, however, SWCNTs have been observed in the gut epithelia of *Mytillus* exposed to SWCNTs in the presence of microalgae, which demonstrates that CNTs can be ingested by filter-feeding bivalves under environmentally relevant conditions (Al-Shaeri et al., 2014).

Lysosomes, subcellular vesicles containing macromolecule degrading hydrolytic enzymes, are responsible for recycling of cellular debris (Brenner *et al.,* 2014). Their structure and membrane stability can be used to detect cellular stress in an organism following exposure (Cheung *et al.,* 1998). Stress, such as that caused by exposure to environmental contamination can cause an increase in lysosome production to help combat and protect cells from cell death (Johansson et al., 2010). When lysosomes are overloaded the semi-permeable membrane surrounding them can become unstable and leak the lysosomal enzymes and contaminants into the cytoplasm (Kohler *et al.,* 2002). Lysosomal damage also occurs under continued oxidative stress caused by an overproduction of reactive oxygen species (ROS) resulting in an imbalance between ROS and cellular antioxidants and also an increase in cytoplasmal free radical by-products (Martinez-Gomez *et al.,* 2015). Contaminant or stress related damage manifested in observable changes in membrane permeability have led lysosomes to be used as biomarkers of poor cell and organism health (Brenner *et al.,* 2014). Neutral Red (NR) dye is a cationic stain used mainly in histology. It is taken up into the cells by diffusion where it is then trapped inside the lysosomes. The dye is only taken up and retained by lysosomes present in healthy cells. Using a light microscope the dye can be seen to leak out of the lysosomes in to the cytosol of the cell indicating damage and breakdown in stability of the lysosomal membranes, and quantified by measuring the time this process takes. This technique is known as the lysomosal neutral red retention assay (Lowe *et al.,*

1992; Mamaca *et al.,* 2005), henceforth referred to as (NRR). However, this is a very time consuming technique prone to operator bias. An alternative sprectrophotometric NR method also relies on the ability of the cell to take up and retain the dye (NRU). The lysosomes of non-viable cells are not able to do this and the dye diffuses out of the cell during incubation, and the spectrophotometric absorption values obtained are lower than for viable cells (Babich and Borenfreund, 1990). Therefore, the aim of this study was to explore the possibility of increasing the screening throughput of mussels exposed *in vivo* to different types of CNTs by optimizing the microplate assay (NRU) and comparing the results with the microscope method already established in our lab (NRR).

CNTs were purchased from SIGMA Aldrich (SWCNTs: catalog 704121 - manufacturer's specifications: diameter 1.1 nm; length 0.5– 100 µm; MWCNTs: 724769 – manufacturer's specifications: diameter 5.5 nm; length 5 µm). CNTs were characterized according to Al-Shaeri *et al.* (2013) using TEM and DLS/zeta potential to estimate the diameter of agglomerates and surface charge, respectively (Figure 1; Table 1). *Mytilus edulis* were collected from mussel beds 6 miles east of Edinburgh, in Musselburgh. The beds are found at the mouth of the River Esk, on the south shore of the Firth of Forth (55°57'145''N 3°3'549''W) during low tide. Mussels of similar sizes (5 cm ± 0.5) were collected and taken immediately back to the Heriot-Watt aquarium to be cleaned on the outside before being held in an aerated moulded glass tank to acclimatize in filtered seawater (salinity of $32^{0}/_{00}$, dissolved O_2 8.6mg/L, temperature 16°C, measured using a YSI 85 Salinity, Conductivity, Dissolved Oxygen & Temperature Meter; pH 7.86, Thermo Orion 420^{A+}). The mussels were tested individually and examined to ensure only healthy specimens were used, weak or dead animals were discarded.

The *in vivo* experiment was set up in the Heriot Watt aquarium and conducted using three litre jars containing one litre of aerated seawater. Four experiments were run in total, two each with three independent exposures to multi-walled carbon nanotube (MWCNT stock prepared in 0.2% BSA) and two each with three independent exposures to single walled carbon nanotubes (SWCNT stock prepared in 0.02% Suwannee River natural organic - SRNOM). Mussels were picked randomly from the storage tank and placed in each jar, left for 24 hours to acclimatise, after which three concentrations of carbon nanotubes (50, 250 & 500µg L^{-1}) were introduced to the respective jars. The remaining three jars acted as controls containing either no CNTs or appropriate amounts of SRNOM or BSA as dispersant controls. The use of BSA and SRNOM as dispersants helped prevent excessive agglomeration of CNTs in the stock preparation and kept them in suspended in the exposure medium long

enough for mussels to clear them from the water column. In order to enhance dispersion, CNT stock preparations were sonicated (Megason Ultrasonic Bath Cleaner) for 2 hours prior to spiking the exposure systems (Al-Shaeri *et al.*, 2013). Following 24 hours of exposure the mussels were removed and haemolyph extracted according to Coughlan et al. (2002). Briefly, each mussel was prised open in turn using a pair of scissors, the water drained and the scissors used to prop up the mussel in order to provide access to the abductor muscle for haemolymph extraction. 200µl of haemolymph was extracted using a 1ml syringe/21 gauge needle that had been rinsed with physiological saline (PS) solution (0.4M NaCl, 0.027M $MgSO_4$, 0.01M KCl, 0.01M $CaCl_2$, 0.02M Hepes, pH 7.3). Each haemolymph sample was collected and mixed with an equal volume of PS solution and placed in Eppendorf tubes on ice until use on the day. The microscope assay was based on the protocol of Lowe et al. (1992) and performed according to Coughlan et al. (2009). Briefly, 40µl of each sample was pipetted on to Poly-L-Lysine (PLL) coated microscope slides and incubated for 15 minutes in a light proof humidity chamber. Use of the latter ensured a humid environment for the cells to prevent drying out and to stop the light-sensitive dye degrading. After incubation, excess cell suspension was tipped off and the edge of the slide wiped before pipetting 40µl of working Neutral Red working solution (2% w/v in DMSO) onto the slide and a coverslip applied. The microscope slides were then incubated in the light proof humidity chamber for 15 minutes after which cells were observed under a light microscope (Olympus BX50; x400 magnification) to assess initial staining. The slides were then coded and scored blindly to avoid operator bias. Cells were observed every 15 minutes for the first hour and every 30 minutes for a further two hours. The time in minutes was recorded when 50% of the cells began to show lysosomal leakage of dye into the cytoplasm. This event can also be accompanied by excessive swelling of the lysosomes. The experiment was halted after 180 minutes as it is thought that after this time the neutral red itself can be damaging to the lysosomes and can cause instability (Moore *et al.*, 2009).

The microplate assay (NRU), originally developed by Borenfreund & Puerner (1985), was adapted from Amachree *et al.* (2013). Briefly, 50µl of haemolymph was pipetted in quadruplets into the right side of a well of a 10% PLL-treated 96 well microplate, agitated (400 rpm for 1 minute), covered and incubated in the dark at 15°C for 50 minutes. Using a multi-channel pipette the excess haemolymph was then carefully removed from the left to avoid disturbing adhered cells. Neutral red solution (200µl; 0.004%) was pipetted into the left of each well and incubated in the dark for 3 hours at 15°C. Once incubated, using a multiple channel pipette, the supernatant was carefully removed from the left and 200µl of PS

solution used to gently wash the cells and remove any extracellular NR residue. 200µl of acidified ethanol was added to each well to aid the breakdown of the cell membranes and the release of the intracellular dye and protein. The microplate was then left to incubate in the dark for a further 10 minutes at room temperature. After incubation the microplate was agitated in the plate reader (Spectramax & Softmax Pro software) for 30 seconds and read at 550nm. 10µl of supernatant from each well was transferred to a new untreated microplate and total protein determined according to Bradford (1976) . Neutral red retention was expressed as OD_{550} mg^{-1} protein.

Statistical analysis was carried out using SIGMA-STAT 2.03. Where appropriate, data were log_{10} transformed and then analysed by parametric ANOVA followed by a Tukey multiple comparison test; significance was accepted at $p<0.05$ (Dytham, 2011).

Positive controls using 50% H_2O_2 and dispersant controls (SRNOM and BSA) were performed *in vitro* on extracted haemocytes and the results are shown in Figure 2. The positive controls for both assays were significantly lower than the respective controls. In contrast the dispersants SRNOM and BSA did not show any significant effects.

Neutral red retention (NRR) time (the time at which 50% of the cells showed lysosomal leakage) indicating the level of lysosomal damage following carbon nanotube exposure is shown in Figure 3. The control samples showed no lysosomal leakage or excessive swelling at the experimental cut off point of 180 minutes. A statistically significant concentration-dependent decrease in the NRR time was observed for both SWCNTs and MWCNTs. The NRR results are consistent with the underlying theory of the technique that an increase in cellular stress, brought about by contaminant exposure, was compromising the lysosomal membrane integrity (Lowe *et al.,* 1992). This is likely to decrease cell viability and ultimately lead to cell death (Hauton *et al.,* 1998). There was no significant difference in NRR times between the two CNT treatment groups, except at the highest exposure concentrations, where SWCNTs appeared to be more toxic (Figure 3). This is in agreement with the findings of a recent review by Jackson *et al.* (2013) which concluded that SWCNTs were generally more toxic in environmentally relevant exposure media than MWCNTs. The toxicity of CNTs is mainly determined by surface characteristics and agglomerate size. Although the capacity for MWCNTs to adsorb co-contaminants from the water is generally lower than that of SWCNTs with equivalent functionalization, it is unlikely that the increased toxicity of SWCNTs was caused by metal impurities residual or acquired in theses batches and at the concentrations used as previously demonstrated by Al-Shaeri et al. (2013). Rather, mussels are known to filter bacteria from the water column whose size range can coincide with that of the

agglomerates of SWCNTs at higher concentrations and mussels have also been shown to filter SWCNTs dispersed in seawater (Al-Shaeri *et al.*, 2013). It is therefore conceivable that the differences in toxicity between SWCNTs and MWCNTs are down to the size of the agglomerates at the concentrations used and the ability of mussels to better remove the former from the water column, thus achieving a higher degree of physical contact with gill epithelia.

The NRU technique using microplates relies on the measurement of the dye retained by the cell following lysosomal leakage, with the healthier cells recording a higher optical density, expressed as OD_{550} mg^{-1} protein. This technique is less labour intensive than the NRR technique and also notionally more objective. However, it has not been applied as extensively as the NRR method especially within the marine environment. The expected result would be for cell samples to show an OD inversely proportional to the CNT exposure concentration (Amachree *et al.*, 2013). The NRU results in the present study are presented in Figure 4. ODs following exposure to 250µg L^{-1} of both forms of CNTs and 500µg L^{-1} of SWCNTs were higher than the respective controls, and the results were lower than the controls following 50µg L^{-1} for both forms of CNTs and also for 500µg L^{-1} of MWCNTs. However, no concentration dependent relationship was observed and the results of exposures to both SWCNTs and MWCNTs were not significantly different from the respective controls. Comparison of the two methods by converting the results to % change and also by plotting NRR against NRU for both SWCNTs and MWCNTs confirmed the observation that, unlike the NRR method, the NRU microplate method was not able to detect a concentration-dependent toxic insult to mussel haemocytes following *in vivo* exposure (Table 2). Moore et al. (2009) showed that, whilst the NRR assay produced consistent results following *in vitro* exposure to C_{60}, the technique did not yield concentration dependent toxicity data for CNTs. This was partly explained by the observation that CNTs, unlike C_{60}, were not entering the cells. The results of the present study, however, are consistent with the notion that CNTs exhibit cytotoxicity through the generation of reactive oxygen species (ROS) (Pacurari et al., 2008), rather than direct contact with lysosomal membranes (Al-Shaeri et al., 2013). The CNT concentrations in the present study were also far lower than those used by Moore at al. (2009), and represent a range of exposure scenarios. To the best of our knowledge there are currently no accepted predicted environmental concentrations (PEC) for CNTs in seawater, mainly because of the lack of suitable methods of detection. The few published examples of PECs for freshwater, sewage sludge and sediments (Gottschalk et al., 2013; Gottschalk et al., 2009) are derived from models based on probabilistic material flows. The lack of detection methods of

CNTs (and most engineered nanomaterials) in environmental media makes it currently impossible to determine and verify reliable PECs. We conclude that, although we have shown that SWCNTs and MWCNTs show a cytotoxic effect in mussels following *in vivo* exposure under environmentally relevant conditions using the microscope method (NRR), the microplate assay (NRU) is simply not sensitive enough, even to unrealistically high concentrations of pristine CNTs. For the foreseeable future the more established NRR method (Martinez-Gomez *et al.,* 2015) should continue to be the preferred endpoint assay for assessing CNT ecotoxicology using mussels.

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References

Al-Shaeri, M., Ahmed, D., Mc Cluskey, F., Turner, G., Paterson, L., Dyrynda, E.A., Hartl, M.G.J., 2013. Potentiating toxicological interaction of single-walled carbon nanotubes with dissolved metals. Environ. Toxicol. Chem. 32, 2701-2710.

Al-Shaeri, M.A.M., Paterson, L., Stobie, M., Cyphus, P., Fernandes, T.F., Hartl, M.G.J., 2014. Green algae interacting with single-walled carbon nanotubes affect the feeding behaviour of mussels, mitigating nanotube toxicity., SETAC Europe Meeting, Basel, Switzerland, Basel, 11-15 May, 2014.

Amachree, D., Moody, A.J., Handy, R.D., 2013. Comparison of intermittent and continuous exposures to cadmium in the blue mussel, Mytilus edulis: Accumulation and sub-lethal physiological effects. Ecotox Environ Safe 95, 19-26.

Babich, H., Borenfreund, E., 1990. Applications of the neutral red cytotoxicity assay to in vitro toxicology. ATLA 18, 129-144.

Borenfreund, E., Puerner, J., 1985. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). Journal of Tissue Culture Methods 9, 7-9.

Bradford, M.M., 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72, 248-254.

Brenner, M., Broeg, K., Frickenhaus, S., Buck, B.H., Koehler, A., 2014. Multi-biomarker approach using the blue mussel (Mytilus edulis L.) to assess the quality of marine environments: Season and habitat-related impacts. Mar. Environ. Res. 95, 13-27.

Cheung, V.V., Wedderburn, R.J., Depledge, M.H., 1998. Molluscan lysosomal responses as a diagnostic tool for the detection of a pollution gradient in Tolo Harbour, Hong Kong. Mar. Environ. Res. 46, 237-241.

Coughlan, B.M., Hartl, M.G.J., O'Reilly, S.J., Sheehan, D., Morthersill, C., van Pelt, F.N.A.M., O'Halloran, J., O'Brien, N.M., 2002. Detecting genotoxicity using the Comet assay following chronic exposure of Manila clam *Tapes semidecussatus* to polluted estuarine sediments. Mar. Pollut. Bull. 44, 1359-1365.

Coughlan, B.M., Moroney, G.M., van Pelt, F.N.A.M., O'Brien, N.M., Davenport, J., O'Halloran, J., 2009. The effects of salinity on the Manila clam (*Ruditapes philippinarum*) using the neutral red retention assay with adapted physiological saline solutions. Mar. Pollut. Bull. 58, 1680-1684.

Dytham, C., 2011. Choosing and using statistics : a biologist's guide, 3rd ed. Wiley-Blackwell.

EC, 2011. Commission recommendation on the definition of nanomaterials. European Commission, p. 3.

Gottschalk, F., Kost, E., Nowack, B., 2013. Engineered nanomaterials in water and soils: A risk quantification based on probabilistic exposure and effect modeling. Environ. Toxicol. Chem. 32, 1278-1287.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. Modeled Environmental Concentrations of Engineered Nanomaterials (TiO2, ZnO, Ag, CNT, Fullerenes) for Different Regions. 43, 9216-9222.

Grieger, K., Redmon, J., Money, E., Widder, M., van der Schalie, W., Beaulieu, S., Womack, D., 2015. A relative ranking approach for nano-enabled applications to improve risk-based decision making: a case study of Army materiel. Environ Syst Decis 35, 42-53.

Hauton, C., Hawkins, L.E., Hutchinson, S., 1998. The use of the neutral red retention assay to examine the effects of temperature and salinity on haemocytes of the European flat oyster Ostrea edulis (L). Comp. Biochem. Physiol. B 119, 619.

Jackson, P., Jacobsen, N.R., Baun, A., Birkedal, R., Kuhnel, D., Jensen, K.A., Vogel, U., Wallin, H., 2013. Bioaccumulation and ecotoxicity of carbon nanotubes. Chem Cent J 7.

Johansson, A.-C., Appelqvist, H., Nilsson, C., Kågedal, K., Roberg, K., Öllinger, K., 2010. Regulation of apoptosis-associated lysosomal membrane permeabilization. 15, 527-540.

Kahru, A., Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicol. 269, 105-119.

Kohler, A., Wahl, E., Soffker, K., 2002. Functional and morphological changes of lysosomes as prognostic biomarkers of toxic liver injury in a marine flatfish (Platichthys flesus (L.)). Environ. Toxicol. Chem. 21, 2434-2444.

Lowe, D.M., Moore, M.N., Evans, B.M., 1992. Contaminant Impact on Interactions of Molecular Probes with Lysosomes in Living Hepatocytes from Dab Limanda-Limanda. Mar Ecol Prog Ser 91, 135-140.
Mamaca, E., Bechmann, R.K., Torgrimsen, S., Aas, E., Bjornstad, A., Baussant, T., Le Floch, S., 2005. The neutral red lysosomal retention assay and Comet assay on haemolymph cells from mussels (Mytilus edulis) and fish (Symphodus melops) exposed to styrene. Aquat. Toxicol. 75, 191-201.
Martinez-Gomez, C., Bignell, J., Lowe, D., 2015. Lysosomal membrane stability in mussels. International Council for the Exploration of the Sea, p. 46.
Moore, M.N., Readman, J.A.J., Readman, J.W., Lowe, D.M., Frickers, P.E., Beesley, A., 2009. Lysosomal cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: An *in vitro* study. Nanotoxicol. 3, 40 - 45.
Nowack, B., Bucheli, T.D., 2007. Occurrence, behavior and effects of nanoparticles in the environment. Environ. Pollut. 150, 5-22.
Nowack, B., Gottschalk, F., 2011. The release of engineered nanomaterials to the environment. J Environ Monitor 13, 1145-1155.
Pacurari, M., Yin, X.J., Zhao, J.S., Ding, M., Leonard, S.S., Schwegier-Berry, D., Ducatman, B.S., Sbarra, D., Hoover, M.D., Castranova, V., Vallyathan, V., 2008. Raw single-wall carbon nanotubes induce oxidative stress and activate MAPKs, AP-1, NF-kappa B, and Akt in normal and malignant human mesothelial cells. Environ. Health Perspect. 116, 1211-1217.
Shepard, J.L., Bradley, B.P., 2000. Protein expression signatures and lysosomal stability in Mytilus edulis exposed to graded copper concentrations. Mar. Environ. Res. 50, 457-463.
Sørensen, S.N., Hjorth, R., Delgado, C.G., Hartmann, N.B., Baun, A., 2015. Nanoparticle ecotoxicity—physical and/or chemical effects? 11, 722-724.
Stone, V., Pozzi-Mucelli, S., Tran, L., Aschberger, K., Sabella, S., Vogel, U.B., Poland, C., Balharry, D., Fernandes, T., Gottardo, S., Hankin, S., Hartl, M.G.J., Hartmann, N., Hristozov, D., Hund-Rinke, K., Johnston, H., Marcomini, A., Panzer, O., Roncato, D., Saber, A.T., Wallin, H., Scott-Fordsmand, J.J., 2014. ITS-NANO - Prioritising nanosafety research to develop a stakeholder driven intelligent testing strategy Part Fibre Toxicol 11.
Tedesco, A.C., Sheehan, D., 2010. Nanomaterials as Emerging Environmental Threats. Curr Chem Biol 4, 151-160.
Tsai, C.S.J., 2015. Contamination and Release of Nanomaterials Associated with the Use of Personal Protective Clothing. Ann Occup Hyg 59, 491-503.
Wigger, H., Hackmann, S., Zimmermann, T., Koser, J., Thoming, J., von Gleich, A., 2015. Influences of use activities and waste management on environmental releases of engineered nanomaterials. Sci Total Environ 535, 160-171.

Table 1 CNT characterization data in exposure medium[a]

	DLS (nm)		Zeta potential	
µg L^{-1}	SWCNTs	MWCNTs	SWCNTs	MWCNTs
50	1740	960	-10.13	-1.91
250	nd	nd	nd	nd
500	6206	1114	-13.73	-6.65

[a] nd: no data; DLS: dynamic light scattering; a: pH 8.3; salinity 32 ‰

Table 2 Comparing the neutral red retention time (NRR, microscope) with the microplate (NRU) method.

	SWCNTs				MWCNTs			
	% change[a]				% change[a]			
µg L^{-1}	NRR	OD	R^2	*p*	NRR	OD	R^2	*p*
50	64	27			42	41		
250	68	71	0.49	0.304	67	18	0.07	0.725
500	88	49			79	31		

a: % change from respective control; NRR: neutral red; OD: optical density (550nm mg $protein^{-1}$); SWCNTs: single-walled carbon nanotubes; MWCNTs: multi-walled carbon nanotubes.

Figure captions

Fig 1: Transmission electromicrographs (TEM) of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) prepared in distilled water with 0.02% SNORM and 0.2% BSA, respectively.

Fig 2: Positive controls. A: Neutral red retention (NRR) and B: Neutral red uptake (NRU). *: significantly different from respective control ($P < 0.05$).

Fig 3: Mean (± SD) lysosomal retention time (mins) of neutral red dye in *M. edulis* haemocytes exposed to increasing concentrations of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) CNTs. Data is mean NRR time The control animals all gave the same NRR time reading and therefore SD was calculated as 0. Individual animals were used for each sample (n = 6 CNT exposed samples: n = 12 for controls) Columns not sharing a letter are significantly different $P < 0.05$.

Fig 4: Mean (± SD) lysosomal retention (OD) of neutral red dye in *M. edulis* haemocytes exposed to increasing concentrations of single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) CNTs. Data is mean NRR time the control animals all gave the same NRR time reading and therefore SD was calculated as 0. Individual animals were used for each sample (n = 6 CNT exposed samples: n = 12 for controls) Columns not sharing a letter are significantly different $P < 0.05$.

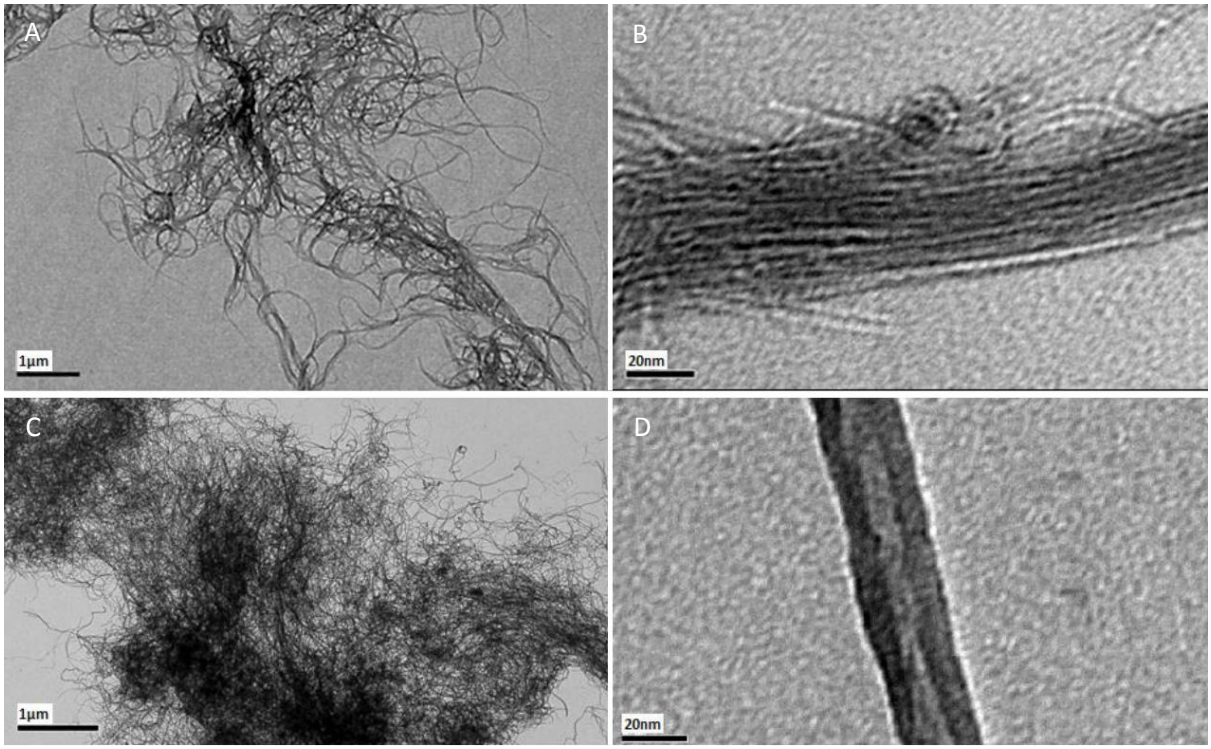
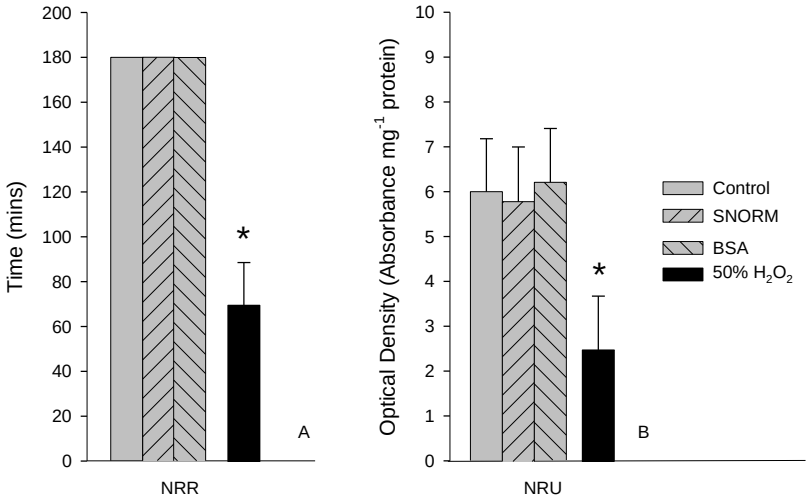


Fig 1


Time (mins)
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A
NRR
Optical Density (Absorbance mg-1 protein)
10
9
8
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6
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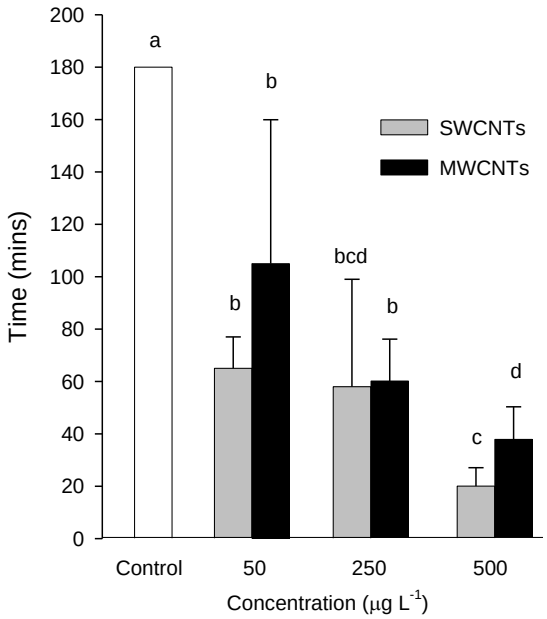
Control
SNORM
BSA
50% H2O2

Fig 2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50
250
500
Concentration (μg L-1)

Fig 3

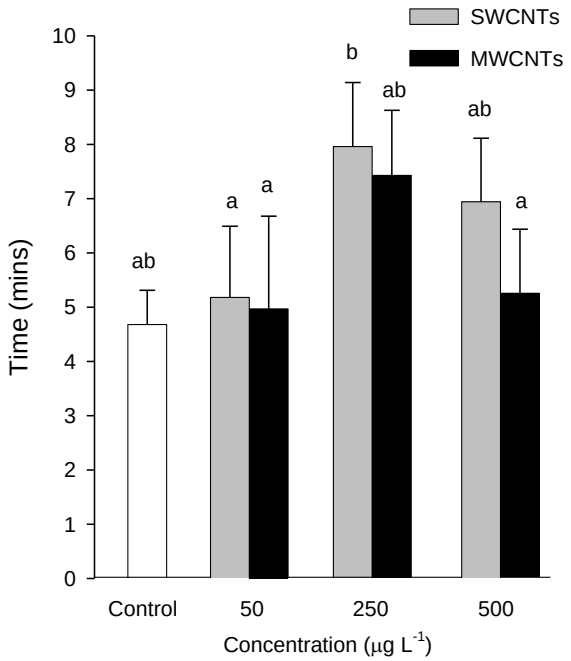
SWCNTs
MWCNTs
Time (mins)
10
9
8
7
6
5
4
3
2
1
0
ab
a
a
b
ab
ab
a
Control
50
250
500
Concentration (μg L-1)

Fig 4