



## Nanoecotoxicity testing with *Lymnaea stagnalis*

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Investigating the effects of nanoparticle exposure in the aquatic environment is of significant importance, since it ultimately receives run-off and wastewater from domestic and industrial sources and is being targeted with nanoscale environmental remediation techniques. A recent review of nanoecotoxicology literature indicates that further aquatic invertebrate testing will be key in developing this field (Baun et al. 2008). As sensitive and relevant test species, both short-term acute and long-term chronic aquatic invertebrate tests will be required to investigate the bioavailability and toxicity of nanoparticles in aqueous suspensions.

The great pond snail *Lymnaea stagnalis* is an aquatic snail being used as one model species in Napier University's nanoecotoxicity research programme in Edinburgh. The study described in this article focussed on investigating the toxicity of both microscale and nanoscale carbon black particles to *L. stagnalis*, as part of an honours project at Napier University in 2008. This species inhabits stagnant fresh waters and ponds and has been found to reproduce easily under standard laboratory conditions (Bohlken and Joosse 1982), making it a particularly suitable species for investigating life-cycle effects from nanoparticle exposure.

*L. stagnalis* have previously been used as a model organism to investigate the effects of nanoparticles on immunological defence mechanisms through haemocyte (blood like cells) responses (Russo et al. 2007). The kinetics of haemocytes of *L. stagnalis* have been found to resemble those of Reactive Oxygen Species (ROS) generated by mammalian leukocytes (Adema et al. 1993). Thus they also could provide an ideal model for investigating nanoparticle enhancement of ROS production in haemocytes and the relationship with lysosomal damage. It has also been suggested that markers of nanoparticle exposure in species such as aquatic gastropods would be useful as a non-invasive means of monitoring nanoparticle exposure and effects in the aquatic environment (Moore et al. 2006).

The rationale for the research stemmed from the hypothesis that nanoparticles could present an oxidative pressure in aquatic organisms (Moore 2006). Nanoparticles have been implicated in causing oxidative damage in the brains of largemouth bass (Oberdorster 2004). At the cellular level, lysosomes are a known target for toxicity exerted by ROS with oxyradicals having been implicated in lysosomal membrane damage in mammalian cells (Olsson et al. 1989). Studies on marine mussels revealed that oxyradicals can be generated both externally and internally to lysosomes of haemocytes (Winston et al. 1996).

Moreover, a relationship between lysosomal and antioxidant responses to environmental pollutants has been proposed in marine bivalves where lysosomal damage is in part dependent on intra- and extra-lysosomal generation of reactive oxygen species and the efficiency of antioxidant mechanisms (Regoli 2000). Therefore, a similar relationship may exist in response to nanoparticles. Furthermore, Moore et al. (2006) have suggested that lysosomal membrane stability is a predictive indicator for cell injury and pathology; and supporting evidence indicates that this parameter is generic in the eukaryotes. The authors also suggest that lysosome fragility could be used a generic biomarker of nanoparticle exposure that would be useful in ecotoxicological monitoring of nanoparticle exposure in the aquatic environment.

To investigate these responses, a comparison of the toxicity of nanoscale (14nm) and microscale (260nm) CB particles at concentrations of 1, 10 and 100mgL<sup>-1</sup> was carried out in a chronic, 21 day exposure of *L. stagnalis*. Nanoparticulate CB provides a greater surface area to volume ratio than fine CB and this study allowed an investigation into the difference in toxicity that this property may give to carbon black. The endpoints growth, feeding and reproduction were examined in order to assess any difference in effect of chronic exposure on the physiological state of *L. stagnalis*.

In addition, cellular stress responses were investigated after both chronic and acute exposure to CB nanoparticles alone. The response of haemocytes isolated from snails in the chronic study to nanoparticle exposure were investigated using the Neutral Red Retention Time (NRRT) assay to assess lysosome fragility. The NRRT assay makes use of the fact that lysosomes retain the neutral red dye after uptake. However, the dye will leak from the lysosomes into the cytoplasm more rapidly when membrane damage has occurred. The NRRT assay has been used to assess the integrity of lysosomal membranes after exposure to different xenobiotics. Svendsen and Weeks (1995) assessed the effects of copper on the freshwater snail *Viviparus contectus*. The technique has also been applied to the terrestrial snail *Helix aspersa* in studies assessing exposures to copper oxychloride (Snyman et al. 2000).

Lastly, an acute exposure was carried out to investigate further haemocyte responses, in relation to the oxidative potential of nanoparticles to *L. stagnalis*. ROS production in haemocytes was measured after acute in vivo exposure to CB nanoparticles at concentrations of 10, 100 and 1000mgL<sup>-1</sup>. The fluorescent probe dihydrorhodamine 123 (DHR) was used to measure reactive oxygen species (ROS) production within the lysosomal compartment of the haemocytes. Haemocytes were treated with the probe and viewed under the confocal microscope to determine the location and strength of fluorescence as a indication of reactive oxygen species production. The fluorescence of the oxidized probe as R123

was observed within isolated haemocytes in three replicates of each 10mgL<sup>-1</sup>, 100mgL<sup>-1</sup> and 1000mgL<sup>-1</sup> group at x62 magnification and with fluorescein isothiocyanate (FITC) excitation and emission using the confocal microscope.

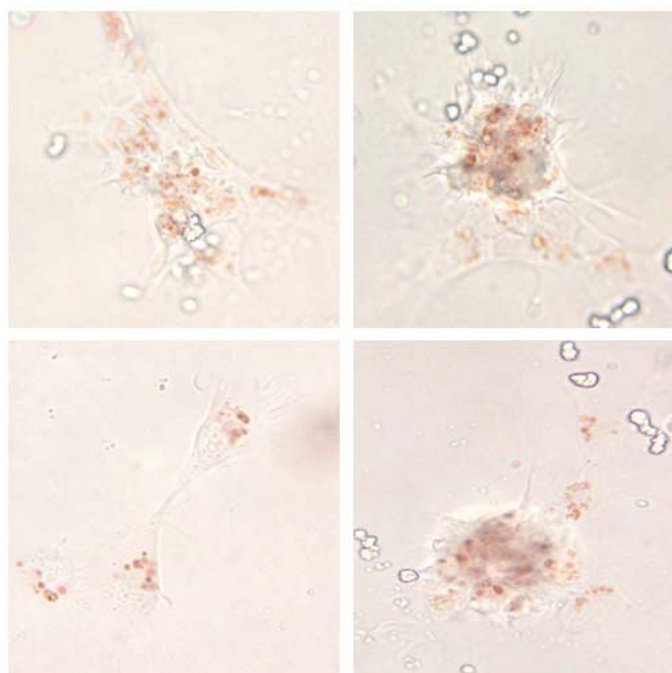


Fig. 1: Photographs at x100 magnification showing haemocytes isolated from the control group, 10 minutes after addition of the neutral-red dye.



The initial toxicity study provided some interesting results, with chronic exposure to CB shown to cause significant effects on feeding, growth and reproduction. The results of this part of the investigation implying that CB nanoparticles are more toxic to *L. stagnalis* than fine CB particles, with a significant reduction in growth at 10mgL<sup>-1</sup> and significant increase in reproduction at 100mgL<sup>-1</sup> of nanoparticle CB exposure. However, a significant reduction in feeding at 10mgL<sup>-1</sup> of fine CB was also shown.

In the cellular response studies, the NRR times obtained suggest there was a decrease in the length of time neutral red was retained in the lysosomes of haemocytes isolated from snails exposed to nanoparticle CB compared to those in the control group. The 1mgL<sup>-1</sup> treatment group showed a decrease compared to the control, followed by a further decrease in the 100mgL<sup>-1</sup> treatment, while the greatest decrease is seen in the 10mgL<sup>-1</sup> treatment group. Figure 1 shows photographs taken of haemocytes isolated from snails in the control group 10 min after the addition of neutral red dye. The photographs show that the neutral red dye was selectively taken up by the lysosomes which are clearly stained red. Figure 2 shows photographs of haemocytes isolated from snails in the 100mgL<sup>-1</sup> nanoparticle CB group after 3 hours of the addition of neural-red. These haemocytes show that the neutral red dye has leaked from the lysosomes and stained the cytosol.

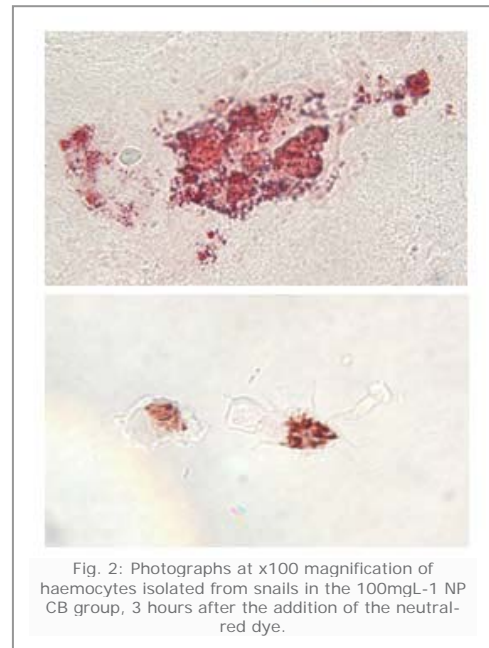


Fig. 2: Photographs at x100 magnification of haemocytes isolated from snails in the 100mgL<sup>-1</sup> NP CB group, 3 hours after the addition of the neutral-red dye.

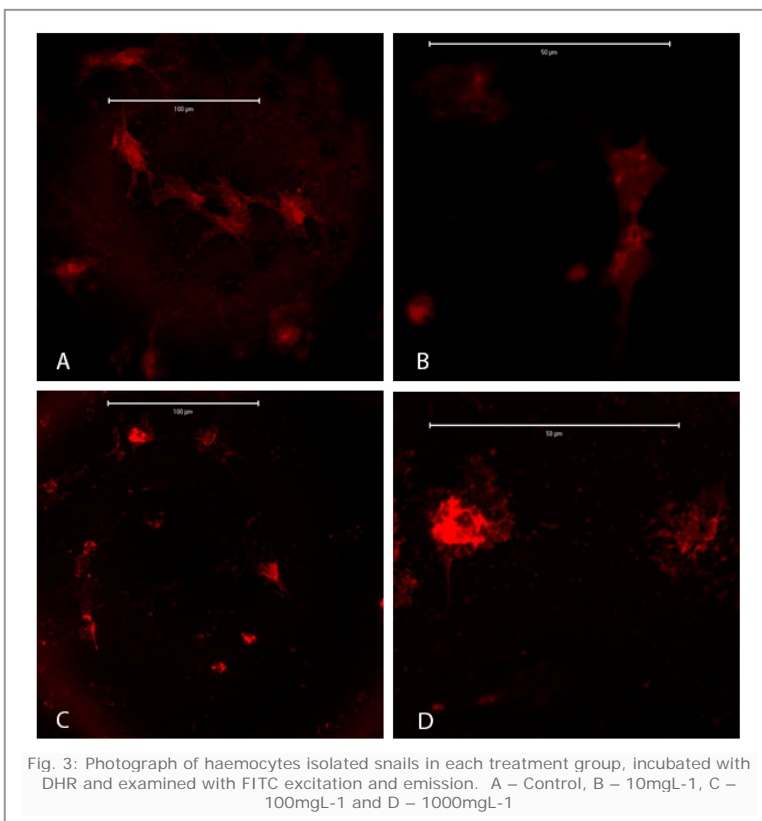


Fig. 3: Photograph of haemocytes isolated snails in each treatment group, incubated with DHR and examined with FITC excitation and emission. A – Control, B – 10mgL<sup>-1</sup>, C – 100mgL<sup>-1</sup> and D – 1000mgL<sup>-1</sup>

In the haemocytes examined under the confocal microscope, fluorescence was found to be absent in haemocytes isolated from the control groups and not treated with the probe DHR. Figures 3 also shows confocal photographs of haemocytes isolated from snails at each treatment group, control, 10mgL<sup>-1</sup>, 100mgL<sup>-1</sup> and 1000mgL<sup>-1</sup> after 10 min of incubation in the presence of 32μM DHR. There was limited evidence of fluorescence specifically within the lysosomes, however there appears to be suggestion of some granular concentration of the fluorescence. Fluorescence was found to be attenuated in the higher concentrations of 100mgL<sup>-1</sup> and 1000mgL<sup>-1</sup> compared to the control, while haemocytes from 10mgL<sup>-1</sup>

group showed similar strength of fluorescence to the control.



These results successfully demonstrate haemocyte responses both to chronic and acute exposure of nanoparticle CB. Haemocyte responses in this study suggested that nanoparticle CB may cross cellular membranes, which in *L. stagnalis* could potentially lead to enhanced ROS production, loss of lysosome integrity and cellular damage. Furthermore, this property may be an important factor in the differences in toxicity between fine and nanoparticle CB in *L. stagnalis*. The results indicate the importance of studying the comparisons between nanoscale and microscale materials, and identify a need for further research on the ability of nanoparticles to cross cellular membranes, and to establish links between particle size and toxic effects. The findings of this study support the view outlined by Moore et. al (2006) that lysosomal membrane fragility could act as a predictive indicator for cell injury and pathology and that it would be a useful biomarker of nanoparticle exposure for ecotoxicological monitoring.

The study also supports the view that long term exposure to nanoparticles in the aquatic environment is of particular concern. Shifts in the feeding, growth and reproductive strategy over the long term could lead to reduction in local populations of *L. Stagnalis*. However, there may be compensatory mechanisms which would minimise the consequences of nanoparticle pollution for population maintenance (Coutellec and Lagadic, 2006). It is clear that there is a need for further testing to determine the distinct threat that nanoparticles pose in the aquatic environment.

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