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Exposure of sticklebacks (*Gasterosteus aculeatus*) to cadmium sulfide nanoparticles: biological effects and the importance of experimental design

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Abstract

Effects of nanoparticles on aquatic organisms have been little studied to date and toxicological data are urgently needed for development of regulatory frameworks for these substances. Here we report the findings of a study exposing sticklebacks to cadmium sulfide (CdS) as bulk material and quantum dots. Fish were exposed for 21 days in a flow through test system to 5, 50 or 500 $\mu\text{g L}^{-1}$ CdS nanoparticles (nCdS) coated in thiol terminated methyl polyethylene glycol (MPEG), bulk CdS or MPEG at 500 $\mu\text{g L}^{-1}$ (nominal concentrations). With the exception of the highest nCdS exposure, predicted concentrations were approximately one order of magnitude below nominal. A single fish from each group (excluding MPEG) was

examined using Energy Dispersive X-ray analysis (EDX) to localise cadmium, however, cadmium could not be detected in whole body sections. Elevated levels of oxidized glutathione were measured in the gills of fish exposed to 50 and 500 μgL^{-1} nCdS. Induction of vitellogenin synthesis was not detected in any of the treatment groups. The number of males engaged in nest-building behaviour following exposure to 500 μgL^{-1} nCdS was reduced and livers of 4/6 fish in the same treatment displayed hepatocellular nuclear pleomorphism. The results are discussed emphasising the fundamental importance of experimental design and the need to understand the behaviour of nanoparticles in the aqueous phase.

Keywords: Nanotoxicology; *Gasterosteus aculeatus*; Biomarkers; Histopathology; Behaviour; Cadmium sulfide; Vitellogenin; Oxidative stress; engineered nanoparticles; ENPs

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Engineered nanoparticles (ENPs) are being developed due to their unique physico-chemical properties, including elevated surface:volume ratio, size-dependant optical properties, highly reactive nature and ability to penetrate cellular barriers (Li et al., 2003; Geiser et al., 2005). However, these same properties have precipitated concerns over their fate in, and effect on, the environment (Owen and Depledge, 2005). Evidence that ENPs can translocate along olfactory neurons (Oberdörster et al., 2005) potentially causing biochemical changes, such as oxidative stress in fish brains (Oberdörster et al., 2004), suggests that the behaviour of exposed individuals may be affected. The use of behavioural assays as biomarkers offers a means of detecting perturbations in the higher-level functions of animals

following contaminant exposure. The following study investigated the biological effects of ENPs (cadmium sulfide quantum dots coated in thiol-terminated methyl polyethylene glycol: nCdS) on the three-spined stickleback at different levels of response.

Nanoparticles were prepared by arrested precipitation of CdCl_2 and Na_2S in the presence of a thiolated polyethylene glycol. Powder X-ray diffraction showed that the material was in the cubic phase and the sample was purified using an ion exchange resin. The nanoparticles were $4.2 \text{ nm} \pm 1 \text{ nm}$ and readily dispersed in water without the requirement for interfering surfactants (Zhu et al., 2006). Six tanks (5 treatment and 1 control) of sticklebacks were exposed for 21 d in brackish water (5ppt) within the flow through system described by Katsiadaki et al. (2007). Each tank comprised six interconnected compartments housing 5 separated males, which had visual access to 8 communally held females. The five treatment tanks received stock solutions via a peristaltic pump producing nominal exposures to 5, 50 or $500 \mu\text{g L}^{-1}$ nCdS, $500 \mu\text{g L}^{-1}$ bulk cadmium sulfide (CdS) and $500 \mu\text{g L}^{-1}$ thiol terminated methyl polyethylene glycol (MPEG). Water samples were collected on days 6, 13, 18 for Cd analysis via ICP-MS. All treatments were tested in duplicate using a different brood of fish for each replicate. Following the 21 d exposure, fish were humanely sacrificed, blood and tissue samples taken for analysis, and whole sticklebacks either underwent cryopreservation for EDX analysis or standard wax embedding. Single hematoxylin and eosin stained longitudinal sections were examined using a Nikon Eclipse E800 microscope and Lucia™ image capture software. Reduced and oxidized glutathione levels were measured in gill tissue according to Stephensen et al. (2002).

Stoica et al. (2007) reported the ability of Cd (II), provided as CdCl_2 , to bind to, and activate, the oestrogen receptor- α *in vitro* at concentrations as low as 10^{-12} M. The lack of vitellogenin induction in male sticklebacks in any of the treatments (ANOVA; $F_{5,49} = 0.2$, $p < 0.05$) suggests that intracellular levels of Cd^{2+} were below this level. Bulk cadmium sulfide

was used as a control so that the degree of dissociation of CdS to Cd²⁺ and S²⁻ ions could be accounted for. With the Cd²⁺ equilibration state being approximately equal in all treatments it was predicted that differences in biological responses, between the bulk and nanoform, would be attributable to properties of nanoparticles not leached Cd ions. The inability to identify intracellular nCdS via EDX, prevents conclusions being drawn on the potential effect such nanoparticle presence might have on intracellular Cd²⁺ levels and cellular homeostasis. The lack of nest building behaviour in all of the treatment levels of one of replicates, suggest that the fish were not in the correct physiological status for a spawning assay. For this reason, we only report the following results for one of the two replicate treatments. Averaged over the three sample points, cadmium sulfide concentrations were 37.6 (± 9.56) $\mu\text{g L}^{-1}$ in the CdS 500 $\mu\text{g L}^{-1}$ exposure and 149 (± 33.8), 6.09 (± 2.50) and 0.67 (± 0.21), in the 500, 50 and 5 $\mu\text{g L}^{-1}$ nCdS treatments. Average cadmium concentration in the MPEG treatment was 0.14 (± 1.14) $\mu\text{g L}^{-1}$ while control levels were below the limit of detection (< 0.8 , $\mu\text{g L}^{-1}$). The insolubility of CdS resulted in accumulation in the tank and delivery tube, necessitating periodic removal and potentially explaining the discrepancy between nominal and measured CdS concentrations. Therefore, the difference in the total Cd between the nCdS and CdS treatments prevents direct comparison on the effects of the two forms. Exposure to all the treatments reduced the number of males building nests (Fig. 1A), with a significant reduction in individuals exposed to 500 $\mu\text{g L}^{-1}$ nCdS (Fisher exact test, OR = 0.00, $p = 0.048$). The livers of exposed sticklebacks exhibited hepatocellular nuclear pleomorphism (Fig. 2), with the most severe cases recorded in individuals exposed to 500 $\mu\text{g L}^{-1}$ nCdS (Fig. 1A); this liver pathology was not observed in the control treatment. Elevated levels of oxidized glutathione were measured in the gills of fish exposed to 50 and 500 $\mu\text{g L}^{-1}$ nCdS but not in the group exposed to bulk CdS or 5 $\mu\text{g L}^{-1}$ nCdS (Fig. 1B); differences were not significant due to low number of individuals measured.

The results of this pilot study indicate liver pathology and compromised reproductive fitness of male sticklebacks is associated with (nominal) exposure to 500 μgL^{-1} nCdS and highlights the need for assessing inter-individual variability and susceptibility to nanoparticle exposure in current assessment methodologies. Future studies, analysing protein expression and nest construction may provide further information on the mechanisms influenced by nCdS. The propensity of many ENPs to agglomerate (eg. carbon nanotubes) in aqueous media raises questions as to their fate in the aquatic environment. The inability of the current study to identify or localise nCdS within the small number of exposed individuals tested using EDX analysis highlights the need for further studies on uptake routes and subsequent translocation and distribution of nanoparticles *in vivo*. Future studies, with higher levels of replication, should consider technical aspects of the dosing route to achieve better parity between nominal and measured exposures and ensure that individuals are of a similar physiological status where behavioural studies are assessed.

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Figure captions

Fig. 1. (A) The number of males that completed a nest following exposure to freshwater (C, control), 500 μgL^{-1} cadmium sulfide (Cds Bulk); 500 μgL^{-1} capping agent (MPEG), 5, 50 or 500 μgL^{-1} cadmium sulfide nanoparticles (nCds); Five males recorded per treatment, except for CdS bulk treatment where four individuals were assessed. Numbers in bold represent the proportion of individuals displaying hepatocellular nuclear pleomorphism out of the total number of fish examined per treatment. (B) Oxidized glutathione (GSSG) expressed as a percentage (%) of total glutathione (GSH) present in gill tissue of sticklebacks exposed to freshwater (C, control), 500 μgL^{-1} cadmium sulfide (Cds Bulk); 500 μgL^{-1} capping agent (MPEG), 5, 50 or 500 μgL^{-1} cadmium sulfide nanoparticles (nCds). Five males analysed per exposure group. Nest building activity and levels of oxidised GSH did not respond in a clear dose dependant manner, however, statistical analysis and interpretation is constrained by the level of replication.

Fig. 2: Haematoxylin and eosin stained liver section showing example of hepatocellular nuclear pleomorphism (arrow) in stickleback exposed to 500 μgL^{-1} nCd. The normal trabecular structure of the liver appears relatively unaffected. Bar = 50 microns.

Figure 1

Figure 2