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Intravenous injection of unfunctionalized carbon-based nanomaterials confirms the minimal toxicity observed in aqueous and dietary exposures in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Highlights

- Trout were injected with carbon-based nanomaterials: nanotubes, C₆₀, carbon black
- Carbon-based nanomaterials were observed in kidneys of fish
- Only carbon nanotubes caused elevated TBARS in spleen
- No treatment-based lesions in tissues of fish were recorded
- Conclude minimal environmentally relevant toxicity of carbon nanomaterials in trout

Graphical abstract

Intravenous injection of unfunctionalized carbon-based nanomaterials confirms the minimal toxicity observed in aqueous and dietary exposures in juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Numerous ecotoxicology studies of carbon-based nanomaterials (CNMs) have been conducted in fishes; however, different approaches have been used to make CNM dispersions and dose tanks for aqueous exposures, and to prepare food containing CNMs for dietary studies. This diversity of experimental methods has led to conflicting results and difficulties in comparing studies. The objective of the present study was to evaluate intravenous injection of unfunctionalized CNMs in rainbow trout (*Oncorhynchus mykiss*), as a means of delivering a known internal dose, on tissue biochemistry and histopathological lesions; then, subsequently, to compare the results with our previous work on aqueous and dietary exposures of rainbow trout to CNMs. Rainbow trout were injected in the caudal vein with corn oil dispersions of 200 μg (approximately $1 \mu\text{g g}^{-1}$) of either the fullerene C_{60} , single-walled carbon nanotubes (SWCNTs), or amorphous carbon black. After 96 h, injected fish were euthanized and tissue samples collected for biochemistry and histology. Histological examination of the kidney of fish injected intravenously indicated the presence of black material consistent with the injected carbon treatments. However, there were no additional lesions associated with CNM exposure compared to controls. There were also no significant changes in haematology, or ionoregulatory disturbance in blood plasma among the intravenously injected fish. Significant elevation in lipid peroxidation (thiobarbituric acid reactive substances TBARS) was detected only in kidney and spleen of fish injected with SWCNTs, but not the other carbon treatments. The elevated TBARS following injection contrasted with CNMs delivered via aqueous or dietary routes in our previous studies, suggesting that the latter exposure routes may not lead to absorption and toxicity of the internal tissues. Comparison of the effects of injected CNMs with aqueous and dietary CNMs exposures indicates that these materials are of minimal environmentally-relevant toxicity in rainbow trout.

Capsule:

Intravenous injection of unfunctionalized carbon-based nanomaterials (CNMs) confirms the minimal toxicity observed in aqueous and dietary exposures of CNMs in rainbow trout

Key words: carbon nanotubes; rainbow trout; toxicity; bioavailability; C₆₀

1. Introduction

For the last fifteen years there has been considerable controversy regarding the potential for engineered nanomaterials (ENMs) and nanoparticles (NPs) to cause adverse effects in organisms after these materials are released into the environment. The physicochemistry of some ENMs suggests some cause for concern e.g., persistence, reactive surface chemistries and high aspect ratio materials. However, despite intensive research on the ecotoxicology of ENMs over the last fifteen years (e.g., see reviews by Handy et al. 2012; Schultz et al. 2014), there is no consistent evidence of specific nanoscale properties being linked to toxic effects. Among the challenges for establishing consistent evidence are the difficulties of making comparisons among different ecotoxicology studies that have been reported in the literature. A critical comparison of the ecotoxicology studies of TiO₂-NPs in rainbow trout (*Oncorhynchus mykiss*) in which similar test endpoints were assessed across multiple routes of exposure have been effective in resolving some uncertainties of individual tests (Boyle et al. 2013a), and similar approaches should be applied for other ENMs.

Among the ENMs in which published ecotoxicology research provide differing and conflicting information are carbon-based nanomaterials (CNMs) that include fullerenes (C₆₀, nC₆₀ in agglomerative state), and single- and multi-walled carbon nanotubes (SWCNT, MWCNT). Initial studies of C₆₀ toxicology in fish reported oxidative damage (peroxidation of lipids) in brain of largemouth bass (*Micropterus salmoides*; Oberdörster, 2004) and oxidative stress in zebrafish embryos (*Danio rerio*; Zhu et al. 2007). Subsequently, as knowledge about methods for testing toxicity of CNMs improved, these previous reports of C₆₀ toxicity have been attributed to decomposition products of the tetrahydrofuran solvent used to improve water solubility of C₆₀ (Henry et al. 2007; reviewed by Henry et al. 2011; Zhang et al. 2009). The methods used to prepare suspensions of other CNMs may have also introduced experimental artefacts into previous studies. For example, carbon nanotubes have

also been shown to chemically alter dispersing agents with potential for generation of toxic degradation products not present in corresponding solvent controls (Wang et al. 2013). Metal impurities adsorbed to CNMs during synthesis may also be mobilised by ultrasonication (Toh et al. 2012) and have been shown to contribute to effects in fish (Hull et al. 2009). As the field of nanoecotoxicology matures, and knowledge widens, there is a need to retrospectively review and contextualise previous results. This process is especially pertinent when advanced characterisation techniques e.g., Raman spectroscopy to measure tissue accumulation of CNMs (Edgington et al. 2014) and testing regimens more suited to maintaining stable aqueous dispersions of CNMs (e.g., Boyle et al. 2015) are developed, become more widely used, and can strengthen previous observations.

Much of the controversy of CNM-induced toxicity has focussed on the capacity of CNMs to generate reactive oxygen species (ROS) and cause oxidative stress in exposed organisms (Henry et al. 2011; Markovic and Trajkovic, 2008). Contrary to expectation, activities of antioxidant enzymes were consistently decreased in goldfish (*Carassius auratus*) exposed to MWCNT dispersed in deionised water (Yan et al. 2016). In contrast, total glutathione levels were shown to be elevated in liver of *Fundulus heteroclitus* exposed to concentrations of up to 10 mg C₆₀ L⁻¹ prepared without solvents (Blickley and McClellan, 2008). Similarly, concentrations of reduced glutathione (GSH) were also decreased in brain of juvenile goldfish (*Carassius auratus*) exposed to nC₆₀ suspended in water after long-term stirring (Zhu et al. 2008). However, Zhu et al. (2008) also measured significantly lower levels of ROS-induced injury (lipid peroxidation) in fish exposed to nC₆₀ compared to controls, suggestive of a possible protective effect of the materials to ROS. Indeed, C₆₀ has been shown to act as both an anti-oxidant and pro-oxidant during a 4 h time-course after spiking into tissue homogenates in vitro (Ferreira et al. 2012). However, the ecological relevance of the findings of Ferreira et al. (2012) is unclear since the approach used presumes C₆₀ is

bioavailable and the effects reported in other in vivo studies were mediated by tissue accumulation of CNMs.

The relationship between bioaccumulation and toxicity of CNMs appears complex. In part, this has been due to challenges in routinely quantifying CNMs against a high background of carbon in biological samples and there has been a paucity of studies reporting both tissue measurements and toxicity endpoints in organisms in the same study.

Agglomerates of nC₆₀ were observed to adhere to, but did not traverse, the chorion of *F. heteroclitus* embryos (Blickley and McClellan, 2008). Shinohara et al. (2009) also demonstrated C₆₀ did not translocate to the brain in carp (*Cyprinus carpio*) during aqueous exposure, challenging previous interpretations of mechanisms of toxicity (e.g., Zhu et al. 2008). Where techniques to detect carbon nanotubes in tissues have been applied e.g., near-infrared fluorescence to detect intestinal uptake, no tissue accumulation has been observed in fathead minnow [*Pimephales promelas* (Bisesi et al. 2014, 2015)]. Nevertheless, some minor histopathological changes in gut morphology of fathead minnow were observed following oral gavage with carbon nanotubes which was suggestive of a surface mediated effect (Bisesi et al. 2014).

Previously, we have reported mixed results on the effects of aqueous and dietary exposures to SWCNTs and C₆₀ in rainbow trout. Ten-day aqueous exposure to SWCNT caused brain lesions in juvenile rainbow trout but was also observed to cause dose-dependent decreases in brain thiobarbituric acid-reactive substances [TBARS, an indicator of lipid peroxidation (Smith et al. 2007)]; however, neither of these effects were reproducible in a later study (Boyle et al. 2014). Six-week dietary exposure to elevated concentrations (500 mg/Kg feed) of C₆₀ and SWCNT also caused no effects on fish growth or survival and there were no histological or biochemical lesions (Fraser et al. 2011). The reasons for the

differences in effects observed in these studies are unclear and it is unknown if the CNMs were bioavailable and accumulated in fish during these exposures.

The aim of the present study was to critically compare previous studies conducted in our laboratory, and other studies in the literature on the ecotoxicology of aqueous and dietary exposures of CNMs in rainbow trout, with new information on the effects of intravenous injection of these materials. In this study we investigated the toxicity of SWCNT, C₆₀ and amorphous carbon (carbon black; an additional control), dispersed in corn oil and injected into the caudal vein of rainbow trout. Whilst not relevant to CNM exposures in the field, injection of a known dose of CNMs into the blood circulation circumvents the issue of absorption at the gill or intestine as an unknown factor, and observations of toxicity after injection will therefore aid interpretation of effects observed in our earlier studies (e.g., Boyle et al. 2014). In addition, we also injected a separate second group of fish with CNMs intramuscularly. Later, histological examination of the muscle at the injection site, would give information on the mobility of CNMs in tissue and the cellular response to these materials. Furthermore, observations of the appearance of CNMs in muscle would aid our identification of CNMs in other internal tissues of the intravenous injected group.

2. Materials and methods

2.1 Experimental animals

Juvenile rainbow trout were obtained from a local supplier (Torre fishery, Watchet, Somerset) and maintained in a semi-closed system with aerated, dechlorinated and recirculating Plymouth tap water (in mM, means $\pm$ SD): 0.34 ± 0.01 Ca²⁺; 0.03 ± 0.01 K⁺; 0.05 ± 0.01 Mg²⁺; 0.40 ± 0.02 Na⁺; pH 6-7. Fish were fed twice daily with a commercial trout diet (EWOS, Westfield, UK) until 48 h prior to the commencement of the experiment to allow evacuation of the gut prior to handling and injection.

2.2 Stock suspensions of carbon materials

Stock suspensions of 2 mg mL⁻¹ SWCNT (Cheap Tubes Inc., Brattleboro, VT, USA), C₆₀ (purity 99.9%, SES Research, Houston, TX, USA) and carbon black (kindly donated by Dr V. Stone, Heriot-Watt University) were prepared in autoclaved corn oil (Mazola®, ACH Foods, Spain). Stocks were dispersed with sonication (6 h, 35 W, 35 kHz frequency, Fisherbrand FB 11010, Germany) and then vortexed (10 sec) immediately prior to injection. These are the same materials as previously characterized and used by Boyle et al. (2014), Fraser et al. (2011) and Henry et al. (2007). The appearance of the prepared corn oil-carbon material suspensions differed, with the C₆₀ preparation having a red/purple colour, while carbon black and SWCNT preparations were black (Figure 1). Trace metal impurities in corn oil stocks of CNMs were measured using inductively coupled plasma mass spectrometry and compared to analytical standards following digestion of 100 µL material (200 µg CNM) in 1 mL trace analysis grade HNO₃ (Fisher). No impurities were measurable in C₆₀ or carbon black preparations, or, in the corn oil vehicle, but traces of Fe (0.08 ng µg⁻¹ SWCNT), Co (0.39 ng µg⁻¹ SWCNT) and Mo (0.19 ng µg⁻¹ SWCNT) were associated with the SWCNT suspension.

2.3 Intravenous and intramuscular injections

Trout (227 ± 38 g, mean ± standard deviation, $n = 35$; $n = 7$ per treatment) were anaesthetised in buffered MS222 (50 mg L⁻¹, pH 7) and injected in caudal vein with corn oil (100 µL, control), SWCNT, C₆₀ or carbon black (200 µg in 100 µL corn oil) with a 1 ml Terumo insulin syringe with 29 gauge needle (Terumo Medical Corporation, Somerset, NJ, USA). A further $n = 35$ fish ($n = 7$ per treatment) were also injected with CNMs (or corn oil, controls) intramuscularly near the anterior insertion of the dorsal fin. Other than the excision and histological examination of muscle tissue immediately surrounding the site of injection after 96 h, no other analyses were performed on these fish. Immediately after injection, trout

were revived in oxygen saturated system water and returned to treatment-marked tanks in a recirculating semi-closed system (see above). No fish exhibited signs of poor health following injection and all fish fed as normal after the exposure. Fish were fed twice daily thereafter until 96 h post-injection.

2.4 Tissue excision and blood analyses

Tissue and blood analyses were performed for $n = 7$ initial un-injected fish (0 h) and $n = 7$ intravenously injected fish per treatment at 96 h post-injection. Fish were terminally anaesthetised in pH buffered MS222, blood withdrawn from caudal puncture avoiding site of injection using a heparinised syringe (1 mg mL^{-1} Li salt) and tissues excised, weighed, snap frozen in liquid N_2 and stored at -80°C until required (see section 2.6). Sub-samples of whole blood were taken for measurement of haematocrit (HCT) and haemoglobin (Drabkin's reagent, Sigma-Aldrich). The remaining blood was centrifuged (13000 rpm, 2 min) and osmolality (Osmomat 030, Gonotec, UK), Cl^- (Jenway chloride meter PCLM3, Bibby Scientific, UK), Na^+ and K^+ (Model 420 Flame Photometer, Sherwood Scientific Ltd, UK) measured in the plasma.

2.5 Histopathology

Tissue samples for histological examination were obtained at the same time as other samples collected at 96 h post-injection. Procedures for preparation of histological section were identical to those previously described (Boyle et al., 2013a). After excision, internal tissues (kidney, and spleen) were collected from intravenous injected fish, and skeletal muscle around and including the site of fish injected intramuscularly, were preserved in 10% neutral buffered formalin and processed for routine histological examination by light microscopy.

2.6 Biochemical analyses

Total glutathione and TBARS in tissues were analysed according to protocols used in our previous publications (e.g., Boyle et al. 2014). In brief, snap frozen tissues were defrosted on ice and homogenised in buffer containing (in mM): 300 sucrose, 20 HEPES, 0.1 EDTA, pH 7.8 (All reagents Sigma-Aldrich, UK), centrifuged at 13000 rpm for 2 min, and supernatants analysed for GSH and TBARS. GSH was measured in solutions with final assay concentrations of (in mM): 76.5 phosphate buffer at pH 7.5, 3.8 EDTA, 0.6 DTNB, 0.2 NADPH and glutathione reductase at 0.12 U/mL with absorbance read at 412 nm for 20 min. TBARS were measured in de-proteinated homogenates (50% trichloroacetic acid) treated with butylated hydroxytoluene (1 mM) according to an original protocol by Camejo et al. (1998). Total protein was quantified with Bradford's reagent according to the manufacturer's protocol (Sigma-Aldrich, UK).

2.7 Data handling and statistical analyses

Statistical analyses were performed using SigmaPlot (v. 13.0 for Windows). All data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Brown-Forsythe test). Where data were not normally distributed, data were $\log_{10}$ transformed, and if $\log_{10}$ transformation did not improve normality of data, suitable non-parametric tests were used for analyses on untransformed data. Statistically significant differences between treatment groups at 96 h were determined by one-way ANOVA with the Holm-Sidak test *a posteriori*. Where significant differences were observed between treatment groups and the control at 96 h, an additional comparison was also made to the 0 h pre-injection controls to further interpret the magnitude of effect observed. Comparison with 0 h pre-injection controls was tested using a Student's *t*-test or Mann-Whitney U test where appropriate. All data are reported as means $\pm$ SEM. A *p* value of < 0.05 was considered significant.

3. Results

3.1 Blood parameters and plasma ions

There were no indications of ionoregulatory disturbances in plasma or haematological perturbations in fish injected with carbon compared to control fish injected with corn oil at 96 h (one-way ANOVA and Kruskal-Wallis tests, $p > 0.05$, Table 1). Observation of the data would suggest subtle changes in several parameters especially haemoglobin concentration and osmolality between 0 and 96 h. These were not assessed with statistical tests but may have arisen due to stress of handling of fish for the injection, temporal fluctuations or been due to the corn oil vehicle; an un-injected control group was not included in the comparison at 96 h.

3.2 Histological observations

Histological examination of the site of intramuscular injection demonstrated the presence of agglomerates of black material for fish injected with C₆₀, SWCNTs, or carbon black that was not observed in corn oil control injected fish (Figure 1). The black material was similar in appearance for each treatment and was found around muscle fibre bundles and within blood vessels in the tissue sections. The distribution of black material varied within the section relative to the location of injection, which was also indicated by presence of haemorrhage and necrosis of individual muscle fibres. There were no inflammatory cells associated with the black material, no black material was observed inside cells and within the blood vessels permeating the muscle blocks, the black material appeared freely dispersed rather than associated with blood cells. The presence of clear/opaque material (COM) in histological sections of injected skeletal material was presumed to be the corn oil used as the dispersant media for the carbon materials. However, this conclusion is based on previous histological examinations of muscle (and kidney and spleen) of trout (e.g., as performed by

Boyle et al. 2013b) and no oil-free sham-injury control fish or specific staining for oil deposits were performed in the present study.

Histological examination of the kidney and spleen indicated normal tissue architecture with no appreciable lesions in control fish relative to the expected morphology of healthy cultured rainbow trout (Figure 2). The normal presence of melanin and melanomacrophage aggregates in rainbow trout was observed and was present in both the kidney and spleen of all sections examined. In fish that received an injection of corn oil, with or without carbon materials, the presence of COM was observed in both kidney (Figure 2) and spleen. Deposits of COM varied in size (tens to hundreds of μm in diameter) but had a consistent bubbly appearance within the parenchyma of tissues and also when observed within blood vessels of the examined spleen and trunk kidney. Occasionally, black material was observed around the margins of the COM and adjacent to tissue cells in kidney, and this black material appeared to have a different texture and colour compared to the melanin deposits present within the tissues. This black material was similar in appearance to that observed in histological sections of skeletal muscle from fish injected with CNMs intramuscularly i.e., as shown in Figure 1. Cells that were observed to be in close proximity to this black material did not appear to differ in morphology relative to other cells except perhaps a minor compression of some cells near the COM. Occasional minor lesions in individual cells (e.g., necrosis) or presence of cellular debris within vessels were observed in all tissue sections examined (controls and carbon injected) with no evidence of differences among treatments.

3.3 Biochemical markers of oxidative injury

There were increased lipid peroxidation products in kidney and especially spleen of fish injected with SWCNT, that were not observed in fish injected with carbon black or C_{60} (Figure 3). In kidney, $0.41 \pm 0.06 \text{ nmol TBARS mg}^{-1} \text{ protein}$ were measured in fish injected

with corn oil compared to 0.71 ± 0.07 nmol TBARS mg^{-1} protein in fish injected with SWCNT (one-way ANOVA, $p < 0.001$). However, there was no significant difference between fish injected with SWCNT and 0 h controls (Student's t -test, $p = 0.227$). In spleen, this effect of SWCNTs was more pronounced and TBARS was elevated to 3.83 ± 0.45 nmol mg^{-1} protein compared to 0.72 ± 0.15 , 1.03 ± 0.17 and 1.69 ± 0.60 nmol TBARS mg^{-1} protein in spleen of fish exposed to corn oil alone, carbon black and C_{60} , respectively. Furthermore, there was a significant elevation compared to pre-injection 0 h values (Student's t -test, $p = 0.001$). There were no significant differences in liver or brain lipid peroxidation among the treatments (Figure 3).

There were minor perturbations in GSH in kidney of fish injected with CNMs but there were no significant differences compared to control fish (Figure 4). Concentrations of GSH in liver were also unaffected. There was a small significant decrease (approximately 20%) in GSH in brains of fish injected with C_{60} when compared with 96 h control fish (one-way ANOVA, $p < 0.001$) but not when compared to pre-injection controls (Mann-Whitney U test, $p = 0.053$). There was also no effect of injection of carbon NPs on activity of acetylcholinesterase in brains of fish (data not shown). Mean activities ranged from 67.1 to 72.4 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ protein in fish in all treatment groups at 96 h.

4. Discussion

The main aim of the present study was to provide additional context to our previous studies of aqueous and dietary exposures to CNMs, primarily SWCNT, in trout and especially the interpretation of effects where CNM bioavailability and accumulation was unknown (i.e. Boyle et al. 2014; Fraser et al. 2011; Smith et al. 2007; see Table 2 for summary of effects observed in each study). The need to provide explanations for conflicting reports of CNM toxicity have been the foci of previous review articles (e.g., Liu et al. 2013),

but this current study is the first to bring together directly comparable effects data of aqueous, dietary and intravenous exposures to provide a hazard assessment for pristine SWCNTs in trout. Single-walled carbon nanotubes injected into rainbow trout caused small elevations in lipid peroxidation in kidney and spleen that were not seen in fish injected with the other carbon allotropes. This pattern of toxicity was also different to our previous reports of aqueous and dietary exposures (Boyle et al. 2014; Fraser et al. 2011; Smith et al. 2007; Table 2).

Deposits of what were likely agglomerates of SWCNTs and carbon black were visible in kidney of intravenously injected trout, but were not apparent in controls. These were also similar in appearance to those observed in muscle of fish injected intramuscularly. These visible agglomerates were almost certainly extracellular and were at the edge of opaque deposits which were likely the corn oil vehicle although specific staining for oil deposits was not performed in this study. Micron sized particulate matter has been shown to partition to the kidney in fish with smaller (including nanoscale) particles accumulating in the spleen (Furukawa et al. 2002). Similar observations that have been made in rodents suggest that the size of particles may determine the distribution to filtration organs (Umbreit et al. 2012). In previous studies where TiO₂-NPs were injected into the caudal vasculature of trout, the kidney was identified as the principle site of accumulation with negligible partitioning observed to other tissues, except spleen (Boyle et al. 2013a; Scown et al. 2009); the high ionic strength physiological saline used in preparing NPs for injection into trout likely promoted larger agglomerates of the material. Scown et al. (2009) reported the presence of agglomerates of TiO₂-NPs between 0.5 - 2 µm in vesicles surrounding the kidney tubules; but the lack of change in concentrations of Ti detected in the tissues through the 90 day experimental period suggested that the NPs did not enter excretory pathways. Whether C₆₀ exhibited similar patterns of accumulation is unclear. No material resembling agglomerates of

C₆₀ was observed in histological sections of kidney with light microscopy, but agglomerates were observed in muscle of fish injected intramuscularly, and it is entirely possible that C₆₀ present in the kidney was overlooked in our analysis. It is also likely that the C₆₀ prepared at 2 mg mL⁻¹ in corn oil was better dispersed than the other CNMs because the suspension had the purple hue associated with the dispersed phase of the material and is similar to the C₆₀ toluene solution (Colvin, 2003).

In our previous study, the accumulation of TiO₂-NPs in the kidney and spleen did not result in changes in biomarkers of oxidative stress, TBARS and GSH, or lesions in the tissues (Boyle et al., 2013a). In the present study, intravenous injection of SWCNT caused elevations in TBARS in kidney and spleen indicating that tissue-specific accumulation (in kidney) was associated with toxicity. No elevations of TBARS in kidney or spleen were observed in fish injected with C₆₀ or amorphous carbon black. It is more likely that these CNMs did not cause lipid peroxidation, rather than that these materials did not accumulate in these tissues, because the presence of carbon black, at least, was confirmed by black aggregates in the kidney.

Much has been written about the contributions of metal contaminants in SWCNT suspensions, especially Co, Mo and Ni, to toxicity (Hull et al. 2009; Liu et al. 2007; Pulskamp et al. 2007). In environmental waters, CNMs including carbon nanotubes are predicted to rapidly adsorb trace metals (e.g., Li et al. 2003) and risk assessment of both CNMs and trace metals should include consideration of these mixtures (Hull et al. 2009). However, the presence of trace metal impurities in tests with CNMs may also obscure effects attributable to the CNMs themselves. While we cannot completely discount a role of Co and Mo in the observed effects of SWCNT (Ni was too low to be quantifiable), acid digestion of the corn oil suspension indicated that the total leachable metallic contaminants were very low, < 0.1% w/w of injected SWCNT, and unlikely to cause toxicity in trout (see Bisesi et al. 2014

and references therein for similar interpretation). Furthermore, where previously we measured Co in fish exposed to aqueous and dietary SWCNT, no change in Co concentration was observed in any tissue (Fraser et al. 2011; Smith et al. 2007).

The importance of morphology to toxicities of CNMs has been explored in vitro and in agreement with the present study, observations in cell culture systems have indicated SWCNT may be more toxic than C₆₀ (e.g., Jia et al. 2005). The SWCNTs used here were 5-30 µm in length and structure activity relationship modelling has indicated that the disparity in toxicity of CNMs may be due to the high aspect ratio of CNTs (Wang et al. 2014). Tubes of this length have been shown to lead to frustrated phagocytosis and pro-inflammatory responses in macrophages in vitro (shorter tubes did not; Boyles et al. 2015). This mode of toxic action is unlikely to be specific to CNMs. In the gastrointestinal tract of zebrafish larvae, long aspect ratio CeO₂ rods (> 1 µm length) caused blunted microvilli and compromised digestive processes while shorter nanorods and nanospheres did not (Lin et al. 2014). The presence in spleen of SWCNTs, but not other CNMs, could therefore increase TBARS in the tissue.

Reports of CNMs causing oxidative stress in brains of fish have attracted much controversy and caution has been suggested when extrapolating biochemical measurements to physiological effects (e.g., Henry et al. 2011; see discussion in Windeatt and Handy, 2013). Declines in the total GSH concentrations in brains of trout injected with C₆₀ could be interpreted as material mediated changes in redox status. However, concentrations of GSH were not different from 0 h controls suggesting that these values may have been within the normal physiological range of rainbow trout. C₆₀ has previously been shown to act as both a pro-oxidant and antioxidant in fish brain homogenates in vitro (Ferreira et al. 2012). Regardless, the accumulation of injected C₆₀ in the brains of trout is unclear. In aqueous exposures with C₆₀, Shinohara et al. (2009) reported no accumulation in brains of carp

(*Cyprinus carpio*). Intraperitoneal injection of hydroxylated fullerenes at much higher concentrations than used in the present study also caused no pathology in the brains of fathead minnow (*Pimephales promelas*) after 72 h, but did result in obvious and measurable changes in structure of the kidney; suggesting either preferential accumulation of C₆₀ in the kidney of minnows over the brain, or simply that the brain is well defended from oxidative stress compared to other internal organs (Jovanović et al. 2014).

Together with our previous studies of aqueous and dietary exposures to SWCNT and C₆₀ in rainbow trout, data presented in this study support a position of minimal environmentally relevant effects that are attributable to pristine CNM exposures (Table 2). The magnitude of effects we observed was small, with data for TBARS in spleen only, raising some concerns over SWCNT, but this was not supported by histopathology results in the same tissue. During aqueous exposure to SWCNT in trout, similar elevations in TBARS in spleen were not observed, and Boyle et al. (2014) reported few effects in trout overall. A similar conclusion of minimal effects of SWCNT and C₆₀ was also made for dietary exposures in trout (Fraser et al. 2011). Where we have previously reported differing levels of effects in tissues of trout during aqueous exposures, the discrepancies observed were likely attributable to the different methods used to produce dispersions of SWCNTs and especially the higher solvent concentration used in our earlier aqueous study in which we reported greater toxicity (Boyle et al. 2014; Smith et al. 2007; Table 2).

In conclusion, there is an urgent need for critical comparison of previous studies on the ecotoxicology of engineered nanomaterials and nanoparticles to enable conclusions to be reached and improve the utility of these studies for environmental risk assessments. Particular attention should be given to the methods used to prepare suspensions of CNMs and the carry-over of impurities including trace metals to toxicity tests. Injection of CNMs prepared in corn oil in trout led to detectable accumulation of the materials in tissues but caused minimal

effects. Critical comparison of these data with previous aqueous and dietary exposures suggest that CNMs may not be bioavailable, and any effects although minor, may have been mediated at external epithelia, such as the gills as observed for TiO₂-NPs (Boyle et al. 2013b) and the gut in dietary studies with SWCNT (Bisesi et al. 2014, 2015).

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Figure legends

Figure 1. Photomicrographs (100 × objective) of histological sections of skeletal muscle of juvenile rainbow trout collected 96 h after intramuscular injection with carbon black (A and B), C₆₀ (C) and single-walled carbon nanotubes (D and E) near the anterior insertion of the dorsal fin. Agglomerates of black material (arrows) were present between the interstitial spaces of skeletal muscle (A, C, D) and within blood vessels near site of injection (B and E). The globular unstained material within the blood vessels to which some black material appears associated is presumably the corn oil vehicle used in the injection. Similar black deposits were not observed in photomicrographs of muscle from corn oil injected control fish. The scale bar in all panels = 100 µm. The visual appearances of the carbon nanomaterial stocks prepared in corn oil are shown in the bottom right panel.

Figure 2. Photomicrographs (100 × objective) of histological sections of spleen and trunk kidney of juvenile rainbow trout collected 96 h after intravenous injection with corn oil containing carbon nanomaterials. The photomicrographs show corn oil injected control spleen with normal appearance of tissue architecture and melanin deposits (A); corn oil injected control trunk kidney with normal appearance of renal tubules and melanin deposits (B); clear/opaque material (COM) present within trunk kidney of two separate corn oil injected control fish (C and D); black material observed at the margins of some areas of COM in trunk kidney in single-walled carbon nanotube injected fish (E), and carbon black injected fish (F). Similar black deposits were not observed in trunk kidney of control fish or fish injected with C₆₀, or, in spleens of fish from any treatment group. The scale bar in all panels = 100 µm.

Figure 3. Concentrations of thiobarbituric acid reactive substances (TBARS; nmol mg⁻¹ protein) in kidney (A), spleen (B), liver (C) and brain (D) of un-injected rainbow trout at 0 h (closed bars) and 96 h after intravenous injection with corn oil (open bars), carbon black (C Black; open, hatched bars), SWCNT (grey bars) and C₆₀ (grey bars, hatched). Data are means $\pm$ S.E.M. ($n = 7$). Bars with different lower case letters are significantly different at day 4 (one-way ANOVA, $p < 0.05$). Where significant differences to time-matched controls at 96 h were detected, treatments were then compared to 0 h controls (#, Student's t -test, $p < 0.05$).

Figure 4. Concentrations of total glutathione (GSH; nmol mg⁻¹ protein) in kidney (A), liver (B) and brain (C) of un-injected rainbow trout at 0 h (closed bars) and 96 h after intravenous injection with corn oil (open bars), carbon black (C Black; open, hatched bars), SWCNT (grey bars) and C₆₀ (grey bars, hatched). Data are means $\pm$ S.E.M. ($n = 7$). Bars with different lower case letters are significantly different at 96 h (one-way ANOVA, $p < 0.05$). GSH levels in brain of fish exposed to C₆₀ were not significantly different to 0 h controls (Mann-Whitney U test, $p > 0.05$).

Table 1. Haematological parameters and plasma ion concentrations in un-injected rainbow trout at 0 h and 96 h after intravenous injection with 100 µl corn oil (control), 200 µg carbon black, 200 µg SWCNT or 200 µg C₆₀ (each in 100 µl corn oil).

	Time (h)	Control	Carbon Black	SWCNT	C ₆₀
Haematocrit (%)	0	36.9 ± 1.0			
	96	35.7 ± 0.9	37.0 ± 1.1	35.1 ± 0.3	36.5 ± 0.8
Haemoglobin (g L ⁻¹)	0	77.1 ± 1.6			
	96	87.4 ± 1.1	96.2 ± 3.6	88.6 ± 2.5	90.4 ± 3.2
Osmolality (mOsm Kg ⁻¹)	0	323.1 ± 4.7			
	96	308.7 ± 3.1	310.0 ± 3.9	312.3 ± 3.1	307.1 ± 5.1
Plasma Cl ⁻ (mmol L ⁻¹)	0	195.7 ± 2.1			
	96	191.7 ± 2.2	189.0 ± 3.4	204.3 ± 4.0	197.4 ± 7.2
Plasma Na ⁺ (mmol L ⁻¹)	0	130.9 ± 1.6			
	96	132.2 ± 3.1	127.5 ± 1.0	128.5 ± 1.0	125.1 ± 4.5
Plasma K ⁺ (mmol L ⁻¹)	0	2.12 ± 0.11			
	96	2.38 ± 0.11	2.23 ± 0.03	2.30 ± 0.16	2.19 ± 0.09

Data are means ± SEM ($n = 6$ or 7 fish/treatment). No significant differences were found between treatments at 96 h (one-Way ANOVA and Kruskal-Wallis tests, $p > 0.05$).

Table 2. Comparative table of statistically significant (compared to time matched controls) effects of aqueous, dietary and intravenous exposures to carbon nanomaterials in rainbow trout.

Study	Exposure	Haematology	Histology	TBARS	GSH
Smith et al. (2007); Aqueous	SWCNT $\leq$ 0.5 mg L ⁻¹ (SDS vehicle)	↓ Hct and Hb	Pathologies in brain, liver	↓ Liver and brain	↑ liver
Fraser et al. (2011); Dietary	SWCNT, C ₆₀ 500 mg Kg ⁻¹ (SDS vehicle)	Not measured*	Mild pathologies in liver	↑ brain (temporal, SWCNT)	No effects
Boyle et al. (2014); Aqueous	SWCNT 0.25 mg L ⁻¹ (SDS vehicle)	No effects	Mild pathologies in kidney	No perturbations	↓ brain
Present (intravenous)	~1µg g ⁻¹ SWCNT, C ₆₀ , carbon black (corn oil vehicle)	No effects	No pathologies observed	↑ kidney, spleen (SWCNT)	↓ brain (C ₆₀)

SWCNT = single-walled carbon nanotubes; SDS = sodium dodecyl sulphate; Hct = haematocrit; Hb = haemoglobin; TBARS = thiobarbituric acid reactive substances; GSH = total glutathione; arrows (↑↓) denote significant increase (↑) or decrease (↓) in measured endpoint. *study measured plasma ions, only.

Figure 1.

Figure 2.

Figure 3.

Figure 4.