Ingestion of metal-nanoparticle contaminated food disrupts endogenous microbiota in zebrafish (Danio rerio)

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1. Introduction

Increased production and incorporation of nanoparticles (NPs) into consumer products will ultimately lead to release of NPs into the environment and potential for unintended NP exposure in humans and other organisms (Nowack, 2007). Some NPs have antimicrobial properties and such properties have been previously established and described for Ag-NPs and Cu-NPs (Sondi and Salopek-Sondi, 2004; Cioffi et al., 2005; Yoon et al., 2007; Ruparelia et al., 2008). The mechanisms behind the anti-microbial activity are partly described and their efficacy varies depending on particle size, particle shape and bacterial species (Panacek et al., 2006; Pal et al., 2007; Ruparelia et al., 2008). As a result NPs have been included in the formulations of consumer products for disinfection purposes [e.g., Ag-NPs (Cho et al., 2005)]. The ability to kill or impede the growth of pathogenic bacteria is an important aspect of human and veterinary medicine; however, if unintended exposure to NPs occurs and affects beneficial bacteria there is potential for negative effects on animal health.

Perhaps the most environmentally relevant route of NP exposure is through the diet, either via ingestion of food contaminated by NPs, or by consumption of food items (e.g., prey) that have accumulated NPs (Petersen and Henry, 2012). Filter-feeding aquatic invertebrates can accumulate NPs from the aqueous phase (Petersen et al., 2009) and substantially increase dietary exposure of consumers (e.g., organisms that eat invertebrates) to NPs (Park et al., 2010). NP contamination of food via deliberate close association between food and NPs is an issue of considerable concern for the food packaging industry, which has embraced incorporation of some NPs into packaging materials to maintain freshness and inhibit growth of microorganisms (Xu et al., 2010). The potential for food items to become contaminated by NPs used in packaging has been recognized and is a concern for human health that requires further investigation (Xu et al., 2010).

Previous research on the toxicity of ingested NPs has focused on determining whether or not NPs are absorbed across the gut, and the consequences for systemic toxicity [e.g., humans (Panessa-Warren et al., 2006), fishes (Ramsden et al., 2009; Fraser et al., 2011)]. Experiments have documented absorption of some NPs (e.g., Ag-NPs) across epithelial membranes (e.g., Scown et al., 2010); however, accumulation appears to be minimal in internal tissues,
and whether the accumulation is because of NPs or metal ions is unresolved (e.g., Ag-NPs, Scown et al., 2010; TiO2-NPs, Ramsden et al., 2009; Cu-NPs, Al-Bairuty et al., 2012). A neglected area of research in both animals and humans are the effects of ingested NPs on the major physiological functions of the gastrointestinal tract (GIT). For nutrition, these functions are gut motility, the secretion of fluids into the digestive tract, digestion, and the absorption of nutrients. Accumulation of some NPs within the gut lumen can influence gut motility in organisms (e.g., Heilman et al., 2011), and some lesions in epithelial mucosa of the gut associated with dietary NP-exposure have been reported (Smith et al., 2007; Federici et al., 2007).

The microbial community within the gut is an important contributor to organism health and the ingestion of NPs with antimicrobial properties may disrupt this community. The intestinal microbiome in organisms including humans is now recognized to form a complex symbiotic relationship that influences metabolism, physiology and gene expression (Kinross et al., 2009). A previous study reported that NPs may influence the gut microbiota of vertebrates: specifically, increased populations of lactic acid bacteria were detected in quail (Coturnix coturnix japonica) after ingestion of Ag-NPs (Sawosz et al., 2007). This preliminary study is interesting but the results are limited due to the culture-based microbiological approach used and the lack of a suitable Ag control (i.e. the experimental design does not allow for the differentiation of an Ag effect and an Ag-NP effect).

The zebrafish (Danio rerio) has become an important model species for the study of microbial communities in vertebrate intestines (Rawls et al., 2004, 2007), and this model has also been useful for assessing the toxicity of NPs (Henry et al., 2007; Park et al., 2010, 2011). Therefore, the primary objective of the present study was to make an initial assessment of the effect of dietary Cu-NPs or Ag-NPs on gut microbial communities in adult zebrafish. In addition to evaluation of the gut microbiota, the presence of lesions in the intestinal epithelial mucosa after exposure to NPs was assessed by electron microscopy. While uses of Cu-NPs are emerging they do not currently present substantial exposure risk in organisms; however, Cu is an important environmental toxicant and selection of Cu-NPs for this study was based on the interest to compare results with Ag-NPs, evaluate differences between nano and bulk forms of Cu, and to enable comparison with previous studies of Cu-NPs (e.g. Grifftit et al., 2007; Shaw et al., 2012).

2. Materials and methods

2.1. Experimental diets

A basal diet was formulated to provide ca. 40% protein and 6% lipid (Table 1). This diet was used for the control group. Treatment diets containing NPs (Fig. 1) were prepared by adding the unmodified powdered form of each NP (no stabilizing coats or surface functionalization) to the basal ingredients of the diet to ensure even distribution of NPs within the feed pellets (see below) at 500 mg NP kg⁻¹ feed. Selection of this exposure concentration was based on our previous experiments with dietary NP exposures in fish (e.g., Fraser et al., 2011; Ramsden et al., 2009). The Cu-NPs (Copper nanopowder < 50 nm, Sigma Aldrich) were identical and from the same batch as reported in Shaw et al. (2012) with mean particle diameter of 87 ± 27 nm (mean ± S.D., n = 50; Fig. 1d); and the Ag-NP (Silver nano < 100 nm, Sigma Aldrich) were identical and from the same batch as reported in Bradford et al. (2009), with mean particle diameter 58.6 ± 18.6 nm (mean ± S.D., n = 64, Fig. 1a). As positive controls, and to distinguish the difference between nanoparticulate forms and elemental forms, 500 mg kg⁻¹ Ag (as AgCl Sigma–Aldrich) and 500 mg kg⁻¹ Cu (as CuSO₄·5H₂O Sigma–Aldrich) were added to the basal ingredients to produce diets for treatments labelled AgCl and CuSO₄ respectively. This approach ensured that levels of relevant metals remained equal (i.e. 500 mg kg⁻¹) within each diet. Dietary ingredients were mixed with warm water until a soft slightly moist consistency was achieved. This was then cold pressed extruded to produce 2 mm diameter pellets that were dried at 40 °C for 24 h. Pellets were then ground and sieved to produce a crumbly of consistent size (~1 mm) suitable to feed adult zebrafish. All food was prepared once and the same food was used throughout the experiment.

Concentrations of metals in prepared diets were evaluated by inductively coupled plasma optical emission spectrometry (ICP–OES) analysis. Briefly, feed pellets were digested in aqua regia (HCl and HNO₃ in a ratio of 3:1) and then diluted with pure water prior to ICP–OES. Analysis by ICP–OES indicated that metal concentrations were within 20% of nominal in feed preparations (Table 2). In addition, feed pellets were assessed using Field Emission SEM (FESEM). Pellets were mounted on aluminium stubs, carbon coated (Emitech K550X, Kent, UK) and examined with a FESEM Joel-7001F (Joel, Japan) using backscatter images to look at composition. FESEM confirmed even distribution of NPs in pellets, and indicated that NPs were present from nano scale sizes (<100 nm) to larger agglomerates (500–1000 nm) (Fig. 1b and e).

2.2. Experimental fish

Wild type zebrafish (D. rerio) were obtained from the Zebrafish Research Facility at Plymouth University (Plymouth, UK), and fish were treated humanely in accordance with the regulations on animal use within research in the UK. Adult zebrafish (33–39 mm total length; 343–610 mg) were held in 40-l glass aquaria (photoperiod, 14 h light) with partial re-circulating water (30% water change per day) and measurements of water quality indicated that conditions during the experiment did not differ from normal for the facility (i.e., mean ± SE (N): pH, 6.8 ± 0.1; temperature, 27 ± 1 °C (85%); percent oxygen saturation, 89–92% (85%); ammonia, <0.01 mg l⁻¹; nitrite, <0.4 mg l⁻¹; nitrate, <3.0 mg l⁻¹). The electrolyte composition of system water was 0.3, 0.04, 0.08 and 0.4 mM for Ca, K, Mg, and Na, respectively.

2.3. Experimental design

Zebrafish were randomly assigned to 15 glass aquaria (4 l recirculating water as above: 12 fish/aquarium) that were designated as control, Ag-NP, Cu-NP, AgNO₃, CuSO₄ (triplicate tanks per treatment); and fed the control diet for 7 d prior to initiation of the exposure. Fish were fed twice per day the prepared diets at a total of 3% body weight d⁻¹, fish were observed during feeding, and all food was consumed within 5 min of adding to aquarium. Aquarium water samples analyzed by ICP–OES (as above) for Ag and Cu indicated that there was no detectable release of these metals into the water during the experiment (data not shown). Exposure to the experimental diets continued for 14 d. At the end of the exposure fish were processed as described below.

2.4. Assessment of intestinal microbiota

After 14 days of dietary exposure, 6 fish per tank were sampled for isolation of intestinal material. After aseptic dissection, the digestive tract in its entirety (ca. 30 mg per fish) was removed, pooled per tank (i.e. 3 replicate samples, each representing 6 fish) and homogenised. DNA extraction, PCR amplification of the 16S rRNA V3 region and DGGE were conducted as described by Merrifield et al. (2009). Several phylotypes were excised for sequence analysis. DNA was eluted from excised DGGE banding patterns were analysed using Quantity one® 46.3.0 software (Bio-Rad laboratories, CA, USA). A Bray Curtis similarity matrix was used to represent the relative similarities between treatments and replicates using Primer V6 (Clarke and Gorley, 2006) after Dimitroglou et al. (2009).

2.5. Examination of intestine by transmission electron microscopy (TEM)

Samples of intestine collected for TEM were fixed in glutaraldehyde (2.5% pH 7.2, 0.1 M), post-fixed with osmium tetroxide (1%, in buffer pH 7.2, 0.1 M), embedded in...
resin, sectioned with a diamond knife (80 nm), and ultrathin sections were placed on copper grids prior to staining (uranyl acetate and Reynold's Lead citrate) following routine procedures (after Dimitroglou et al., 2009). Ultrathin sections were screened with a JSM 1200 EX transmission electron microscope (JEOL, Japan) at 120 kV and digital images were analysed with Image J 1.36 (National Institutes of Health, USA). Multiple sets of images (20–40) were examined to observe the integrity of the epithelial brushborder membrane.

3. Results

3.1. Survival and gross observations

During the experiment only one fish died (control group) and there were no clinical indications of disease, abnormal behaviour, or changes in feeding activity in any of the experimental fish. The fish from all treatments and controls showed normal feeding behaviour; all of the allocated food was consumed by fish within 5 min. No external or internal (peritoneum) gross lesions were observed in any fish (n = 150) euthanized and dissected for examination of intestine by TEM or evaluation of intestinal microbiota.

3.2. Morphology of the epithelial mucosa of the zebrafish intestine

Examination of electron micrographs of the epithelial cells of the intestinal mucosa indicated normal morphology and no differences among fish fed the control or treatment diets. Sections of the intestine that were examined included regions with short or long microvilli (reflecting regional variation within the intestine; Fig. 2A–D), and all treatment comparisons were based on regions...
having similar morphology. Gross morphology of the epithelium was not affected and there were no indications of inflammation or damage. All fish examined had a similar appearance with intact intestinal mucosal epithelium, no signs of junctional rupture, well organised microvilli and no cellular debris in the lumen (Fig. 2). No lesions were observed in fish fed either control or treatment food.

### 3.3. Endogenous microbiota

The microbial profiles within replicates from each treatment were highly similar and displayed the same key trends; specifically, the observed taxonomical units (OTUs) Z1, Z2, Z4 and Z6 were the most prominent OTUs present in all control, AgCl and Ag-NP replicates (refer to Table 3 and Fig. 3). However, OTUs Z1 and Z2 were absent in all replicates for CuSO4 and Cu-NP. Additionally, OTUs Z4 and Z6 were not detected in any Cu-NP replicates. The inter-animal variability in microbial profiles within each treatment

<table>
<thead>
<tr>
<th>Experimental diet</th>
<th>Nominal concentration (mg kg⁻¹)</th>
<th>Measured concentration (mg kg⁻¹)</th>
<th>Percent of nominal (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.2 ± 1.1</td>
<td>1.7 ± 0.06</td>
<td>na</td>
</tr>
<tr>
<td>CuSO₄</td>
<td>500</td>
<td>509.5 ± 28.3</td>
<td>101.9</td>
</tr>
<tr>
<td>Cu-NPs</td>
<td>500</td>
<td>489.2 ± 23.8</td>
<td>97.8</td>
</tr>
<tr>
<td>AgCl</td>
<td>500</td>
<td>401.0 ± 63.3</td>
<td>80.2</td>
</tr>
<tr>
<td>Ag-NPs</td>
<td>500</td>
<td>462.5 ± 29.3</td>
<td>92.5</td>
</tr>
</tbody>
</table>

(n = 12). na = not applicable.

Detection limits (3 * S.D. of procedural blank).

Cu = 0.018055 mg l⁻¹.

Ag = 0.035236 mg l⁻¹.

* Data are mean ± S.D.
Table 3
Isolated bacterial bands and their closest relatives (BLAST) from PCR–DGGE of the endogenous communities of exposed zebrafish.

<table>
<thead>
<tr>
<th>Code</th>
<th>Accession number</th>
<th>Groups present</th>
<th>Closest ID</th>
<th>Closest ID source</th>
<th>Closest ID accession #</th>
<th>Closest ID %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z1</td>
<td>HQ003236</td>
<td>Control, AgCl, AgNp</td>
<td>Bacterium clone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GIT of zebrafish</td>
<td>AY537952.1</td>
<td>97</td>
</tr>
<tr>
<td>Z2</td>
<td>HQ003237</td>
<td>Control, AgCl, AgNp</td>
<td>Bacterium clone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GIT of zebrafish</td>
<td>AY537952.1</td>
<td>96</td>
</tr>
<tr>
<td>Z4</td>
<td>HQ003238</td>
<td>Control, AgCl, AgNp, CuSO4</td>
<td>Cetobacterium somerae</td>
<td>GIT of fish</td>
<td>AB353124.1</td>
<td>98</td>
</tr>
<tr>
<td>Z5</td>
<td>HQ003239</td>
<td>Control, AgCl, AgNp, CuSO4</td>
<td>Cetobacterium somerae</td>
<td>GIT of fish</td>
<td>AB353124.1</td>
<td>97</td>
</tr>
<tr>
<td>Z6</td>
<td>HQ003240</td>
<td>CuSO4 (1), CuNp (1)</td>
<td>Citrobacter freundii</td>
<td>Estuarine sediments</td>
<td>FN997639.1</td>
<td>95</td>
</tr>
<tr>
<td>Z9</td>
<td>HQ003241</td>
<td>CuSO4 (1), CuNp (1)</td>
<td>Bacterium clone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GIT of zebrafish</td>
<td>AY537951.1</td>
<td>97</td>
</tr>
<tr>
<td>Z10</td>
<td>HQ003242</td>
<td>CuSO4 (1), CuNp (1)</td>
<td>Bacterium clone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GIT of zebrafish</td>
<td>AY537951.1</td>
<td>94</td>
</tr>
<tr>
<td>Z13</td>
<td>HQ003243</td>
<td>Control (1)</td>
<td>Sphingobacteriales bacterium</td>
<td>Soil</td>
<td>AY466725.1</td>
<td>100</td>
</tr>
</tbody>
</table>

*Present in all replicates from the group unless indicated by the number of replicates in parenthesis.

<sup>a</sup>Uncultured bacterium clone.

was minor (i.e. the occasional OTU was present in some replicates but not consistent within the treatment; e.g. Z7, Z9, Z10 and Z13). A representative set of replicates is displayed in Fig. 3. Sequence analysis from the DGGE bands revealed that most OTUs were uncultured and not identified to species level (Table 3). OTUs Z1 and Z2, which were sensitive to dietary Cu (i.e. not detected in any CuSO4 or Cu-NP replicates), displayed greatest similarity (97% and 96%, respectively) to uncultured bacteria previously isolated from the GIT of zebrafish (Rawls et al., 2004; Roeselers et al., 2011); showing 92–94% taxonomical similarity to various Exiguobacterium sp. and Bacillus sp., likely placing them in the Firmicutes order and Bacilli class. OTUs Z4 and Z6 displayed greatest similarity (98 and 97%, respectively) to Cetobacterium somerae.

PCR–DGGE profiles from a representative set of replicates showed that the control, AgCl and Ag-NP displayed similarity coefficients of >91% to one another (Fig. 3). However, compared to the control, profiles of CuSO4 and Cu-NP were only <65% and <43% similar, respectively. Cu-NP had the greatest effect on the microbial profile as was demonstrated by <46% similarity to the profiles of any other group. This trend was observed in all sets of replicates. The control group displayed the lowest number of OTUs, while the effect of CuSO4 and Cu-NP was greater than that of AgCl and Ag-NP, and displayed the greatest number of OTUs.

4. Discussion

Dietary exposure of organisms to NPs is a likely outcome of the release of NPs into the environment, and the use of nanotechnology in food (review (Chaudhry et al., 2008)) will also result in exposure via ingestion. The present study is the first to report NP induced (as opposed to metal induced) changes in microbial communities in the GIT of any vertebrate animal after dietary NP intake. Zebrafish fed NPs had a feeding response that was consistent with fish fed the control diet suggesting that there were no obvious effects of NPs on food palatability. Previous studies with fish have also indicated that fish readily consume both formulated food pellets containing NPs (Ramsden et al., 2009; Fraser et al., 2011) and invertebrates that have accumulated NPs via filter feeding (Park et al., 2010).

Results of the present study demonstrate that dietary exposure to NPs can cause changes in the microbial community of the zebrafish intestine. Bacterial species richness and diversity were greatest in fish exposed to Cu-NPs, but there were some key species missing from the community in zebrafish exposed to Cu-NPs. The bacterium C. somerae (OTUs Z4 and Z6) is a common member of the microbial community of fish intestine (for review see Tsuchiya et al., 2008); and this species was absent in zebrafish fed food containing Cu-NPs. It is possible that the loss of C. somerae in fish fed Cu-NPs enabled colonization by other bacterial species, which could explain the increased diversity and species richness in this treatment (i.e. competitive release), as we have seen in previous studies when a dominant member of the gut microbial community...
has been suppressed (Ferguson et al., 2010). Food containing CuSO₄ with the same amount of copper metal as the Cu-NP treatment did not impact *C. somerae*, indicating that the elimination of this species was an effect associated with Cu-NP rather than metal ion exposure. In the fish intestine, the presence of food rich in SH-ligands and the neutral to alkaline pH of the intestine is likely to severely limit free Cu²⁺ and thus a metal ion dissolution-effect from the Cu-NPs to the epithelium is unlikely. How a Cu-NP effect will inhibit that the growth of *C. somerae* is unclear, but in fishes *C. somerae* produces vitamin B₁₂ at high efficiency, and has been suggested to be an important commensal organism in the provision of vitamin B₁₂ to the host (Tsuchiya et al., 2008; NRC, 2011). Therefore, we speculate that the suppression of this vitamin producing species may have negative consequences for fish health during long term exposure. Further studies are necessary to validate this hypothesis.

OTUs Z1 and Z2 displayed highest similarity to uncultured phylotypes previously isolated from the GIT of zebrafish (Rawls et al., 2004; Roeseler et al., 2011) and are apparently highly specific to this environment. These phylotypes were sensitive to dietary Cu (i.e. were not detected in fish fed either CuSO₄ or Cu-NP amended food), but further research is required to elucidate the identity of these organisms and assess their significance to the host. The mechanisms driving the changes in microbial community structure in zebrafish fed food containing Cu-NPs are not understood and should be investigated.

Zebrafish fed food containing either Ag-NPs or an equivalent amount of Ag (as AgCl) had similar microbial communities in their intestine and only minor changes in community richness and diversity compared to fish fed the control diet. The absence of an Ag-effect may relate to Ag speciation in the gut lumen. Fishes in freshwater have ~70 mmol/l chloride in the intestinal fluid (Handy et al., 2002), and free silver ions are therefore likely to be readily precipitated as insoluble silver chloride. A similar explanation for a lack of microbial biodiversity effects of Ag-NPs in seawater was offered by Bradford et al. (2009). The –SH residues on any organic matter from food will also avidly scavenge Ag⁺ ions. Because of the many ligands within the gut lumen that are available to bind dissolved Ag⁺ it is likely that the dissolved concentrations of Ag⁺ were low and this could explain the relatively minor differences in microbial community observed in fish fed Ag amended diets and the control. It has also been reported from previous studies that some bacterial strains are more sensitive to Cu-NPs than Ag-NPs and although no in vitro sensitivity studies are available for the dominant bacteria present in the GIT of zebrafish these results may reflect the fact that the communities comprise a greater proportion of Cu-NP sensitive strains that Ag-NP sensitive strains. It should also be noted that the method used in this preliminary study (DGGE) is semi-quantitative and therefore possible subtle changes in the levels of non-dominant phylotypes might not have been observed. Future studies should incorporate quantitative analyses.

The apparent lack of a specific NP effect with regards to the Ag treatments in the present study might have connotations to the prior study of Sawosz et al. (2007) which revealed that ingestion of Ag-NPs could significantly influence certain bacterial groups in the cecum of quail. Given the findings of the present study, and the lack of an Ag control in the prior study, it is not clear if reported changes in quail gut microbiota were a NP or Ag effect.

Concern regarding the toxicity of NPs after dietary exposure has generally been based on absorption of NPs, distribution to tissues (e.g., Scown et al., 2010), and toxicity driven by “nano effects” at the tissue and body systems level (Handy et al., 2008, 2011). It was not the objective of the present study to evaluate the absorption or toxicity of NPs in fish tissues or body systems (i.e., if NPs were absorbed), but some studies, despite lack of evidence of NP presence in tissues, have reported subtle biochemical changes in tissues of organisms exposed to dietary treatments containing NPs (e.g., Ramsden et al., 2009; Fraser et al., 2011). It is possible that NP-induced effects on the microbial community within the lumen of the intestine could be a contributory factor to the observed changes in tissues previously reported.

5. Conclusions

The present study indicates that the microbial community of a vertebrate intestine can be disrupted by dietary exposure to NPs. The disruption of gut microbiota differed between Ag- and Cu-NPs and compared to the bulk metal controls, and indicated unique effects associated with ingestion of NPs. Although the presence and distribution of NPs in food pellets were documented, the forms of NPs during passage within the lumen of the gut are unknown (methods not available with current technology), and the effects of complex physical, chemical, and biological processes of the fish digestive system on NPs in food require further investigation. The potential for NPs to disturb microbial communities of the intestine and the importance of maintaining healthy intestinal microbiota is an additional concern for animal exposure to NPs. Future studies should be directed towards evaluation of dose dependent effects, assessments of the autochthonous and allochthonous microbiota, duration of disruption after exposure, and the implications of microbial disruption on host health.

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