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Comparison of Molecular and Histological Changes in Zebrafish Gills Exposed to Metallic Nanoparticles

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Research has demonstrated that metallic nanoparticles produce toxicity in aquatic organisms that is due largely to effects of particulates as opposed to release of dissolved ions. The present research examined the interplay of nanoparticle composition and dissolution on response of the zebrafish gill following exposure to toxic (nanocopper or nanosilver) or nontoxic (nano-TiO_2) nanometals. Female zebrafish were exposed to 48-h no observable effects concentration concentrations of nanocopper and nanosilver or to soluble Cu and Ag that matched the concentration of dissolved metals released during nanoparticle exposure. Both nanocopper and nanosilver exposures increased metal content associated with gill tissue, though silver concentrations were much higher following nanosilver exposures suggesting that intact silver nanoparticles are associated with the gill. Morphological and transcriptional responses of the gills differed among various nanomaterials and between nanoparticulate and soluble species. Nanocopper increased mean gill filament width by three to fourfold between 24 and 48 h, whereas nanosilver did not alter gill filament width at either time point. Global gene expression analysis demonstrates that the exposure to each nanometal or soluble metal produces a distinct gene expression profile at both 24 and 48 h, suggesting that each exposure is producing biological response by a different mechanism. The differences in responses among the exposures indicates that each particle is having a distinct biological effect that does not appear to be driven solely by release of soluble metal ions into the water column. Based on these results, care should be taken when inferring toxicity of nanomaterials from data on a different material.

Key Words: **zebrafish; microarray; nanoparticles; toxicogenomics.**

Engineered nanomaterials have at least one dimension less than 100 nm and consequently, have very high surface areas and high percentages of their component atoms on the surface. This produces unusual reactivity of many nanoparticles, leading to their use in electronics, medicine, environmental remediation, catalysts, and consumer products. The same unique physical and chemical properties of nanomaterials that make them of interest in industrial applications have also increased concern that nanomaterials may have unique biological properties resulting in potential toxicity in the event of unintended use or release into the environment (Lovern and Klaper, 2006; Moore, 2006). Following environmental release, engineered nanomaterials are likely to deposit in aquatic systems and represent a possible danger to aquatic life (Moore, 2006).

Metal and metal oxide nanomaterials comprise a large segment of the growing nanotechnology market. Nanoparticles have been made from many metals, including gold, silver, copper, nickel, cobalt, zinc, and titania. Nanocopper preparations are widely used for bacteriocidal purposes (Cioffi *et al.*, 2005) and are also commonly used for air and liquid filtration, as coatings on integrated circuits and batteries, and to increase thermal and electrical conductivity in coatings and sealants. Silver nanoparticles are also used as bacteriocides and in making stain and odor resistant clothing, sensors, inks, and catalysts (Baker *et al.*, 2005; Tang *et al.*, 2007). Soluble forms of many of these metals are toxic to aquatic organisms, implying that the potential exists for nanoparticulate formulations of these metals to induce toxicological effects in aquatic species. Although regulations exist for protecting aquatic life from dissolved forms of these metals, it is unclear if they are appropriate for use with metallic nanomaterials which may produce quantitatively or mechanistically different toxicity.

It has been demonstrated that copper and silver nanoparticles are acutely toxic to across a wide spectrum of aquatic species including zebrafish (Griffitt *et al.*, 2008). This toxicity is largely manifest at the gills and does not seem to be explained simply by particle dissolution (Griffitt *et al.*, 2007). Studies on metallic nanomaterials are confounded by the twin effects of particle aggregation and dissolution, which simultaneously increase particle size and release dissolved metal ions. These factors make proper characterization of exposure conditions essential, as well as making it paramount that the effect of dissolved metals be considered in any study of nanometal toxicity to aquatic organisms.

The goal of the present study was to determine if the response of the gill to nanoparticulates depends on nanoparticle composition and, if so, to what degree responses to various

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metals are driven by dissolution. To that end, adult female zebrafish were exposed to toxic (nanocopper and nanosilver) and nontoxic (nano-TiO_2) nanoparticles, as well as soluble forms of the toxic nanometals. At various times, gills were examined for changes in global gene transcriptional activity, gill morphology, and total metal content. The results demonstrate that the gill responds differently to each nanomaterial and the response can differ considerably from that elicited by soluble forms of the metals.

MATERIALS AND METHODS

Particle characterization. Copper and silver nanoparticles were provided gratis by Quantum Sphere (Santa Ana, CA) as dry powders. These materials were prepared by gas phase condensation and comprise a metal core with a thin metal oxide coating. Nanoparticulate TiO_2 (P25) was obtained from Degussa (Essen, Germany). Nanomaterials were characterized as dry powders by scanning electron microscopy and Brunauer-Emmett-Teller method (BET) to determine particle size distribution and specific surface area. The mean unaggregated particle diameters (determined from SEM micrographs) were 26.6 ± 8.8 nm for nanosilver, 26.7 ± 7.1 nm for nanocopper, and 20.5 ± 6.7 nm for nano-TiO_2 (Fig. 1). Specific surface areas (measured using BET method) were 14.53, 30.77, and 45.41 m^2/g for nanosilver, nanocopper, and nano-TiO_2, respectively. Zeta potentials were −27.0 mV for nanosilver, −0.69 mV for nanocopper, and −25.1 mV for nano-TiO2. Particle size distributions and dissolution were also determined for suspensions prepared under exposure conditions as described previously (Griffitt *et al.*, 2008). Under exposure conditions, particles aggregate significantly producing polydisperse suspensions containing a range of sizes from single nanoparticles to aggregates of several hundred nanometers (Griffitt *et al.*, 2008). Primary peak sizes in suspension were 44.5 and 216 nm for nanosilver, 94.5 and 447 nm for nanocopper, and 220.8 and 687.5 nm for nanoparticulate TiO2.

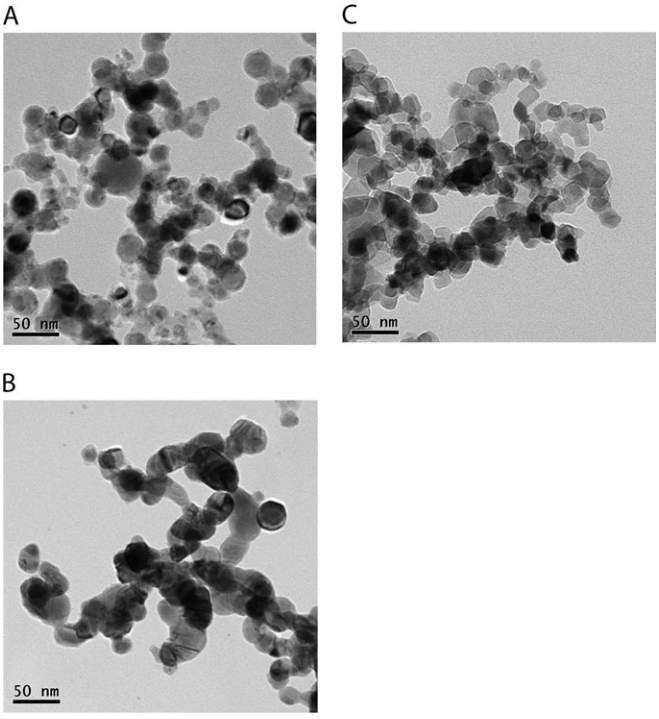

FIG. 1. Scanning electron micrographs of the nanoparticulate metals used in this study. (A) Silver; (B) copper; and (C) titanium dioxide.

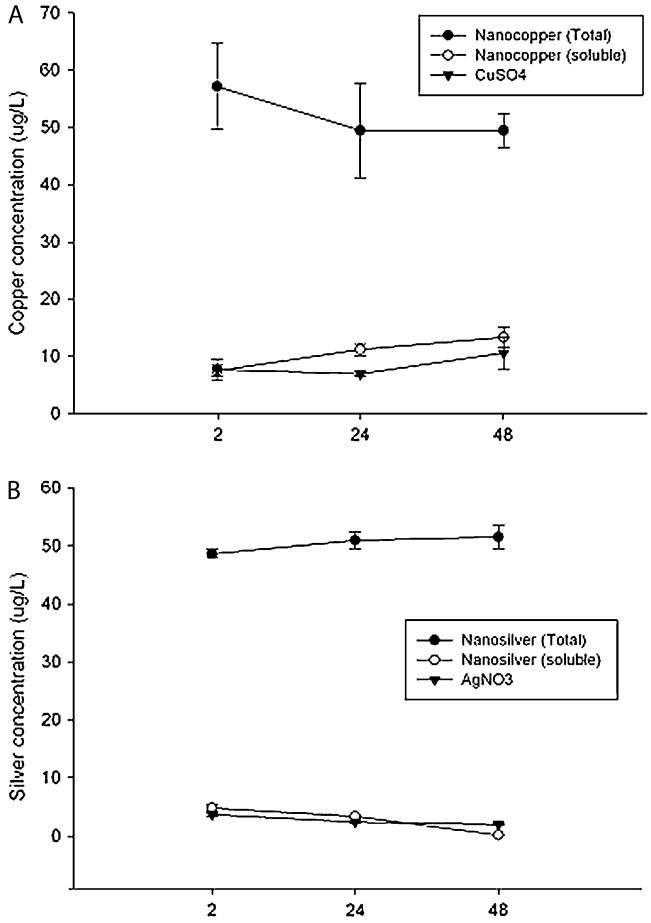

FIG. 2. Concentrations of particulate and soluble metals during exposures. Data for particulate and soluble metal for each nanoparticle exposure as well as the matching soluble metal exposure are shown. Soluble metal concentrations in nanoparticulate and matching soluble exposures are similar. (A) Copper; (B) silver.

Exposure conditions. Wild type zebrafish were purchased from Ekk-Wil (Gibsonton, FL) and housed in the Aquatic Toxicology Facility at UF as described (Griffitt *et al.*, 2007). Exposures were performed as static renewal assays in beakers containing 2 l of 0.22-μm filtered moderately hard facility water, with four adult female zebrafish per beaker, and four replicate beakers per exposure condition. Fish were allowed to acclimate overnight before addition of nanoparticulate metals. Dosing was performed by suspending dry nanometal powder in MilliQ water (18.2 mΩ), sonicating for 6 s in 1-s bursts at 6 W output using a probe sonicator with a frequency of 22.5 kHz, and adding the requisite volumes of stock suspension to each exposure tank. Exposures were conducted at 25°C with an ambient light:dark cycle (approximately 14:10).

Six separate exposures were performed: nanocopper (100 μg/l, corresponding to 0.003 m^2/l if monodispersed), nanosilver (1000 μg/l, corresponding to 0.014 m^2/l if monodispersed), nano-TiO_2 (1000 μg/l, corresponding to 0.045 m^2/l if monodispersed), soluble copper and soluble silver exposures that matched the dissolution of each nanoparticle, as well as concurrent no-dose controls. Concentrations of nanoparticulate copper and silver were chosen to reflect approximate 48-h no observable effects concentration (NOEC) concentrations for each metal based on lethality, whereas nano-TiO_2 concentrations were chosen to match the highest concentration of toxic

TABLE 1
Sampling Plan for the Experiments Described in this Paper

Exposure	2 h	24 h	48 h
Ctrl	W	W, GM, GW, M	W, GM, CM, GW, M
Nanocopper	W	W, GM, GW, M	W, GM, CM, GW, M
$CuSO_4$	W	W	W, GM, CM, GW, M
Nanosilver	W	W, GM, GW, M	W, GM, CM, GW, M
$AgNO_3$	W	W	W, GM, CM, GW, M
Nano-TiO_2	W	W	W, GM, CM, GW, M

Note. W = water column metal measurements (total and soluble). GM = gill tissue metal analysis. CM = carcass tissue metal analysis. GW = gill filament width measurements. M = Microarray analysis.

nanometals used. Soluble copper and silver exposures were performed to produce exposures that mimicked the soluble portion of each nanoparticle exposure. Preliminary experiments were conducted to determine the time course of dissolution for nanosilver and nanocopper during exposures at these concentrations. Soluble copper (as copper sulfate) and soluble silver (as silver nitrate) were added to tanks at 0, 2, 24, and 48 h to match the concentration of soluble metals released by dissolution of each nanoparticle over the course of the 48-h exposure (see Fig. 2). Preliminary studies demonstrated that nano-TiO_2 did not produce lethality during 48-h exposures in zebrafish at concentrations up to 100 mg/l. Five fish were removed from control, nanocopper, and nanosilver treatments at 24 h after exposure, and all treatments were sampled after 48 h (Table 1).

Metal analysis. Water samples were taken from each exposure chamber at 2, 24, and 48 h, and analyzed for soluble and nanoparticulate metal water concentrations. In each case, duplicate 5 ml of water samples were removed from the middle of the exposure container water column. One sample was filtered through a 0.02-μm filter (Anotop 25, Whatman), acid digested to 2% HNO3, and was considered to represent soluble metal. The second duplicate was acid digested without filtration, and represents total metal. Concentrations of the nanoparticulate form of each metal were calculated as the difference between total and soluble at each time point.

Individual gills and whole carcasses (minus gills) from copper and silver exposures were analyzed separately for tissue metal concentration. Tissues were analyzed for total metal content via acid digestion. Entire gill arches were removed from zebrafish at 24 and 48 h, and immediately frozen until analysis. Gills were digested with 1 ml of concentrated HNO_3 (Optima grade, Fisher, Waltham, MA) for 1 h at 120°C, after which 0.25 ml of 30% H_2O_2 (Ultrex II, Mallinckrodt Baker, Phillipsburg, NJ) was added. Samples were evaporated to 200 μl, then reconstituted in deionized water for a final concentration of 2% HNO_3. Whole body samples were treated similarly, except that HNO_3 was increased to 2 ml, and H_2O_2 was increased to 1 ml.

The reconstituted samples were analyzed for total metal content on a Thermo Electron XSeries inductively coupled plasma - mass spectrometry (Waltham, MA) using indium as an internal standard, and quantified against a seven-point standard curve for each metal.

Gill histopathology. Gills were fixed in 4% paraformaldehyde prepared in 10mM phosphate-buffered saline (PBS) for 24 h at 4°C. The tissues were rinsed twice in 10mM PBS and dehydrated in an increasing concentration of ethanol, cleared in Citrisolv (Fisher Scientific, Waltham, MA), and embedded in paraffin wax. Tissue blocks were cut at 7 μm and the orientation of the tissue was lamellar cross sections and filamental sagittal sections. These sections were placed on Superfrost Plus slides (Fisher) and baked overnight (30°C).

To examine morphological changes in filament and lamellae structure, tissue sections were dewaxed in two changes of Citrisolv, and rehydrated through a series of decreasing ethanol washes (100–35% ethanol) and placed in 10mM PBS for 5 min. These slides were then run through a series of histological stains

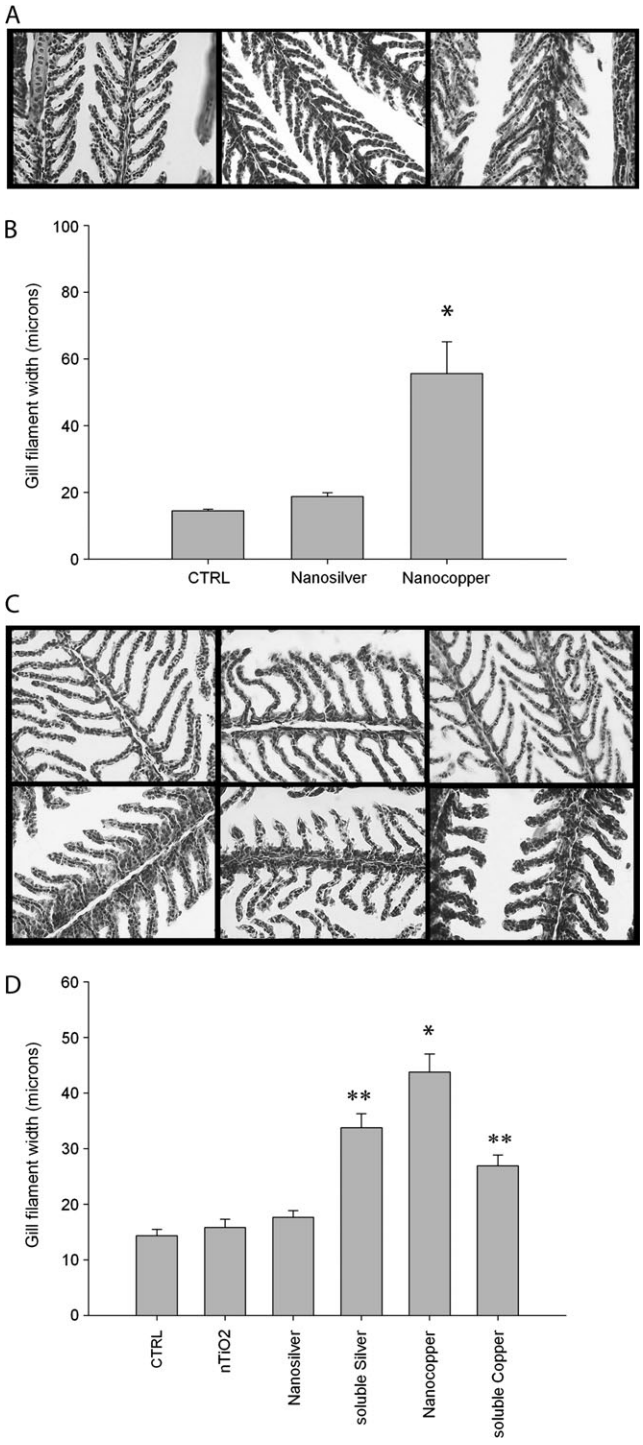

FIG. 3. Effect of nanoparticulate or soluble metals on gill filament width after 24 and 48 h of exposure. (A) Representative pictures of gill filaments from 24-h control, nanosilver, and nanocopper exposures (panels from left to right). (B) Filament width in gills of fish exposed to control, nanosilver and nanocopper for 24 h. (C) Representative pictures of gill filaments from 48-h control, nano-TiO_2, nanosilver, silver nitrate, nanocopper, and copper sulfate exposures (panels clockwise from top left). (D) Filament width in gills of fish exposed for 48 h. Values are mean ± SEM (N = 5 individuals). *Indicates values that are significantly different than control ($p < 0.05$). **Indicates that values where nanoparticulate and soluble forms of the metal differ significantly ($p < 0.05$).

following a modification of the Masson Trichrome (Presnell and Schreibman, 1997).

To determine filamental width, four fish in each treatment were analyzed. Filament width is defined as the width of the epithelial cells in the interlamellar region from the gill vasculature of cartilage. This number was then doubled to represent total filamental width. This way, any size differences due to cartilage, vessel or other artifacts from embedding, did not influence the filamental width. For each fish, a complete series of slides (about 20 slides per gill) were analyzed by randomly measuring five filaments/slide. On each filament, five randomly chosen filament widths, in the interlamellar region, were measured for a total of 500 measurements per fish.

Microarray processing. Transcriptome responses were analyzed with the Agilent 1 × 22k zebrafish microarray (Santa Clara, CA), using a two-color reference design. Three biological replicates for each exposure were performed for each sampling point, for a total of 27 arrays. Gill samples were removed at 24- and 48-h postexposure, immediately frozen in liquid nitrogen, and stored at −80°C until the samples were processed. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), following manufacturer's protocols. Quality and concentration of the RNA samples was assessed using a 2100 Bioanalyzer (Agilent, Santa Clara, CA) and Nanodrop ND-1000 (Thermo Fisher Scientific, Wilmington, DE), respectively. RNA samples with RNA Integrity Number greater than 8.0 and showing no visible evidence of RNA degradation were chosen for microarray analysis. The reference pool was constructed by pooling equal amounts of RNA from all available samples, and diluting to a final concentration of 100 ng/µl. For each sample, equal amounts of total RNA from three separate fish were pooled together. cRNA synthesis and in vitro transcription were performed according to manufacturers protocols using a two-color Low RNA Input Linear Amplification Kit PLUS (catalog number 5184-3523) and Gene Expression Hybridization Kit (catalog number 5188-5242), both from Agilent. Due to low levels of Cy3 incorporation in the reference samples, the amount of sample loaded on each array was increased to 1000 ng for both sample and reference. The pooled samples were hybridized to the arrays for 17 h at 65°C, washed and immediately scanned using a G2505 B Microarray Scanner, and individual spots extracted using Feature Extraction 9.5 (both from Agilent). Individual spots flagged by feature extraction as absent or marginal were removed from the analysis, and any spot that was present in less than 75% of the arrays was removed. This left a high quality data set consisting of 13,555 spots. Any missing values were calculated using the Missing Value Imputation function in JMP Genomics (SAS Institute, Cary, NC) as the median of the Treatment by Time interaction. Using this dataset, a ratio analysis was performed, normalizing the sample signal to the reference signal, and performing a within chip Loess normalization.

Statistical analysis. Statistical analysis of gill filament width, and gill and carcass metal burden were performed in SigmaStat. Significant differences between treatment means were determined by ANOVA followed by Dunnet's test, with a p value of 0.05. For microarray data, differentially expressed genes were identified by ANOVA implemented in JMP Genomics 3.0 (SAS Institute), using the Benjamini and Hockenberg FDR multiple test correction. To identify relationships between the different exposures, the significantly differentially expressed genes were hierarchically clustered using the freely available programs Cluster and Treeview (http://rana.lbl.gov/EisenSoftware.htm).

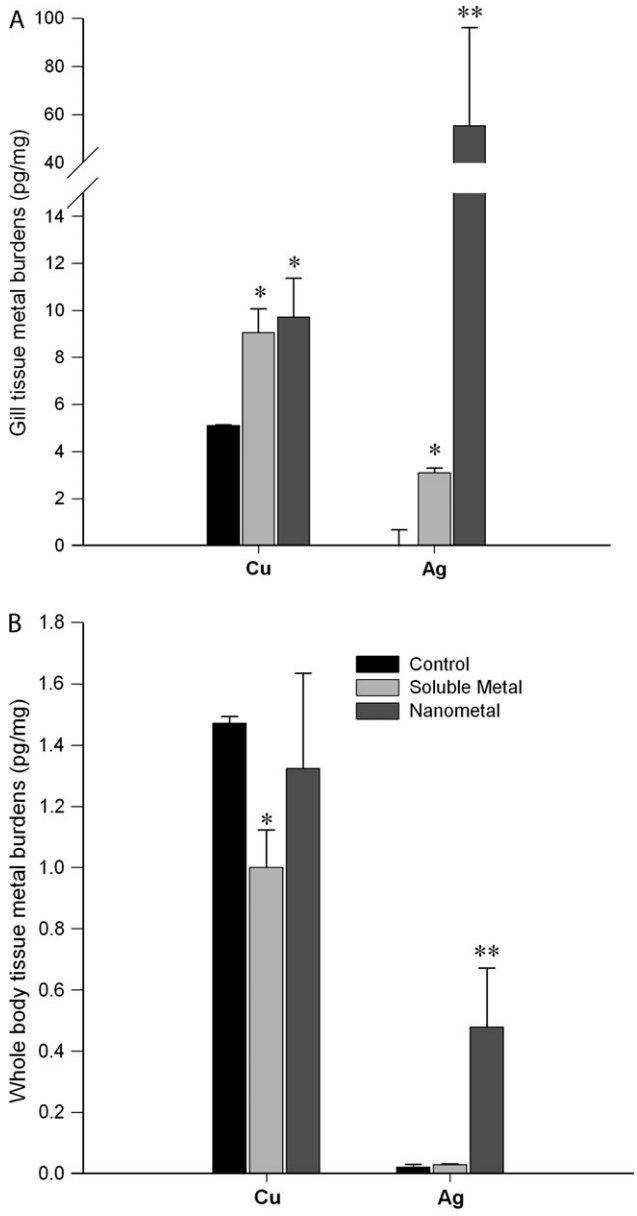

FIG. 4. Tissue metal content of zebrafish exposed to nanoparticulate and soluble forms of copper and silver. Bars represent means ± SEM ($N = 5$). Similar letters indicate means that are not significantly different at a p value of 0.05. (A) Gill metal content; (B) carcass metal content.

RESULTS

Exposure Characterization

In accordance with use of NOEC concentrations, no mortality occurred in any treatment during the course of the exposure, and there were no alterations in zebrafish appearance, behavior or movement patterns (e.g., no visible lesions, uncoordinated swimming, or air-breathing). In all nanometal exposure containers, ingestion and excretion of nanometal particle aggregates was visually observed. Two hours postdosing, only 57% of the nanocopper added to exposures remained in the water column (Fig. 2A). In nanosilver exposures the fraction of metal lost from the water column was even larger, as only 4.9% of the original mass added was retained in the water column (Fig. 2B). The sedimented particles were visible on the bottom of the exposure tank within 15 min following nanoparticle addition. Beyond two hours, loss of nanoparticulate metals from the water column decreased, presumably as the

TABLE 2
Distribution of Genes Significantly Differentially Expressed in the Different Treatments

	24 h	48 h
Nanocopper	49 upregulated; 77 downregulated	192 upregulated; 221 downregulated
$CuSO_4$	NA	72 upregulated; 116 downregulated
Nanosilver	66 upregulated; 82 downregulated	126 upregulated; 336 downregulated
$AgNO_3$	NA	24 upregulated; 104 downregulated
Nano-TiO_2	NA	60 upregulated; 111 downregulated

large particle aggregates were lost, and only smaller, truly nanosized particles were left. At 24-h postdose, the nanocopper and nanosilver exposures retained 49 and 5.1% of the initial dose, respectively; at 48 h those numbers were constant at 49 and 5.1%. Particle dissolution followed a similar pattern, with most of the dissolution occurring during the initial 2-h period following addition of the nanoparticles to the water. By 48-h postdose, the nanocopper treatments contained an average of 13.3 μg/l soluble copper; 7.4 μg/l (55%) of this was released in the first 2 h. In nanosilver exposures, soluble silver decreased over time, reaching a peak concentration of 4.9 μg/l, 2 h into the exposure; this value decreased to 0.2 μg/l by 48 h.

Gill Histopathology

Morphological responses of the gills to metal exposure were characterized by increased cellularity in the interlamellar space (Fig. 3). However, changes in gill filament width following metal exposure varied significantly with metal and form of the metal. At 24 h, copper nanoparticle exposure caused a 3.5-fold increase in gill filament width, whereas only slight changes were observed with silver nanoparticles (Fig. 3). After 48 h of exposure, gill filaments were threefold thicker than control in nanocopper exposed fish, but were not significantly altered by nanotitania or nanosilver (Fig. 3). Soluble silver and copper exposure both increased gill filament widths by approximately twofold over control values. Gills taken from nanoparticulate copper-exposed fish were significantly wider than those from soluble copper exposures, highlighting the presence of a copper particle-specific effect. In contrast, gill filament widths were higher in soluble silver exposures than in nanosilver exposures, despite both tanks containing highly similar concentrations of soluble silver.

Metal Tissue Concentration

Exposure to both nanocopper and nanosilver increased metal burdens in gill tissue compared with controls at 48 h (Fig. 4A). A similar pattern was observed at 24 h (Fig. S1). However, the amount of copper associated with gill tissues was virtually identical between particulate and soluble exposures at 48 h, whereas zebrafish exposed to nanoparticulate silver had significantly higher amounts of silver associated with gill than fish exposed to soluble silver. Metal tissue burdens in whole carcasses followed a similar pattern. At 48 h, silver tissue burdens were significantly higher in zebrafish exposed to nanosilver than in fish exposed to soluble silver, whereas copper levels between the two treatments were similar (Fig. 4B). The analysis used to determine tissue metal levels (ICP-MS) cannot differentiate between nanoparticulate and soluble metals.

Bioinformatics Analysis

Transcriptional analysis of gene expression patterns revealed significant differences between treatments. At 24 h, 237 genes were significantly differentially expressed in at least one treatment; that number increased to 918 at 48 h (Table 2). The number of genes significantly affected by nanocopper exposure increased from 126 at 24 h to 413 by 48 h. In nanosilver exposed fish, the number of significantly differentially expressed genes increased from 148 at 24 h to 462 at 48 h. Exposure to nano-TiO_2 resulted in 171 significant genes at 48 h (Table 2). Genes that were significantly differentially expressed and demonstrated a mean treatment difference in excess of 1.5 are listed in Tables 3 (nanocopper) and 4 (nanosilver).

There was little commonality between gene sets affected by the different exposures. At 24 h, there were only 37 genes that were significantly affected by both nanocopper and nanosilver (Fig. 5A). At 48 h, when the nano-TiO_2 exposed fish were included, there were 53 genes affected by all three nanometals (Fig. 5B). Similarly, 95 genes were affected by both nanocopper and nanosilver, 20 genes overlapped between nanosilver and nano-TiO_2, and 32 were commonly affected by nanocopper and nano-TiO_2.

Hierarchical cluster analysis of the significantly differentially expressed genes at both 24 and 48 h revealed that each treatment yielded a unique expression profile, with all replicates of each exposure clustering separately (Fig. 6). At 24 h, both nanocopper and nanosilver exposures produced expression profiles very different from control profiles, as well as very different from each other (Fig. 6A). In the 48-h data set, all three nanometal expression profiles clustered separately from each other (Fig. 6B). For both copper and silver, the particulate and soluble forms of the metals induced different transcriptional profiles, despite having very similar levels of soluble metal in each exposure.

Table 3 lists a subset ($p < 0.001$) of the genes altered by nanocopper exposure at 48 h. A subset of 34 of the altered genes could be matched to human homologues using a combination of the zebrafish to human ontology mapping file provided on the ZFIN database (http://zfin.org/zf_info/downloads.html#orthology) and manual annotation. Pathway

TABLE 3
Differentially Expressed Genes Identified by Microarray Analysis of Zebrafish Gills Exposed to Nanocopper for 48 h

Agilent probe ID	Gene ID	GenBank accession no.	Function	Diff of Treatment	−log10 *p* value
A_15_P100804	*Danio rerio* cDNA clone IMAGE:7038661	BC078368	NA	−1.57	3.06
A_15_P100899	*Danio rerio S*-adenosylhomocysteine hydrolase	NM_199218	Adenosylhomocysteinase activity	1.56	3.31
A_15_P102602	*Danio rerio* hairy/enhancer-of-split	NM_212561	DNA binding	−2.18	3.14
A_15_P103675	*Danio rerio* cathepsin L	NM_212584	Cysteine-type endopeptidase activity	1.85	4.86
A_15_P103682	*Danio rerio* cDNA clone IMAGE:6525439.	BC067158	NA	−2.22	3.05
A_15_P104502	*Danio rerio* period homolog 4	NM_212439	Signal transducer activity	−2.13	3.31
A_15_P104523	*Danio rerio* cDNA clone IMAGE:5071840	BI708697	NA	−2.25	3.94
A_15_P105186	Pendulin		NA	2.09	3.39
A_15_P105204	*Danio rerio* phosphoglycerate mutase 1	NM_198804	Catalytic activity	1.72	3.12
A_15_P105312	*Danio rerio* invariant chain-like protein 2	NM_131372	NA	−2.78	3.66
A_15_P105316	*Danio rerio* zgc:63990	NM_214763	Molecular function unknown	−2.24	3.06
A_15_P105807	O93584 (O93584) ZfY1, partial		NA	3.18	4.45
A_15_P106093	*Danio rerio* si:rp71-30i22.4	NM_200092	Apoptosis	2.28	4.05
A_15_P106239	*Danio rerio* cyclin B1	NM_131513	Regulation of cell cycle	2.95	4.18
A_15_P108453	*Danio rerio* ATPase, Na+/K+ transporting, beta 1b polypeptide	NM_131671	Sodium:potassium-exchanging ATPase activity	−1.71	3.03
A_15_P108968	FXYD domain-containing ion transport regulator 8 precursor	BC090514	NA	−2.37	3.02
A_15_P109513	*Danio rerio* zgc:63534	NM_201035	On channel activity	−2.56	3.24
A_15_P110501	*Danio rerio* zgc:55620	NM_212775	Molecular_function unknown	2.37	3.17
A_15_P110743	*Danio rerio* selenoprotein W	NM_178287	NA	−2.69	3.18
A_15_P112212	D86628 family-2 cystatin (Oncorhynchus keta)		NA	−1.77	3.05
A_15_P112425	*Danio rerio* invariant chain-like protein 2	NM_131372	NA	−2.40	3.25
A_15_P112461	Q7SZC6 (Q7SZC6) Regulator of G-protein signaling 4		NA	3.21	5.41
A_15_P113547			NA	2.86	3.28
A_15_P116930	*Danio rerio* cDNA clone IMAGE:3738975	AW128435	NA	4.35	3.23
A_15_P117333	*Danio rerio* cDNA clone IMAGE:3742320	AW058716	NA	2.36	3.27
A_15_P119881	*Danio rerio* tubulin, alpha 8 like	NM_212772	GTPase activity	2.89	3.19
A_15_P120275	*Danio rerio* Sjogren syndrome antigen B	NM_199547	Nuclear mRNA splicing, via spliceosome	2.23	3.35
A_15_P120784	*Danio rerio* interferon gamma inducible protein 30	NM_001006057	NA	−2.92	3.28

Note. Difference of treatment is $\log_2$ of fold-change values. Positive values indicate upregulation, negative values indicate downregulation; NM nanometal.

analysis performed using Pathway Studio (Ariadne Genomics, Rockville, MD) revealed a large number of these genes were involved in the cellular processes apoptosis, mitogenesis and proliferation, as well as in cancer progression (Fig. 7A). The downregulated subset was not well annotated and only nine genes were able to be mapped to human homologues. Analysis of these genes indicated that they were involved in cellular processes such as myogenesis and differentiation, as well as cancer progression (Fig. 7B).

Table 4 contains a list of the genes most strongly affected ($p < 0.001$) by nanosilver exposure at 48 h. gene ontology (GO) analysis performed revealed no significantly over or underrepresented gene clusters. This was largely due to poor annotation of the probes altered by nanosilver exposure, rendering GO and pathway analysis ineffective.

Although nano-TiO_2 has not been observed to be toxic to zebrafish, there is a clear effect on transcription patterns due to that exposure at 48 h. The nano-TiO_2 arrays cluster together and distant from any other exposure, and there is a small cluster of genes that are highly downregulated. When interrogated to identify possible similarity of function, this cluster contained a number of genes involved in ribosome structure and activity, including ribosomal proteins L23, L4, L9, S29, and S3a, as well as eukaryotic translation elongation factors.

DISCUSSION

Adult female zebrafish were exposed to sublethal concentrations of toxic and nontoxic NM to determine if there was commonality of response between different nanomaterials, suggesting a common mechanism of action for nanoparticulates: or between nanoparticulate and soluble forms of a metal, indicating that release of soluble metal ions into the water

TABLE 4
Differentially Expressed Genes Identified by Microarray Analysis of Zebrafish Gills Exposed to Nanosilver for 48 h

Agilent probe ID	Gene ID	GenBank accession no.	Function	Diff of treatment	−log 10 *p* value
A_15_P100804	*Danio rerio* cDNA clone IMAGE:7038661	BC078368	NA	−1.75	3.40
A_15_P101419	*Danio rerio* secreted acidic cysteine rich glycoprotein	NM_001001942	Calcium ion binding	−2.16	3.46
A_15_P106835	GBG2_HUMAN (P59768) guanine nucleotide-binding protein G(I)		Activation of MAPK	−1.75	3.08
A_15_P107031	*Danio rerio* guanine nucleotide-binding protein (G protein)	NM_213481	Signal transducer activity	−1.72	3.19
A_15_P107751	*Danio rerio* myxovirus (influenza) resistance A	NM_182942	GTPase activity	−1.81	3.77
A_15_P108453	*Danio rerio* ATPase, Na+/K+ transporting, beta 1b polypeptide	NM_131671	Sodium:potassium-exchanging ATPase activity	−1.84	3.25
A_15_P109228	*Danio rerio* spp1 protein (spp1)	NM_001002308	NA	−1.56	3.03
A_15_P110871	*Danio rerio* peripheral myelin protein 22	NM_201311	Integral to membrane	−2.49	3.07
A_15_P112461	Q7SZC6 (Q7SZC6) Regulator of G-protein signaling 4		NA	2.08	3.80
A_15_P116606	*Danio rerio* cDNA clone IMAGE:7038661	BC078368	NA	−2.48	4.11
A_15_P119500	Q8WV51 (Q8WV51) FBXW2 protein		Ubiquitin conjugating enzyme activity	−1.58	3.33

Note. Difference of treatment is $\log_2$ of fold-change values. Positive values indicate upregulation, negative values indicate downregulation; NM nanometal.

column drives observed effects. The results of this study indicate that there are significant differences in the response of zebrafish gills to different nanometals, as well as significant differences between soluble and nanoparticulate forms of a single metal.

Exposure to both dissolved and nanoparticulate metals increased the metal content of gills. Branchial uptake of ionic silver and copper has been well documented in freshwater fish and appears to occur primarily through apical membrane sodium channels and the copper transporter protein (Bury and Wood, 1999; Bury *et al*., 1999). The amount of copper associated with the gill after exposure to either copper nanoparticles or soluble copper was greater than control, but was the same in both cases. This suggests that gill is primarily taking up dissolved copper released from particles and that cellular uptake of intact copper nanoparticles or enhanced dissolution of copper nanoparticles is not a major factor. In contrast, exposure to silver nanoparticles produced significantly higher levels of silver associated with the gills than did exposure to only the soluble fraction. This suggests that the nanoparticles themselves are contributing to the gill burden of silver. There are several mechanisms by which nanoparticulates may increase the gill silver levels. Nanoparticles may be trapped in the mucus layer of the gill as demonstrated for larger particles (Sanderson *et al*., 1996; Tao *et al*., 1999). Nanoparticles trapped in this manner may not actually enter the cells, but mucus entrained particles can also increase intracellular metal content by enhanced dissolution due to changes in water chemistry in the gill microenvironment including mucus complexation (Tao *et al*., 2002). It is also possible that nanoparticles are actually taken up by gill epithelial cells. Previous studies demonstrate that sediment particles less than 500 nm are present intracellularly in salmonid gill epithelial cells (Martens and Servizi, 1993). Further work is required to ascertain the mechanism by which silver and copper nanoparticles interact with the gill, though these results demonstrate that not all nanoparticles will interact with the gill in the same manner.

Exposure to silver nanoparticles also significantly increased whole body silver content. It is unclear whether this is due to translocation of silver from the gills to the rest of the body, as has been shown previously in rainbow trout (Morgan *et al*., 2004), or due to ingestion of particulates and gastrointestinal (GI) absorption. Previous studies have demonstrated that exposure to carbon nanotubes caused stress induced drinking and ingestion of nanomaterials in rainbow trout (Smith *et al*., 2007) and there was evidence of ingestion of nanomaterials in our study. Once in the GI tract, metallic nanoparticles, like silver and copper, can dissolve rapidly resulting in local high concentrations of copper ions sufficient to disrupt copper homeostasis as seen in studies with mice orally exposed to nanocopper (Chen *et al*., 2006; Meng *et al*., 2007). Unlike silver, exposure to copper nanoparticles did not significantly increase the whole body burden of copper despite evidence of ingestion.

The histological response of the gill to these treatments varied among metal oxide nanoparticles and between nanoparticulate and soluble forms of a given metal. Of the three nanometals tested, only nanocopper induced a significant increase in gill filament width, whereas nanosilver and nano-

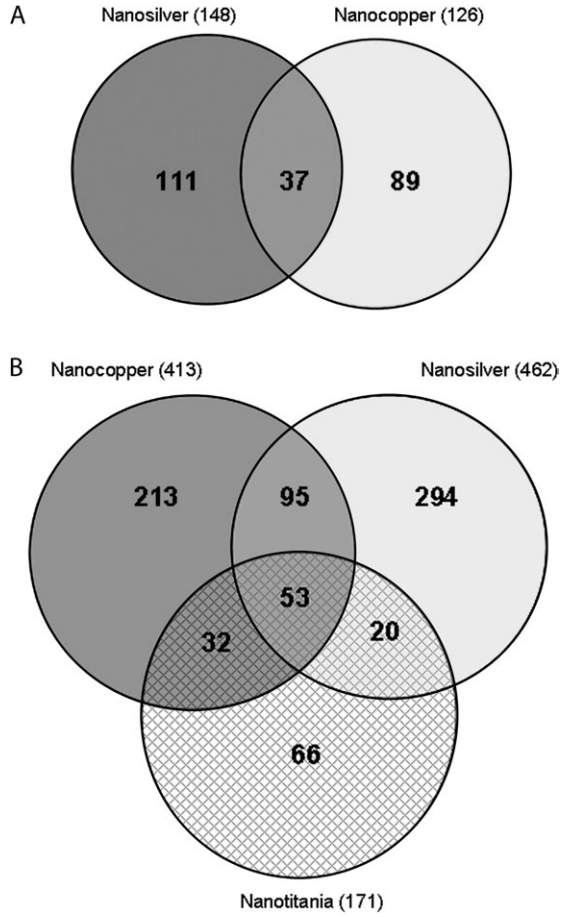

FIG. 5. Venn Diagram analysis of genes identified by microarray analysis as significantly differentially expressed following exposure to nanosilver, nanocopper or nanotitania. (A) 24 h; (B) 48 h.

TiO_2 failed to do so. This pattern was also evident at 24 h, with nanocopper but not nanosilver inducing a significant increase in gill filament width (Fig. 3). Although these types of changes in gill morphology are a common response to many toxicants (Evans, 1987), the current results suggest that the changes in gill morphology observed with copper nanoparticles are not a generic response to small particle exposure. This is consistent with earlier studies on suspended solids which show that changes in gill morphology in response to suspended solid exposure typically do not occur until concentrations exceed 1 g/l, though results vary with particle composition and fish species (Goldes *et al*., 1988; Sutherland and Meyer, 2007). It is also possible that duration of the exposure is a critical determinant of gill morphology to small particles (Newcombe and Jenson, 1996). Work by Federici *et al*. (2007) demonstrates that exposure to nanotitania for 14 days alters gill morphology at concentrations between 0.1 and 0.5 mg/l in rainbow trout.

Substantial differences in gill morphology were observed between nanoparticulate and soluble forms of silver and copper. Soluble copper is a well-known gill toxicant in freshwater fish species (Mazon *et al*., 2002a,b), and in the present study, soluble copper produced significant thickening of the gill filament. Exposure to nanocopper produced significantly more thickening of the gill filaments than did soluble copper. Nanocopper and soluble copper exposures contained nearly identical concentrations of soluble copper, suggesting that the effects of nanocopper and gill morphology are due to a combination of dissolution and particulate effect. A similar pattern of response is observed for Na+/K+-ATPase inhibition in the gill following exposure to nanoparticulate copper (Griffitt *et al*., 2007). Because nanocopper exposures produce similar gill copper burdens as soluble copper exposures, this suggests that nanocopper is acting exterior to the gill.

Zebrafish exposed to soluble silver (as silver nitrate) in the present experiment exhibited significant thickening of the gill filament as was observed for soluble copper and nanoparticulate copper. Silver is among the most potent gill toxicants in freshwater fish, causing highly specific inhibition of Na+/K+-ATPase (Morgan *et al*., 1997). The morphological response of gills to silver exposure is not well defined in freshwater fish, but would be expected to be similar to other toxicants that cause ionoregulatory imbalances. This change in morphology has been observed in spiny dogfish, a marine elasmobranch, exposed to silver (De Boeck *et al*., 2001). No change in filament width was observed in zebrafish exposed to nanoparticulate silver. This is surprising, because similar concentrations of soluble silver were present in both soluble and nanoparticulate exposures. Although there is no clear explanation for this result, exposure to nanoparticulate silver must inhibit or prevent the morphological changes caused by soluble silver. It is possible that the nanoparticulate silver prevents cell proliferation or counteracts the physiological changes induced by soluble silver. Further studies on the physiological response of fish to nanoparticulate silver are necessary to understand these differences.

Gene expression profiling is another method to determine if various compounds are acting on a biological system in a similar manner. A central assumption of toxicogenomics is that chemicals that produce toxicity by the same mechanism will produce similar gene expression responses under a given set of conditions. In this study, nanoparticulates were added at equitoxic concentrations and equal amounts of soluble metals were present in both soluble and nanoparticulate exposures. Under these conditions, nanocopper, nanosilver and nanotitania produced markedly different gene expression profiles as measured by hierarchical cluster analysis. These results suggest that each of the nanoparticulates is interacting with the gill in a different manner. Very little overlap was found among the sets of genes affected by each nanometal exposure (Fig. 5, indicating that there is very little in the way of a ''generic particle'' response at the gill. Comparison of the gene expression profile of nanocopper and nanosilver with the corresponding soluble only metal exposure revealed little similarity, demonstrating that the effects of both nanometals is not due solely to release of metal ions into bulk water. Further analysis of the genes affected by

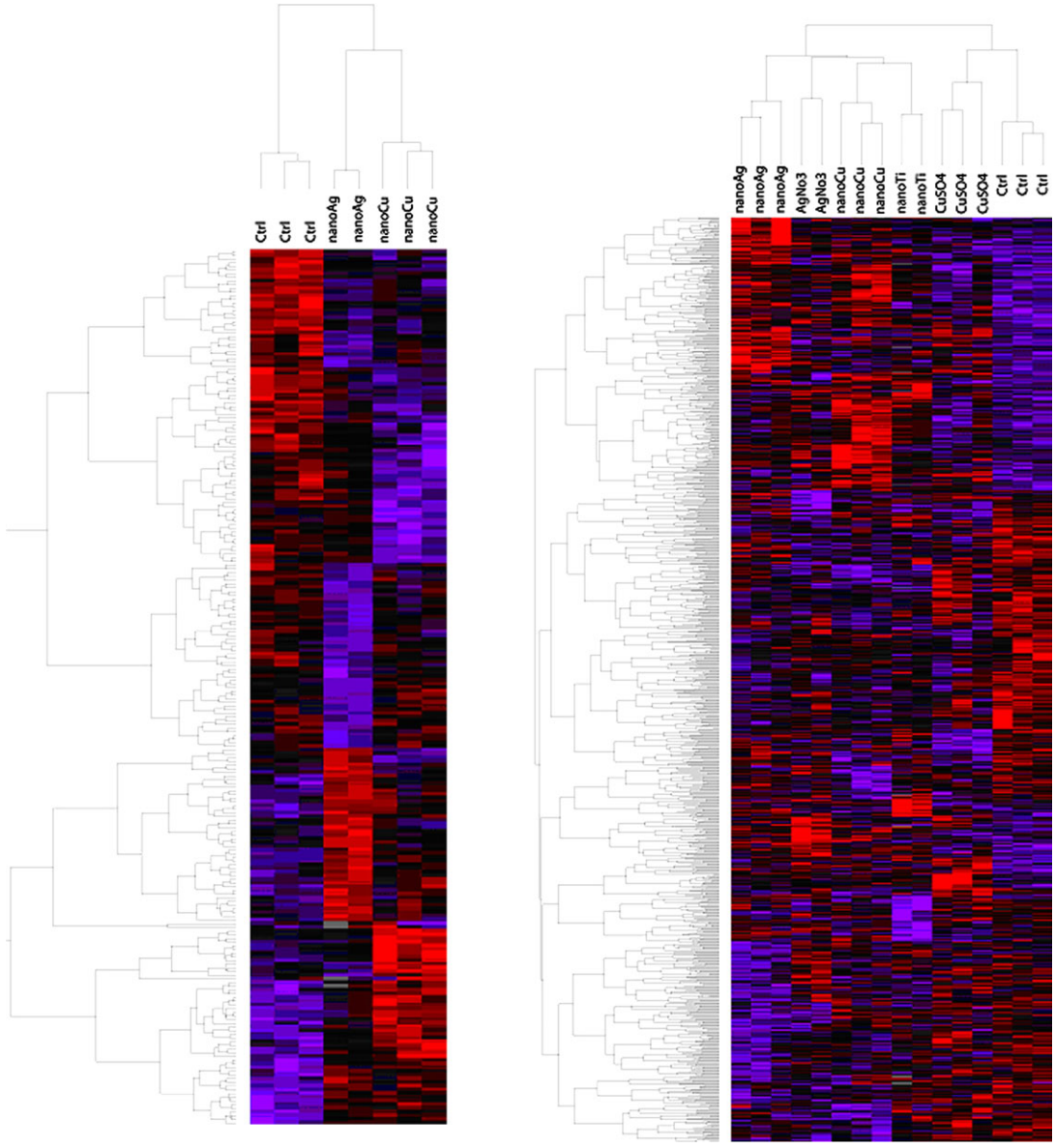

FIG. 6. Hierarchical clustering results for genes identified by microarray analysis as significantly differentially expressed. Hierarchical clustering was performed using the program Cluster and visualized using Treeview. Red cells indicate genes that are overexpressed in relation to the reference pool, whereas blue cells indicate genes that are underexpressed to the reference pool. (A) 24 h; (B) 48 h.

nanocopper exposure indicates that many of the genes are involved in apoptosis, cell proliferation and differentiation. This is in agreement with the morphology observed in the gills and is likely due to the gill remodeling that is occurring. Of interest, nano-TiO_2 exposure altered expression of a number of genes involved in ribosomal function that were not affected by other treatments. Similar responses have been observed in flounder caught in polluted estuaries (Williams *et al*., 2003) and may be related to inhibition of protein synthesis by cellular stress (Patel *et al*., 2002). This raises the possibility that, although not overtly toxic within the time frame studied, exposure to nano-TiO_2 may be having effects on zebrafish gills that are not overt until longer exposures are performed. This is consistent with the results of long term (14-day) exposure to TiO_2 in trout (Federici *et al*., 2007) and suggests that longer term studies are an absolute necessity in assessing risk from nanomaterials in aquatic organisms.

Although little is known about the mechanisms of nanomaterial toxicity, other researchers have provided evidence for nanoparticle-mediated production of reactive oxygen species and generation of oxidative stress as a possible mechanism of toxicity (Oberdorster, 2004; Pickering and Wiesner, 2005; Zhu *et al*., 2007) for carbonaceous nanoparticles (i.e., fullerenes, fullerols, and carbon nanotubes) and nanoparticulate titanium

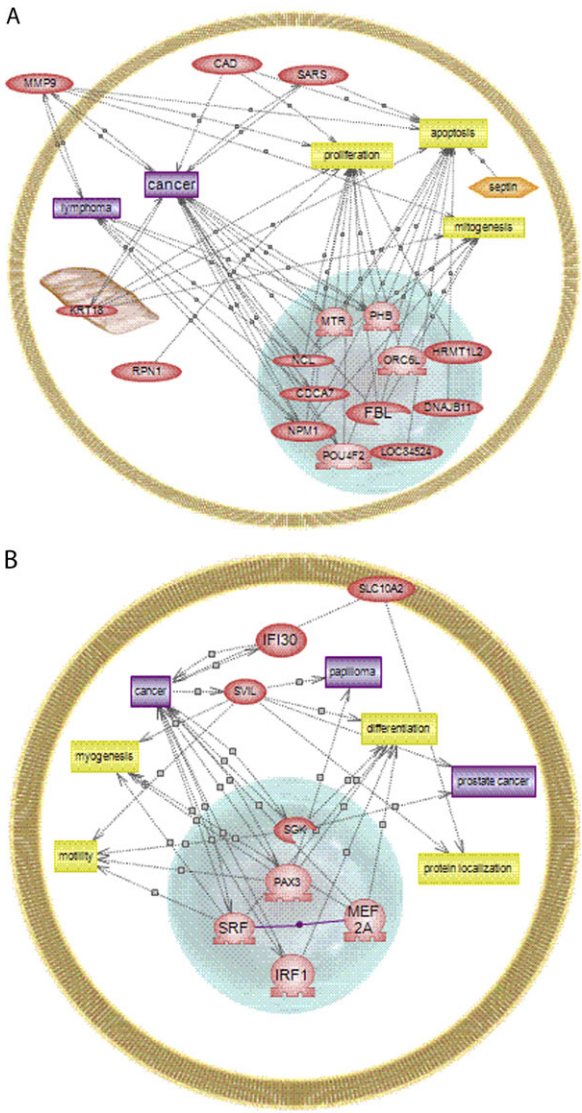

FIG. 7. Pathway analysis of genes altered by exposure to 100 μg/l nanocopper for 48 h. Where possible, zebrafish genes were mapped to human homologues, and queried for similarity of function using the Pathway Studios program from Ariadne Genomics. (A) Upregulated genes. (B) Downregulated genes.

dioxide (Reeves *et al.*, 2008; Xia *et al.*, 2006). Rainbow trout exposed to 1.0 mg/l nano-TiO_2 had small but statistically significant increases in gill thiobarbituric acid reactive substances and gill glutathione levels after 14 days of exposure, suggesting that there was ongoing oxidative stress though perhaps not of sufficient magnitude to cause the observed pathology (Federici *et al.*, 2007). The gene expression data in the present study finds that no genes known to be involved in oxidative stress regulation were affected by any treatment, suggesting that nanoparticle-mediated induction of oxidative stress may be an incomplete explanation for the observed toxic effects.

Accurate characterization of exposure conditions and appropriate dosimetry is a serious issue for nanotoxicologists (Teeguarden *et al.*, 2007), particularly in aquatic exposures. Metallic nanoparticles added to water tend to simultaneously aggregate to form larger particles, dissolve to release soluble metal ions, and sediment out of the water column. Each of these will alter exposure conditions, meaning that the actual conditions to which organisms are exposed may bear little or no relation to the initial added dose. The results of this study demonstrate that much of the mass of nanoparticles added to the test solution were quickly removed from the water column via sedimentation, rendering them nonbioavailable to the zebrafish. Most dissolution occurred in the first 2 h after suspension which may indicate dissolution of very fine particulates or release of relatively soluble metals from the particle surface despite the presence of a metal oxide shell. It is probable that there is formation of relatively insoluble metal oxide/hydroxide layers on the surface of the particles after suspension in water that limits subsequent dissolution. After release, some metal ions will complexed by organic matter in the water (e.g., fecal material) and subsequently removed from the water column. In most aquatic environments, nanoparticles that are removed from the water column through sedimentation are likely to be sediment associated, reducing the release of the soluble ions back into the water column and minimizing the role of dissolution in nanometal toxicity to pelagic aquatic organisms.

Although there are no estimations of environmentally likely concentrations of nanoparticulate silver and copper, the concentrations used in these experiments are relatively high. They were used to investigate interactions of the particles with organisms at toxic levels and it is not clear that aquatic exposures will ever reach concentrations sufficient to generate the reported effects. However, recent research (Benn and Westerhoff, 2008) observed that nanosilver impregnated socks could release up to 1300 μg/l silver following washing, with at least some of that present as nanoparticulate silver. As nanomaterials become more prevalent, methods to accurately quantify their distribution in the environment are critical.

In summary, the results of these studies demonstrate that nanocopper, nanosilver, and nanotitania do not produce similar responses in the zebrafish gill after acute exposure. They also demonstrate that the effects of nanocopper and nanosilver are not due solely to release of soluble metals into the water column. This highlights the need for further studies focused on understanding the mechanisms of nanoparticle toxicity in aquatic organisms as dissolution and the presence of a generic "nanoparticle" response are not sufficient to explain the observed effects. Until this mechanistic data is available, researchers should be very cautious when attempting to extrapolate toxicity information from one nanometal (NM) to another.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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