Attributing Effects of Aqueous C₆₀ Nano-Aggregates to Tetrahydrofuran Decomposition Products in Larval Zebrafish by Assessment of Gene Expression

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BACKGROUND: C₆₀ is a highly insoluble nanoparticle that can form colloidal suspended aggregates in water, which may lead to environmental exposure in aquatic organisms. Previous research has indicated toxicity from C₆₀ aggregate; however, effects could be because of tetrahydrofuran (THF) vehicle used to prepare aggregates.

OBJECTIVE: Our goal was to investigate changes in survival and gene expression in larval zebrafish Danio rerio after exposure to aggregates of C₆₀ prepared by two methods: a) stirring and sonication of C₆₀ in water (C₆₀–water); and b) suspension of C₆₀ in THF followed by rotovaporing, resuspension in water, and sparging with nitrogen gas (THF–C₆₀).

RESULTS: Survival of larval zebrafish was reduced in THF–C₆₀ and THF–water but not in C₆₀–water. The greatest differences in gene expression were observed in fish exposed to THF–C₆₀ and most (182) of these genes were similarly expressed in fish exposed to THF–water. Significant up-regulation (3- to 7-fold) of genes involved in controlling oxidative damage was observed after exposure to THF–C₆₀ and THF–water. Analyses of THF–C₆₀ and THF–water by gas chromatography–mass spectrometry did not detect THF but found THF oxidation products γ-butyrolactone and tetrahydro-2-furanal. Toxicity of γ-butyrolactone (72 hr lethal concentration predicted to kill 50% was 47 ppm) indicated effects in THF treatments can result from γ-butyrolactone toxicity.

CONCLUSION: This research is the first to link toxic effects directly to a THF degradation product (γ-butyrolactone) rather than to C₆₀ and may explain toxicity attributed to C₆₀ in other investigations. The present work was first presented at the meeting “Overcoming Obstacles to Effective Research Design in Nanotoxicology” held 24–26 April 2006 in Cambridge, Massachusetts, USA.

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Nanoscience focuses on investigations of phenomena at the nanoscale and is the foundation for nanotechnology, which develops practical applications for nanomaterials (U.S. Nanotechnology Initiative 2006). Because nanomaterials have unique properties and are potentially biologically active, there is significant concern that harm to ecosystem and human health could occur after environmental contamination. According to a recent report, carbon nanomaterials (fullerenes and nanotubes) have the highest relative frequency of occurrence in consumer products already on the market (Nanotechnology Consumer Products Inventory 2006), and these materials may contaminate the environment in the future (Colvin 2003).

Fullerenes (i.e., Buckminsterfullerene, or “Bucky balls”) are nanomaterials that gained attention after the first preparation of C₆₀ a novel allotrope of carbon consisting of 60 carbon atoms joined to form a cage-like structure (Kroto et al. 1985). The unique structure of C₆₀ facilitates absorption of light and transfer of this energy to triplet oxygen, thereby forming the highly reactive singlet oxygen state (Arbogast et al. 1991). High yield of singlet oxygen with consequential generation of free radicals suggests that presence of C₆₀ in the environment may cause oxidative damage in exposed organisms.

Release of C₆₀ into the environment may lead to contamination of aquatic ecosystems and presence of bioavailable nanoparticles. C₆₀ is exceedingly insoluble in water (2 × 10–24 mol/L; Nakamura et al. 1996); however, nanoparticles (< 220 nm) consisting of aggregates of C₆₀ can occur (Andrievsky et al. 1999; Scharff et al. 2004), and these aggregates are relevant for exposure in aquatic organisms (Lovern and Klaper 2006; Oberdörster 2004). Aqueous aggregates of C₆₀ can be generated by mixing pure C₆₀ in water or by use of vehicle solvents. Tetrahydrofuran (THF) has been used as a vehicle to generate aqueous aggregates of C₆₀ (Deguchi et al. 2001) in toxicology studies (Lovern and Klaper 2006; Oberdörster 2004), and yields water with a persistent amber color (Fortner et al. 2005). However, THF can alter surface charges of C₆₀ particles, and THF may be retained between adjacent C₆₀ molecules within aggregates (Brant et al. 2005). Thus, there is potential that toxicity attributed to C₆₀ (e.g., Lovern and Klaper 2006; Oberdörster 2004) could actually result from the presence of THF or THF degradation products (Brant et al. 2005). Following our initial presentation of the present research (“Overcoming Obstacles to Effective Research Design in Nanotoxicology,” Cambridge, Massachusetts, USA, 24–26 April 2006), subsequent studies (e.g., Oberdörster et al. 2006; Zhu et al. 2006) have prepared aqueous C₆₀ aggregates without THF or other organic solvents.

The objective of the present research was to investigate toxicity of aqueous C₆₀ nanoparticles in larval zebrafish Danio rerio, and to determine if THF or THF degradation products used to prepare aqueous C₆₀ can be responsible for toxic effects. End points of toxicity included survival, behavior, and changes in global gene expression.

Materials and Methods

Fish. Zebrafish D. rerio were obtained from the Zebrafish Research Facility at the University of Tennessee in Knoxville, Tennessee, and all experiments were conducted with approval from the University of Tennessee Institutional Animal Care and Use Committee. In all experiments, zebrafish were treated humanely and with regard for alleviation of suffering. Water for holding fish and conducting experiments (designated “fish water”) was prepared with purified MilliQ water (Millipore Corp., Bedford, MA) with ions added: 19 mg/L NaHCO₃, 1 mg/L sea salt (Instant Ocean Synthetic Seawalt, Mentor, OH), 10 mg/L CaSO₄, 10 mg/L MgSO₄, 2 mg/L KCl. The “fish water” had the following characteristics: pH 7.3–7.9; dissolved oxygen > 6 mg/L; total alkalinity, 30–40 mg/L (as CaCO₃); and total hardness, 15–20 mg/L (as CaCO₃). All larvae used in experiments were obtained (Westerfield 1993) at the same time and were the same age (i.e., fertilization).
follows: approximately 12.5 mg of C60 was obtained from a previously reported method (high purity) nitrogen to remove oxygen. The C60 was added to 500 mL of fresh THF in a 1-L Büchi Rotavap system (Büchi Labortechnik AG, Flawil, Switzerland) was used to remove any visible aggregates that might have formed on the bottom of the bottle. The procedure for generating aqueous C60 with THF was modified from a previously reported method (Deguchi et al. 2001; Forster et al. 2005) as follows: approximately 12.5 mg of C60 was added to 500 mL of fresh THF in a 1-L amber bottle and sparged with UHP ultra high purity nitrogen to remove oxygen. The sealed amber bottle [THF–C60 and THF–water (vehicle control)] were stirred for 24 hr at ambient temperature. Fish water (350 mL) was added to each bottle and sparged with UHP nitrogen for 1 hr, then stirred for 24 hr at room temperature. A Büchi Rotavap system (Büchi Labortechnik AG, Flawil, Switzerland) was used to remove THF, in the dark, at 65°C, and the resulting solutions (THF–C60 and THF–water) were sparged with nitrogen for 2.5 days before use in toxicity tests. Solutions of C60–water were allowed to settle and were carefully pipetted to avoid resuspension of any visible aggregates before addition of fish water to prepare dilutions for exposures. The THF–C60 stock solution did not have any visible (naked eye) aggregates, and dilutions with highest concentrations of THF–C60 had a visible amber color. Examination of THF–C60 solution with an enhanced dark-field microscopy system (Cytoviva, Auburn, AL) designed to resolve particles < 100 nm, demonstrated particle aggregates approximately equal to 0.5–3 times the size of latex nanoparticles of 100 nm examined under identical conditions (Figure 1A, B). This method of particle analysis evaluates particles as they appear in the exposure solution and avoids artifacts introduced by use of transmission electron microscopy methods, which can alter particle size upon drying (Thundat et al. 1993).

Water chemistry analysis. All THF-treated water samples were analyzed for presence of THF with a Hewlett Packard 6890 gas chromatograph with a 5973N mass spectrophotometer (Agilent Technologies, Foster City, CA, USA) equipped with an inert ion source. A 1-μL volume of sample water was injected into a J&W DB-5MS column (30 m × 0.25 mm ID, 0.25-μm film thickness; Agilent Technologies). Helium (UHP grade) was used as a carrier gas, and a constant flow rate (0.6 mL/min) was maintained by electronic pressure control. Injection temperature was 280°C and splitless injection was employed. The oven temperature program started at 30°C, for 4 min, increased to 100°C at 5°C/min, for 2 min, then to 300°C at 50°C/min and maintained for 3 min. MS detection was monitored at full-scan EI mode (m/z 25–100). The intensity of ion m/z 86 was used for quantification of γ-butyrolactone.

Fish exposures. Two dose–response toxicity tests were conducted simultaneously with the test to evaluate changes in gene expression. All fish were exposed in 400-mL glass beakers containing 100 mL exposure water, and exposure began when larvae were 75 hr old and ended when larvae were 147 hr old postfertilization. In dose–response toxicity tests, each beaker contained 9–13 larvae and the following concentrations of either THF–C60 or THF–water: 0%, 1%, 5%, 10%, 20%, and 25% (vol/vol). In the test conducted to evaluate changes in gene expression, each beaker contained 37–45 larvae and the following concentrations (each with six replicate beakers) were tested: control; C60–water (100%); THF–C60 (2.5%); THF–C60 (5%); THF–fish water (2.5%); THF–fish water (5%). Fish mortality was assessed 72 hr after exposure was initiated and behavior of fish in exposure solutions was recorded. Water quality characteristics (temperature, pH, and dissolved oxygen) were measured in treatments at initiation of the experiment, and values were within the range reported for fish water control (see above).

Two additional dose–response tests were conducted separately from experiments described above. The first test chemical was γ-butyrolactone (d150 = 1.1286) at the following concentrations (vol/vol): 0%, 0.0001%, 0.0005%, 0.001%, 0.003%, 0.005%, 0.01%, 0.05%, and 0.1%. Procedures for these dose–response tests were identical to those described above.

Total RNA extraction and microarray analyses. Pairs of the six replicate beakers for each treatment were combined to make three replicates for analysis of differential gene expression by microarray (three arrays per treatment). Total RNA was extracted from fish larvae that survived exposure using the RNA easy mini kit for animal tissues (Qiagen, Valencia, CA, USA). RNA was extracted from larvae using RLT buffer, purified using RNeasy columns, and included DNase digestion according to the procedure described in the RNeasy Mini Handbook for animal tissues (Qiagen 2006). Total RNA was processed by the Affymetrix Core Facility at the University of Tennessee (Knoxville, TN, USA) according to Affymetrix GeneChip Expression Analysis Technical Manual (Affymetrix 2004). Processing of total RNA included synthesis of cDNA (One-Cycle cDNA synthesis kit; Affymetrix, Santa Clara, CA, USA), biotin labeling (3’IVT Labeling Kit; Affymetrix), hybridization, and scanning according to standard Affymetrix protocols. For each

Figure 1. Photomicrographs of nanoparticles (arrows) in aqueous solution obtained by dark-field microscopy (100x objective, oil immersion) under identical conditions with enhanced resolution system. (A) Aqueous aggregates of C60 generated by THF vehicle (THF–C60) (B) FluoSpheres (Invitrogen, Carlsbad, CA, USA) (carboxylate modified microspheres, 100-nm diameter) in 2 mM Na2NO3 solution. Comparison of C60 aggregates indicates they were 0.5–3 times the size of the FluoSpheres. Scale bars = 1 μm.
zebrafish array, an equal amount of labeled cRNA (5 μg) was used. All arrays were assessed for quality measurements including assurance that IVT (in vitro transcription) glyceraldehyde-3-phosphate dehydrogenase (GADPH) 3′/5′ values were < 3, and all internal spike in controls were present at anticipated levels. The Affymetrix GeneChip Zebrafish Genome Array contains approximately 15,509 probe sets that represent 14,900 D. rerio gene transcripts. Probe sets for the array were designed by Affymetrix, members of the zebrafish community, and the National Institutes of Health investigators using public data sources: RefSeq (July 2003; http://www.ncbi.nlm.nih.gov/RefSeq/), GenBank (Danio rerio (zebrafish) genome; release 136.0, June 2003; http://www.ncbi.nlm.nih.gov/mapview/map_search.cgi?taxid=7955), dbEST (Expressed Sequence Tag; July 2003; http://www.ncbi.nlm.nih.gov/dbEST), and UniGene (Build 54, June 2003; http://www.ncbi.nlm.nih.gov/UniGene/UGOrg.cgi?TAXID=7955). Sixteen pairs of oligonucleotide 25-mer probes are used to measure the level of transcription of each sequence. Detection sensitivity is estimated at 1:100,000. Housekeeping and control genes are GADPH and alpha 1 actin. Hybridization controls included are bioB, bioC, bioD, and cre. The Affymetrix Probe Set ID and the gene title and symbol (UniGene; http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) are reported for annotated genes along with their GenBank accession number (http://www.ncbi.nlm.nih.gov/Genbank/index.html).

Data analysis. The 72-hr lethal concentration predicted to kill 50% of larval zebrafish (LC50) was computed by Trimmed Spearman-Karber method (version 1.5; U.S. Environmental Protection Agency 1993). Mortality of fish in exposures used for gene expression analyses was modeled by analysis of variance (ANOVA) after arcsine transformation of mortality data (Zar 1984), and significant (p < 0.05) differences among groups were assessed by Tukey test (Proc GLM, Statistical Analysis System, version 9.1; SAS Institute, Cary, NC).

Statistical assessment of differential gene expression data was conducted according to standard procedures by the University of Tennessee Affymetrix Core Facility. Intensity of expression of individual gene transcripts was obtained from scanned images of zebrafish arrays (Affymetrix 3000 7G) and signal values were obtained using the GCRMA algorithm (ArrayAssist; Stratagene La Jolla, CA, USA). Genes with a fold change in expression of < 1.75 were removed from the data set along with genes that had low signal intensity (< 16 on a scale of 65,536) indicative of minimal (i.e., background) expression. Expression of the resulting set of genes was assessed by an ANOVA model with Benjamini-Hochberg (false discovery rate, FDR) correction (ArrayAssist; Stratagene). After determination of significance (p < 0.05) of the overall ANOVA model, pairwise comparisons of each experimental treatment with the control were conducted by t-test.

Results

Water chemistry analyses. Each water sample was analyzed for presence of THF and impurities, and THF was found in all samples collected immediately after vacuum extraction. However, in water that was sparged with nitrogen gas (N2) for 2.5 days after vacuum extraction, THF was not detected. γ-Butyrolactone and tetrahydro-2-furanol were detected in all THF-treated samples (Figure 2). Tetrahydro-2-furanol was confirmed by comparison with NIST Mass Spectral Library (2005) because authentic standard was not available, and γ-butyrolactone was determined by comparison with an authentic standard (Figure 3).

Immediately after preparation, the concentration of γ-butyrolactone was approximately 103 ppm and approximately 158 ppm in THF–water and THF–C60 treatments, respectively, and after 72 hr, concentrations of γ-butyrolactone increased to approximately 164 ppm in THF–water and approximately 175 ppm in THF–C60. γ-Butyrolactone and tetrahydro-2-furanol were detected by (gas chromatography–mass spectrometry (GC-MS) in some freshly opened THF bottles prior to use in experiments.

Larval fish survival. No fish died during 72-hr exposure in the control, and mortality increased with concentration in both THF–water and THF–C60 with LC50 values of 6.3% [95% confidence interval (CI), 5.1–7.8%] and 3.1% (95% CI, 2.3–4.2%) respectively. At higher concentrations (> 5%) in THF–water and THF–C60, lethal effects were observed within 60 min, and fish typically had arched backs and occasionally had severe yolk-sac and pericardial edema. In concentrations with significant partial mortality (i.e., 5% exposure concentration), surviving fish were lethargic, laterally recumbent, and typically had minor to severe yolk-sac and/or pericardial edema.

Figure 2. TIC traces obtained from GC-MS analysis of THF-treated water samples. Abbreviations: 2-OH-THF, tetrahydro-2-furanol; GBL, γ-butyrolactone. (A) THF, time = 0; (B) THF–C60, time = 0; (C) THF, time = 75 hr; (D) THF–C60, time = 75 hr.

Figure 3. Mass spectrum of γ-butyrolactone obtained from (A) THF–C60-treated sample at 72 hr; (B) spectrum obtained from authentic standard. Intensity of each peak was adjusted to base peak (m/e 42).
Differential expression of genes identified by microarray analysis of larval zebrafish after exposure to C60-water in comparison to control.

**Table 1.** Differentially expressed genes identified by microarray analyses of larval zebrafish after exposure to C60-water in comparison to control.

| Affymetrix Probe set ID | Gene title (gene symbol) | GenBank accession no. | Fold difference | p-Value | Description/function |
|-------------------------|--------------------------|-----------------------|-----------------|---------|---------------------|
| Dr.Affx.3.1.A1 | CDNA clone MGC:113968 | BX296557.35 | −6.29 | 0.019 | Transport, nuclear pore |
| Dr.Affx.1.85.A1 | DNA clone MGC:113968 | BX296557.35 | −6.29 | 0.019 | Transport, nuclear pore |
| Dr.2616.1.S1 | RNA binding motif protein, X-linked (rbmx) | AY325285.1 | −2.44 | 0.005 | Unknown |
| Dr.6431.1.S1 | zgc:56537 (zgc:56537) | AY325285.1 | −2.44 | 0.005 | Unknown |
| Dr.691.1.S1 | zgc:101565 (zgc:101565) | AY325285.1 | −2.44 | 0.005 | Unknown |
| Dr.25536.1.A1 | Similar to Heat shock protein HSP 90-alpha (HSP 86) (DKEY-241L7.8) | CR381646.8 | −1.86 | 0.000 | Unknown |
| Dr.3448.1.S1 | Kruppel-like factor 2a, klf2a | NM_199950.1 | −1.90 | 0.005 | Metabolism, oxidoreductase |
| Dr.20524.1.A1 | Matrix metalloproteinase 13, mmp13 | NM_001004641.1 | −1.90 | 0.005 | Metabolism, oxidoreductase |
| Dr.26327.1.A1 | DiGeorge syndrome critical region gene 8 (dgrc8) | NM_001004641.1 | −1.90 | 0.005 | Metabolism, oxidoreductase |
| Dr.7842.1.A1 | Matrix metalloproteinase 9, mmp9 | NM_001004641.1 | −1.90 | 0.005 | Metabolism, oxidoreductase |
| Dr.10314.1.A1 | Matrix metalloproteinase 9, mmp9 | NM_001004641.1 | −1.90 | 0.005 | Metabolism, oxidoreductase |

*Accession numbers are from GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). Genes were also differentially regulated relative to control for THF-water treatment. Fold changes were k−4.53, −2.29, −1.8, −1.76. Genes also differentially regulated relative to control for THF-C60 treatment; fold changes were k−1.76, −1.8, −1.76.*
low toxicity in larval zebrafish ($LC_{50} = 1.73\%$; 15.4 g/L). Thus, any trace amounts of THF in THF–C$_{60}$ and THF–water treatments did not explain the observed fish mortality. However, use of THF as a vehicle resulted in generation of substances in the water that were detected by GC-MS and could be responsible for toxic effects. Notable among these substances was $\gamma$-butyrolactone, which was shown to be acutely toxic at low concentrations (72-hr $LC_{50}$, 0.00477%; ~47 mg/L based on dilution of analyzed prepared solution) in larval zebrafish in this study. Estimated concentrations of $\gamma$-butyrolactone (based on dilution of the analyzed prepared solution) at the $LC_{50}$ for THF–water and THF–C$_{60}$ treatments were 10 and 5 mg/L, respectively, at the end of exposure.

$\gamma$-Butyrolactone is a colorless liquid that is soluble in water (Ciolino et al. 2001) and rapidly absorbed in mammals where it is readily converted into the neurotransmitter gamma amino butyric acid (GABA) in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999). In zebrafish, GABA receptors are responsive to GABA in several steps by enzyme action (Bernasconi et al. 1999).

Figure 5. Numbers of genes in each treatment that differed significantly from the control. Numbers that fall into more than one circle were common to both treatments, whereas numbers in only one circle indicate genes that were unique for the specific treatment.

Table 2. Selected up-regulated genes in larval zebrafish in THF–water and THF–C$_{60}$ treatments compared to control. No significant changes in expression were observed for these genes between THF–C$_{60}$ and THF–water treatments.

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C$_{60}$ and toxicity of THF decomposition products

Most relevant to the present investigation is research in largemouth bass (Oberdörster 2004), which indicated oxidative injury in the brain resulted after exposure to THF–C$_{60}$. Oberdörster (2004) directly determined lipid peroxidation in the brain by the thiobarbituric acid (TBA) assay for malondialdehyde and found higher peroxidation of lipids in fish exposed to THF–C$_{60}$. We did not investigate oxidative injury directly in zebrafish, but analyses of gene expression can provide important insight on organism physiology and be used to deduce affected metabolic pathways (Brazma et al. 2001). Up-regulation of genes with antioxidant activity (including glutathione S-transferase) in THF–water and THF–C$_{60}$ treatments in the present study is consistent with the hypothesis that fish were responding to defend against oxidative injury resulting from exposure to oxidative chemicals. Oxidative injury in the brains of largemouth bass observed by Oberdörster (2004) could have resulted from oxidative substances generated by THF vehicle rather than C$_{60}$ directly.

Figure 6. Fold changes in expression for the 182 common genes in larval zebrafish from THF–water and THF–C$_{60}$ treatments with expression levels that differed significantly from the control. The x-axis is the number of the gene, which was ordered based on the fold change observed in THF–water.

Table 2. Selected up-regulated genes in larval zebrafish in THF–water and THF–C$_{60}$ compared to control. No significant changes in expression were observed for these genes between THF–C$_{60}$ and THF–water treatments.

Affymetrix Probe Set ID | Gene title (gene symbol) | Expression change (fold) | Description/function
--- | --- | --- | ---
Dr.10624.1.A1 | zgc:110343 (zgc:110343) | 7.00 | Peroxidase activity, antioxidant activity
Dr.23788.1.A1 | Glutathione S-transferase (gstp1) | 5.39 | Metabolism, glutathione transferase activity
Dr.9492.1.A1 | zgc:113156, hypothetical protein (zgc:113156) | 3.69 | Oxidoreductase activity

GenBank accession numbers (http://www.ncbi.nlm.nih.gov/Genbank/): *AL954171.10, *NM_131734.3, AF285098.3; *XM_696730.1, NM_001017861.1.
Changes in global gene expression were nearly identical in THF–water and THF–C₆₀ treatments, and these results indicate that fish were responding to similar exposure scenarios in both treatments. Although expression of specific genes that had significant changes in expression could be probed further with additional techniques [e.g., quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)] to clarify how a treatment affects particular genes, changes in expression appear related to the presence of THF or THF degradation products rather than C₆₀. Therefore, further pursuit of these genes (e.g., genes listed in Table 2) as biomarkers of exposure to C₆₀ was not warranted.

THF–C₆₀ was more toxic than THF–water based on larval zebrafish survival (5% concentration) and gene expression patterns (2.5% concentration). The magnitude of the change in gene expression was higher in THF–C₆₀ for 73% of the 182 genes that were in common between THF treatments, and of the 124 genes that differed separately from the control (89 genes THF–C₆₀, 35 genes THF–water) 72% had a higher magnitude of expression change in THF–C₆₀. These results may be explained by either higher concentrations of γ-butyrolactone (or other THF degradation products) in THF–C₆₀, by presence of C₆₀, or possibly by an interaction between the C₆₀ and γ-butyrolactone (or other THF degradation products). The concentration of γ-butyrolactone was higher in THF–C₆₀ (stock solutions after preparation: ~158 ppm in THF–C₆₀; ~103 ppm in THF–water); this could have resulted from differences in evaporation of water during preparation (particularly during N₂ sparging) or, perhaps, the presence of C₆₀ enhanced the formation of γ-butyrolactone. This investigation was not designed to determine if C₆₀ could influence formation of γ-butyrolactone; however, further investigation into this possibility is warranted.

Larval zebrafish exposed to C₆₀ (C₆₀–water treatment) did not die as a result of exposure, and gene expression changes relative to the control were relatively minimal. Of the 10 genes with altered expression, only 2 genes were up-regulated and the function of these genes was unknown (unannotated). Further investigation is required to determine if these two up-regulated genes indicate exposure to aqueous C₆₀ aggregates and the physiologic importance of those biochemical pathways. For the 8 down-regulated genes, 3 were related by electronic annotation to a known function. Modulation of the heat-shock gene indicates a general stress response; however, down-regulation of this gene is difficult to interpret. Similarly, down-regulation of the gene that codes for an RNA binding factor and the gene with homology to a Kruppel-like factor (KLF) is difficult to interpret but could suggest a global modulation of transcription in response to C₆₀ exposure (although changes in global modulation of transcription is not supported by changes in expression in other genes). The KLF family of transcription factors bind to Sp1 sequences, modulations of which have been implicated in growth-related signal transduction pathways as well as apoptosis, angiogenesis, and tumorgenesis (Black et al. 2001). The rather minimal change in global gene expression observed when zebrafish larvae were exposed to C₆₀–water indicates that the exposure scenario used in this investigation had only minimal effects on the fish. Longer exposures to C₆₀ or other exposure scenarios (different species, life history stages) may result in different effects. A final point is as that changes in gene expression were investigated after a 72-hr exposure, presumably the expression of these genes (and likely other genes) will be affected differently after different exposure durations and at different zebrafish life history stages.

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