Transcriptional responses in newly-hatched Japanese medaka (*Oryzias latipes*) associated with developmental malformations following diluted bitumen exposure

Barry N. Madison[1,2], Sarah J. Wallace[2], Jing Zhang[3,4], Peter V. Hodson[1] and Valerie S. Langlois[1,2,5]*

[1] School of Environmental Studies, Queen's University, Kingston, ON, Canada

[2] Department of Chemistry and Chemical Engineering, Royal Military College of Canada, ON, Canada

[3] Department of Diagnostic Imaging, Hospital for Sick Children, Toronto ON, Canada

[4] Neurosciences & Mental Health, SickKids Research Institute, Toronto ON, Canada

[5] Institut National de la Recherche Scientifique, Centre Eau Terre Environnement, Québec, QC, Canada

* *Corresponding author*

Valerie S. Langlois, Ph. D.

Canada Research Chair (CRC) in Ecotoxicogenomics and Endocrine Disruption

Associate Professor, Institut National de la Recherche Scientifique (INRS)

Centre Eau Terre Environnement

490, rue de la Couronne, Québec, QC, Canada G1K9A9

Valerie.Langlois@inrs.ca

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ABSTRACT

Japanese medaka embryos were exposed to water accommodated fractions (WAF) and chemically-enhanced WAF of two types of diluted bitumen (dilbit) at concentrations bracketing the EC50s for developmental malformations. Within these treatments, fish were grouped based on the presence or absence of developmental malformations (e.g., blue sac disease (BSD), and analyzed for novel transcriptomic responses. Microarray analyses identified novel biomarkers and gene networks in dilbit-exposed malformed embryos that were not evident in dilbit-exposed fish without BSD or in dilbit-naïve controls. The top differentially expressed genes (DEGs) included cytochrome P450 transcripts (*cyp1*) in fish from all dilbit treatments (malformed and non-malformed fish), and also: fibroblast growth factor (*fgf7*), AHR repressor (*ahrr*), and squalene monooxygenase (*sqle*). In dilbit-exposed fish that did not develop BSD, the only reported individual DEG was eukaryotic translation initiation factor 3 subunit D (*eif3d*). However, a number of other pathways were enriched, including melatonin effects on circadian clock and the antioxidant response, estrogen and androgen metabolism as well as many receptor signaling pathways. Pathways associated with hedgehog, steroid biosynthesis, and Wnt signalling were significantly altered between low and high concentrations of dilbit exposure. An effect of the dispersant control on swim bladder development was observed at concentrations 10-fold higher than those used to disperse dilbit, and a number of gene targets unique to fish in this comparison were affected. This suggests that the toxic effects of dispersant may involve alternative mechanisms to dilbit, but cause similar phenotypic responses. This study identified novel biomarkers in fish with or without visual malformations exposed to dilbit that can be used to assess the risks of dilbit to aquatic ecosystem health.

1. INTRODUCTION

The transcontinental shipment of diluted bitumen (dilbit) by road, rail, and pipeline from Canada's oil sands region creates a risk of spills to sensitive ecosystems crossed by these routes (Dupuis and Ucan-Marin 2015; Lee et al. 2015; Crosby et al. 2013). The current understanding of dilbit toxicity to aquatic organisms stems primarily from studies of the toxic components of conventional crude and heavy fuel oils (Lin et al., 2015; Adams et al. 2014b; Martin et al. 2014). Most data were generated from studies-of-opportunity following major spills to marine environments, such as the Deepwater Horizon (DWH) in the Gulf of Mexico (Beyer et al. 2016). Freshwater ecosystems present a different set of environmental challenges from those identified in marine ecosystems (King et al. 2014; GOC 2013), and information regarding the behaviour and effects of dilbit is critically lacking for these watersheds (Dew et al. 2015; Lee et al. 2015).

The chronic toxicity of dilbit to fish embryos has been characterized by an array of developmental malformations at the time of hatch, e.g.: pericardial and yolk sac edema, craniofacial, spinal and cardiac malformations, and circulatory failure (Alsaadi et al. 2018a,b; Barron et al. 2018; Alderman et al. 2017a; Madison et al., 2017, 2015). In combination, these abnormalities have been associated with the blue sac disease (BSD), a generalized phenotype occurring as the result of changes associated with the metabolic response to environmental challenges during development. The characterization BSD was used in our previous dilbit studies to initially assess the chronic toxicity of dilbit to medaka embryos to facilitate rapid tissue sampling for additional genomic analyses (Madison et al. 2017, 2015).

As with conventional crude oils, the toxic effects of unconventional complex mixtures like dilbit have been associated with the 3- to 5-ringed polycyclic aromatic compounds (PACs), their alkylated homologs, and related compounds (Hodson 2017). Dilbit differs from conventional oils due to its more rapid loss of low molecular weight (LMW) alkanes and mono-, and diaromatic hydrocarbons during weathering (Barron et al. 2018; Philibert et al. 2016). These compounds are dominant in the oil-gas condensates used as diluents to prepare dilbit from extracted bitumen (King et al. 2014). Due to their

70 volatility, they should have little role in chronic embryo toxicity following a spill. Assuming that the
71 residual PACs are the components causing chronic embryo toxicity, dilbit toxicity is comparable to that
72 of conventional oils. However, weathered dilbit persists in freshwater environments due to the challenges
73 associated with clean-up (e.g., the 2010 Kalamazoo River spill, Dew et al. 2015). Thus, there is a critical
74 need to characterize the chronic toxicity of dilbit to fish embryos and their future recovery.

75 The application of microarray, next-generation sequencing, RNA-Seq and other "-omic"
76 techniques provides unique and detailed insights on the mechanistic pathways that link chemical
77 exposures to gene expression and changes in morphology and physiological function (Martyniuk and
78 Simmons 2016; Williams et al. 2014; Mehinto et al. 2012). These methods identify changes in gene
79 expression to help: i) understand the molecular mechanisms for phenotypic (morphometric; pathological)
80 and physiological effects of contaminants; ii) identify novel targets and pathways that are associated with
81 toxic effects at environmentally-relevant exposures; and iii) distinguish those responses that are most
82 consistent, sensitive, and easy to measure, as biomarkers for diagnosing and monitoring contaminant-
83 specific effects.

84 Because the responses of aquatic organisms to chemical exposures integrate a wide array of
85 genetic and biochemical changes, it is difficult to understand why specific contaminants are toxic. This is
86 particularly complicated with unresolved complex mixtures, such as oils, and even more so with dilbit.
87 Transcriptomic studies have been conducted to establish the physiological effects of toxicity of
88 conventional crude oils to fish, including rainbow trout (*Oncorhynchus mykiss*; Hook et al. 2010),
89 Atlantic cod (*Gadus morhua*; Olsvik et al. 2011), polar cod (*Boreogadus bahia*; Andersen et al. 2015),
90 red drum (*Scianops ocellatus*; Xu et al. 2017), olive flounder (*Paralichthys olivaceus*), and seabass
91 (*Lateolabrax maculates*; Jung et al. 2017). There are no reports of the transcriptomic responses of fish to
92 dilbit. However, proteomic responses related to swimming performance have been reported in juvenile
93 sockeye salmon (*Oncorhynchus nerka*; Alderman et al. 2017b).

The specific objectives of this study were two-fold: 1) to assess sublethal responses in the transcriptome of newly-hatched Japanese medaka (*Oryzias latipes*) to dilbit, and 2) to identify gene targets and pathways that are related to the presence or absence of the BSD phenotype.

2. MATERIALS AND METHODS

2.1 Dilbit Exposure and Malformation Assessment

2.1.1 Animals

Medaka embryos were collected from a colony at Queen's University and maintained according to a protocol approved by the Queen's Animal Care Committee (Langlois-2015-1584). Specifically, parental fish (n = 52; 32 females, 20 males) and eggs were held in dechlorinated city water sourced from Lake Ontario, ON, Canada: 27 ± 0.5 °C, pH 7.8 ± 0.2, 7.2 ± 0.2 mg/L O_2. A low-powered, indirect light source on a 16L:8D photoperiod enabled natural circadian rhythms for developing fish with negligible photo-transformations of petroleum hydrocarbons. Over 4 days, 300 eggs were collected and mixed prior to distribution into experimental vessels (for details on egg collection, refer to Madison et al. 2015; 2017). Embryos were staged, pooled from breeding pairs, and distributed evenly across treatments with corresponding staggered start dates to ensure that all eggs were exposed at the same developmental stages. The total number of eggs per treatment differed from the total number of newly-hatched fish sampled at the end of the 16-day exposure because unfertilized eggs were removed at day 5, prior to hatch.

2.1.2 Dilbit stock preparations and water fraction analyses

Un-weathered Access Western blend (AWB) and Cold Lake blend (CLB) dilbit (from Winter 2013 stocks) and Corexit 9500A oil dispersant (ECOLAB/NALCO, Illinois, USA; supplied by Fisheries and Oceans Canada, Dartmouth, NS) were stored in 5 L air-tight metal drums at 4 °C. A 500 mL aliquot of each dilbit stock was held in an amber jar with a Teflon-lined lid in the dark at 4 °C throughout the experiment. Stock solutions of AWB and CLB water accommodated fractions (WAF) were prepared fresh

daily (Madison et al. 2017) at an oil-to-water ratio (OWR) of 1:9. The same OWR ratio, and an oil-to-dispersant ratio of 1:10 were used to prepare chemically enhanced WAF (CEWAF). A dispersant control (DC) equivalent to 1% CEWAF, was prepared with Nujol (1% v/v mineral oil, ρ = 0.84 g/mL @ 25 °C; Sigma-Aldrich, St. Louis, MO, USA) substituted for dilbit. The 1% v/v Nujol CEWAF as the DC treatment was 10-fold higher in concentration than the dispersant concentrations used in dilbit CEWAF treatments (0.1% v/v). This is because 0.1% v/v Nujol CEWAF in our early experiments did not appear to cause phenotypic effects in medaka. The DC treatment served as a control for the effects of dispersant toxicity. Earlier assessments by Madison et al. (2017, 2015) indicated that medaka typically display a high prevalence of un-inflated swim bladders at hatch at 1% v/v Nujol CEWAF without exposure to dilbit.

Nominal dilutions of dilbit WAFs were estimated to be within the range of toxicity to medaka embryos, as characterized by the frequency of developmental malformations associated with BSD, including those of the swim bladder (Madison et al. 2015, 2017). The dilutions of WAF (Lo: 1%, Hi: 10% v/v) and CEWAF (Lo: 0.001%, Hi: 0.1% v/v) for AWB and CLB bracketed the EC50 range for BSD of 100-200 µg/L total petroleum hydrocarbons measured by spectrofluorometry (TPH-F) estimated from our past studies with dilbit (Madison et al. 2017, 2015). Concentrations of Corexit in the dispersant control were also measured by fluorescence spectroscopy of the fluorescent compounds in Corexit. In our oil treatments, total polycyclic aromatic compounds (TPAC; sum of all measured PAC µg/L; n = 49) were also analyzed by AGAT Laboratories, Montreal, Quebec, Canada using gas chromatography-mass spectrometry (GC-MS; AGAT Protocol No.ORG-170) and the dilbits used in this study were characterized in the Supplemental Information in Madison et al. 2017. Because the embryo toxicity of AWB was comparable to that of CLB (Madison et al. 2017, 2015), fish from the two dilbit treatments and WAF and CEWAF at Lo and Hi exposure concentrations were combined based on presence or absence of malformations for analyses of gene expression (see section 2.1.3 below; Fig. S1).

2.1.3 Exposure conditions and malformation assessment

Within 24 hours post-fertilization, eggs were assigned randomly to treatment groups. Each treatment included duplicate jars of at least 15 eggs (n = 30/treatment) exposed for up to 16 days by a static daily renewal protocol; fresh WAF and CEWAF solutions were made daily. Jars were constantly and gently agitated on a New Brunswick Scientific Innova 2000 platform shaker (Eppendorf, Germany) at 60 rpm to ensure water movement and gas exchange. Newly-hatched fish were individually sampled < 24 h post-hatch across all staggered start dates. Each was transferred rapidly to 100 µL of buffered 100 mg/L tricaine methanesulfonate (MS-222; Sigma-Aldrich, St. Louis, MO, USA) on glass slides, photographed under a microscope (Leica DMBL; Leica, Germany), and scored immediately for developmental malformations (BSD). Malformations included craniofacial (CF) and spinal deformities (SP), the absence of an inflated swim bladder (no SB), and yolk sac- (YE) and pericardial edemas (PE). Note that the presence or absence of SB was included in the assessment of BSD, but if "no SB" was the only malformation scored, it did not indicate the BSD phenotype. A truncated evaluation of BSD (binomial Y/N) used a threshold of two or more malformations to confirm the presence of this phenotype. This allowed the rapid processing of newly hatched embryos as a priority for genomic analyses, as described previously (Madison et al. 2017). The individual malformations associated with BSD were also scored from the photographs by two other researchers (double blind) and the results averaged with the scores obtained during sampling. The same samples (total n = 268) were stored on dry ice at -80°C until further analysis.

2.2 Microarray Analyses

2.2.1 Sample pooling, gene chip setup, and microarray scanning

A subsample of newly-hatched medaka from the exposure experiment was grouped for transcriptomic analyses (Table 1a; Fig S1). This select group of fish were from dilbit types (AWB, CLB) and treatments (WAF, CEWAF), and were pooled based on the prevalence of malformations (e.g., BSD) at the time of hatch. This included Lo fish (n = 12; 4 pools of 3 fish each) that showed no visible signs of BSD (i.e., normal fish, or "oil_norm") and Hi treatment fish (n = 12) that all showed signs of BSD (i.e., malformed

fish, or "oil_malf") with 40% no SB. For fish grouped for additional analyses, no malformations (i.e., normal, or "norm") were reported for water control fish (normal control, or "NC_norm", n = 6 pools). However, all dispersant control fish ("DC_malf", n = 3 pools) had BSD and no SB (6 to 9 pools of three fish each per treatment). Additional details regarding the individual fish that make up the group pools for the microarray analysis can be found in Table SB1.

Four central comparisons of these treatment groups were evaluated by transcriptomic analyses and the contrast between: (1) oil_malf *v.* NC_norm: the responses of fish with dilbit-induced BSD compared to water control fish; (2) oil_malf *v.* oil_norm: fish with dilbit-induced BSD compared to dilbit-exposed fish without observable signs of BSD; (3) oil_norm *v.* NC_norm: the responses of fish without observable signs of BSD compared to a water control fish; and (4) oil_malf *v.* DC_malf: the responses of fish with dilbit-induced BSD compared to dispersant-induced BSD (See Table 1b for additional details). Note that the transcriptomic responses observed from each of these four comparisons are relative to the direction of the individual contrasts. For example, a positive response in gene targets in the oil_malf *v.* NC_norm comparison could indicate that higher gene transcription was observed in oil_malf fish in contrast to the NC_norm treatment group fish; or, it could represent the decrease in response of gene targets in the NC_norm group fish, without the corresponding changes in expression levels in oil_malf group fish. All of the comparative results were analyzed by the order in which they appear in the text.

Total RNA was extracted and isolated from pooled whole embryos using the RNeasy Micro Kit (Qiagen, Mississauga, ON, Canada) following the manufacturer's method. Samples were homogenized for 30 s at 20 Hz with a Retsch Mixer Mill M400 (Fisher Scientific, Ottawa, ON) and added to a spin column with RNase-free DNase I. Isolated RNA was re-suspended in RNase-free water, quantified with a NanoDrop-2000 spectrophotometer (ThermoFisher, Ottawa, ON) and assessed for integrity on a Bioanalyzer 2100 (Agilent Technologies, Mississauga, ON). Samples with an RNA integrity number (RIN) >8.0 were used for further analysis. A custom-designed 4x44K microarray for Japanese medaka from Genotypic Technology (Bangalore, India) was created with 23,382 probes (1417 spike-in controls, 100 duplicate probes; Table SB2; Agilent Technologies, Mississauga, ON). Samples were prepared by

One-Color Low Input Quick Amp Gene Expression Labeling Kits (Agilent Technologies, Mississauga, ON). An aliquot of 100 ng RNA from each sample was spiked with the same amount of positive control mix and used for cRNA synthesis and labeling. Samples were fragmented and hybridized to the arrays at 65 °C for 17 h. Intensity signals were read using a SureScan Microarray Scanner and extracted with Feature Extraction software (Agilent Technologies, Mississauga, ON).

2.2.2 Microarray and raw data processing

The microarray data were pre-processed with the R package limma (Ritchie et al. 2015). Raw expression values were normalized by the quantile method. Probes with a normalized hybridization signal value below 10% of the 95th percentile of the negative control were considered low expression and rejected. Probes with less than three arrays meeting this signal strength criterion were also removed to ensure a valid statistical analysis. A total of 18,487 gene probes were surveyed for further analysis, including some duplicate genes represented by unique probes. Experimental design and microarray analyses were performed following recommendations of Simmons et al. (2015) to control variability. Following the Minimum Information About a Microarray Experiment (MIAME) standards (Brazma et al. 2001), all raw data were deposited to the NCBI Gene Expression Omnibus (GEO) database (**GSE142734**).

2.2.3 Differential expression (DE) analysis

DE analysis by the limma package followed Zhang et al. (2018). Statistical analyses included a combination of linear model fitting and empirical Bayesian tests. The alpha value for each comparison was determined conservatively by reference to spike-in positive controls. For the empirical Bayesian test, a nested F-test evaluated the overall statistical significance within a multi-group comparison. The minimum of the resultant p values (p values for the nested F test, or F.p. values) from all the spike-in probes was extracted and the empirical Bayesian p value that contributed to the minimum F.p. value was located and considered as a potential candidate for the alpha value. However, because the minimum F.p. values for all comparisons exceeded 0.05, the alpha value was set at 0.001 to increase stringency. Using

stringent significance thresholds (FC ≥ 1.5; $p = 0.001$), we analyzed the fifteen top-ranked genes by ascending p value, and then the top six up- and down-regulated genes within the four comparisons by FC (Table SB5a-d). Heat maps were generated using supervised cluster analysis to group individuals based on overall molecular signature of all DEGs for the four main comparisons. An analysis of select DE genes (DEGs) by qPCR was performed independently to validate the trends observed in the microarray. Six transcripts were measured from sample pools representing each of the treatments between the four comparisons resulting in 24 individual contrasts to targets within the microarray data (Table SB3a; for more details see Supplementary Information).

2.2.4 Gene set analysis

A comprehensive gene set (GS) analysis assessed the potential functional impact from the gene expression profiles, previously described by Zhang et al. (2017) (for more details see Supplementary Information). Based on statistics such as fold-change and t value, the GS analysis also identified up- or down-regulation for each gene set using the core functions from the piano package (Väremo et al. 2013). Specifically, the "distinct category" evaluates the "canceling out" effect among the up- and down-regulated genes in a given gene set and provides a general directional impact. For clarity, only the distinct up- (disup) and distinct down-regulated genes (disdn) were highlighted beyond their presentation in the supplemental files. The current GS analysis takes the entire gene list into account, is not restricted to DE genes with a fold change (FC) > 1.5, and removes potential bias from the DE threshold criteria. Kyoto encyclopedia of genes and genomes (KEGG) pathways (Kanehisa and Goto 2000) were enriched by the GS analysis strategy. Among the significant KEGG enrichment results, key pathways were visualized with the DE results masked on the figures (Luo and Brouwer 2013).

Gene set databases from ResNet and Pathway Studio (Elsevier, USA) characterized the functional significance of the gene expression profiling, including pathways for cell processes, cell signaling, immunology, inflammation, metabolism, receptor signaling, and signal transduction. For the ResNet

249 database enrichment, only Gene Set Enrichment Analysis (GSEA) was used due to the Pathway Studio
250 software configurations.

251 **3. RESULTS**

252 **3.1 Dilbit Exposure and Malformation Assessment**

253 The daily water concentrations of dilbit WAF and CEWAF exposures ranged between 63-93 µg/L TPH-F
254 (0.10-0.16 µg/L TPAC) for Lo, and 217-705 µg/L TPH-F (1.4-3.0 µg/L TPAC) for Hi (Fig. 1, Fig. S1,
255 SA1-3). The threshold of phenotypic effects, separating Lo and Hi group fish, was estimated to be
256 between 100-200 µg/L TPH-F. This also overlapped estimated EC50 ranges from our earlier experiments
257 using these dilbits. As this TPH-F concentration range likely encompassed the EC50 for malformations,
258 fish were pooled by exposure to concentrations above (Hi, malformed) or below (Lo, normal) this range.
259 For simplicity, we defined the 100 µg/L TPH-F concentration as a threshold of effect based on the
260 prevalence of the BSD phenotype. Compared to control embryos, the prevalence of malformations at
261 hatch increased markedly with dilbit exposure and chemical dispersion but there was no difference
262 between AWB and CLB treatments (Table SA1).

263 Relative to control embryos, hatching was prolonged by all treatments and was delayed by up to
264 five days in dispersant controls (Table SA2). In both Lo and Hi treatments, more than 40% of fish
265 displayed signs of BSD comprised mostly of CF malformations and yolk sec edemas, with ~ 30% with no
266 SB (Table SA1). Fish in the dispersant control showed no SB in 94% of the total hatched, with a lower
267 prevalence of other developmental malformations compared to dilbit-exposed fish.

268

269 **3.2 Microarray Analysis**

270 *3.2.1 Characterization of the BSD phenotype in dilbit exposed-fish (oil_malf v. NC_norm)*

271 Several gene responses previously assessed as molecular biomarkers for dilbit exposure in medaka were
272 compared between fish with BSD induced by dilbit exposure (oil_malf), and non-malformed control fish
273 (NC_norm) (Table 2, Fig. 2a). Of twelve targets examined, *cyp1a* mRNA from oil_malf fish was eight-

274 fold higher (p = 1.5e-11) than NC_norm fish (Table SB4). Levels of *ahr* transcripts were also elevated
275 (FC: 1.7; p = 9.9e-06) compared to normal fish, ranking at 16th by p value and 48 out of 18487 genes
276 analyzed for malformed oil-exposed fish (Table SB4).

277 In relating the oil-induced BSD phenotype, forty-six DEGs were significantly altered in oil_malf
278 fish in contrast to responses of dilbit-naïve fish without malformations at hatch, with cytochrome P450-1
279 isoforms (*cyp1a*, *cyp1c1*, *cyp1b1*) rounding out the top 4 (FC range: 3.54 to 7.99; Table SB5a). Only
280 *cyp1a* and *cyp1b* appeared within the top six up-regulated DEGs when ranked by FC. Both *ahr* and its
281 repressor (*ahrr*) ranked 16th and 10th, respectively, when comparing dilbit-malformed fish to dilbit-naïve
282 fish. Of the top six genes down-regulated within this comparison, protein-glutamine gamma-
283 glutamyltransferase K (*tgm1*), squalene monooxygenase / epoxidase (*sqle*), and lanosterol 14-alpha
284 demethylase (*cyp51*) were all significantly down-regulated by dilbit exposure.

285 The top gene sets up-regulated in the oil_malf *v.* NC_norm comparison included ~20% of cell
286 signaling pathways from the atlas of signaling (2022 measured entities, ME), apoptosis regulation (319
287 ME) and hedgehog pathway (128 ME). Others included those associated with eicosanoids in
288 inflammation, scavenger receptors in platelet aggregation and the tricarboxylic acid cycle (4-25% of 53-
289 173 ME) (Table 4; SB7a). Biosynthesis of cholesterol (10% of 158 ME) and the metabolism of estrogens
290 and androgens (4% of 195) were also significantly down-regulated. The full set of GSEA results are
291 provided in Tables SB6 and SB7. A number of top KEGG pathways were identified for oil_malf *v.*
292 NC_norm (Fig. SB2a; 8 up-, 10 down-regulated). Up-regulated pathways included metabolism of
293 xenobiotics by cytochrome P450 (p = 0.014), hedgehog signaling, and steroid hormone biosynthesis
294 while down-regulated pathways included cell cycling (p = 0.013), oxidative phosphorylation, and those
295 related to DNA repair (Fig. SB3a).

296

297 *3.2.2 Dilbit-responsive biomarkers in newly-hatched fish*

298 In dilbit-exposed embryos with or without BSD (oil_malf *v.* oil_norm), only *cyp1a* was significantly up-
299 regulated (Table 2); c*yp1a* transcripts in malformed dilbit-exposed fish were ~5 fold (FC) higher than in

non-malformed dilbit-exposed fish. Of the 18,487 total probes assessed in our analysis, *cyp1a* ranked the number one by *p* value and FC across almost all comparisons with oil_malf fish, and 6th by FC in comparisons of oil_malf *v.* oil_norm (Table SB4). A similar trend was observed for *ahr* in the oil_malf *v.* oil_norm comparison, but the difference was slightly below the statistical threshold with a FC of 1.47 (ranked 107th). Large FC responses were observed in *hsp70* transcripts for the three comparisons involving dilbit-exposed malformed fish, but these were statistically variable ($p \geq 0.01$).

Eighty-three novel DEGs were identified for the oil_malf *v.* oil_norm comparison, the largest number of DEGs across all four comparisons. As anticipated, *cyp1* isoforms (*cyp1a*, *cyp1c1*, *cyp1b1*) ranked in the top 5 DEGs by *p* value in oil_malf *v.* oil_norm (*p* range 3.3e-11 to 1.9e-12) and *cyp1a* was 6th by FC (5.22, Table SB5c). Five of the top six up-regulated DEGs by FC represented protease and hatching-enzymes: *mahce*, *hcea*, *hceb*, *hce*, and *lce*. Only two of the top six down-regulated DEGs by FC changed significantly between normal and malformed dilbit-exposed fish: *sqle* and *cyp51a1*.

Cell process pathways for adipokines production by adipocytes (23% of 92 ME) and tight junction (occludin) assembly (18% of 170 ME) were up-regulated in the oil_malf *v.* oil_norm comparison (Table 4), as were immunological pathways for natural killer cell activation, metabolism of amino sugars synthesis and WNT planar cell polarity (PCP) non-canonical signaling (14-22% of 71-130 ME). Highlighted down-regulated pathways for this comparison consisted of those for circadian clock and tryptophan metabolism in 16% of 54 and 292 ME, respectively. In contrast to fish without observable signs of BSD following dilbit exposure, dilbit-exposed malformed fish collectively showed 11 up-regulated and 13 down-regulated KEGG pathways (Fig. SB2b). Clear effects on the hedgehog signaling pathway (up) and steroid biosynthesis (down) were observed for this comparison (Fig. SB3b; both, $p = 0.014$).

3.2.3 Biomarkers without the presence of BSD during dilbit exposure

Overall no significant differences were observed in any of the twelve previously-examined biomarker genes between water control and dilbit-exposed fish without visible malformations at hatch (Table 2).

Most novel biomarkers screened between oil_norm and NC_norm groups failed to meet the stringent significance thresholds. This comparison identified 16 genes with $p < 0.001$ but only eukaryotic translation initiation factor 3 subunit D (*eif3d*) was up-regulated above the FC threshold (Table SB5c).

When gene set (GS) responses were compared between oil_norm and NC_norm groups, notable changes were observed in 36 novel genes across six pathways (Table 4), including: cell processes and the role of melatonin in cell survival and antioxidant response (18% of 190 ME), and the CD8+ T-cell activation of immune pathways (14% of 225 ME). Receptor signaling and transduction pathways for number of critical growth factors were also down-regulated (24-46% of 24-155 ME), e.g., transforming growth factor beta (TGF-β), epidermal growth factor (EGF), fibroblast growth factor (FGF), nuclear factor kappa-light chain-enhancer of activated B cell (NF-κB), growth hormone (GH), and tumor necrosis factor alpha (TNF-α). However, despite the general inhibition of a number of general growth and developmental pathways by oil-exposure, no significant KEGG pathways were identified by the consensus GS enrichment method ($p = 0.09$).

3.2.4 Dilbit-specific indicators of BSD

In contrast to fish exposed to the dispersant control with observable malformations, *cyp1a* transcripts in newly-hatched dilbit-exposed increased by ~5 fold (Table 2); no other previously identified targets were altered. However, 52 novel DEGs were identified from the newly-hatched medaka between oil_malf and DC_malf groups. *Cyp1a*, *cyp1b1*, and *cyp1c1* transcripts were 1st, 2nd and 4th, ranked by *p* value, and 1st, 2nd (and 6th; duplicate probes), and 4th by positive FC (Table SB5d). Ubiquitin-conjugating enzyme E2 A (*ube2a*) was the only other non-P450 target identified in the most up-regulated FC DEGs at the threshold of significance. Moreover, insulin-like growth factor-binding protein 1 (*igfbp1*) and alpha-2-macroglobulin-like protein 1 (*a2ml1*) were the second- and third-highest down-regulated DEGs within this comparison. Compared to DC_malf, another isoform of insulin-like growth factor-binding protein, *igfbp4*, was significantly up-regulated (1.58 FC, $p = 2.9e\text{-}5$) in oil_malf. Though *igfbp4* was differentially

expressed in oil_malf *v.* DC_malf it did not meet the 1.5 FC threshold in other comparisons. Two of the three genes (alpha-2-macroglobin-like protein 1, or *a2ml1*, and *igfbp1*) that were significantly down-regulated in the oil_malf *v.* DC_malf comparison at ~4 FC; the highest of all comparisons, were not differentially expressed in any other pairings.

A number of down-regulated gene sets for oil_malf *v.* DC_malf were observed (Table SB6,7d); these included: cell processes for male sex determination, inflammation pathways for vascular endothelial cell activation by blood coagulation factors, the anti-inflammatory response of hypothalamic-pituitary-adrenal axis, metabolism of fatty acids, and receptor signaling of prostaglandins (14-25% of 70-204 ME). A smaller number of gene sets associated with the metabolism of estrogens and androgens was also down-regulated (8 of 195 ME). In oil_malf *v.* DC_malf, no significant up-regulated KEGG pathways were identified. However, cardiac muscle contraction - a known target of oil toxicity, ranked at the top of the list (Fig. SB3c; up, $p = 0.11$). Four down-regulated KEGG pathways were identified for this comparison (Fig. SB2d), including top-ranked arachidonic acid metabolism (Fig. SB3c; down, $p = 0.037$).

3.2.5 Dilbit-responsive DEGs across comparisons

Venn diagrams for the total number of DEGs across the 4 comparisons indicated that expression of 139 genes (87 up-regulated; 53 down-regulated; 1 uncharacterized) were significantly affected by dilbit exposure out of 18,487 probes measured (Fig. 3). The highest number exclusive to any one comparison was oil_malf *v.* oil_norm (38 up-, 12 down-regulated), while none were measured between oil_norm *v.* NC_norm. The largest quantity of shared DEGs between comparisons was 22 (including one uncharacterized probe): 19 up-regulated and 3 down-regulated between oil_malf *v.* NC_norm and oil_malf *v.* oil_norm. Beyond these, oil_malf *v.* NC_norm and oil_malf *v.* DC_malf shared 9 DEGs total, 8 of which were up-regulated.

The top four DE individual genes ranked by *p* and FC from each comparison were selected for further evaluation for their potential to convey dilbit-responsiveness across all comparisons (Table 3). In oil_norm *v.* NC_norm, only *eif3d* of the four genes identified in this comparison met the significance

requirements. The three other genes had FCs between 1.3 and 1.4 despite ranking within the top seven by *p* value (*p* range: 8.6e-4 to 2.1e-6). Across comparisons, low-ranking values were reported for other genes in oil_malf *v.* NC_norm, but *eif3d* was the only significant response. No other effects were observed across the oil_malf *v.* oil_norm or oil_malf *v.* DC_malf comparisons for the four genes selected. The 6th-ranked DEG of the oil_malf *v.* NC_norm comparison, fibroblast growth factor 7 (*fgf7*), was significantly up-regulated across the two other comparisons involving dilbit-exposed malformed fish. No significant change was observed in this gene in the oil_norm *v.* NC_norm comparison, but *fgf7* was up-regulated more than 2-fold in all others; making it the only DEG across three of the four central comparisons. Squalene monooxygenase (*sqle*) in oil_malf *v.* NC_norm and oil_malf *v.* oil_norm comparisons was the only down-regulated DEG to pass significance thresholds in more than one comparison. Sterol-O-acyltransferase 2 (*soat2*) and transmembrane protein 144 (*tmem144*) were significantly up-regulated in oil_malf *v.* NC_norm. Though *igfbp4* was differentially expressed in oil_malf *v.* DC_malf it did not meet the 1.5 FC threshold in the other comparisons. A number of genes passed significance thresholds across other comparisons involving oil_malf fish but did not meet the FC criterion, e.g., *ahrr*, suppressor of cytokine signaling 3b (*socs3b*), angiopoietin-2 (*angpt2*), and multidrug resistance-associated protein 4 (*abcc4*; Table 3). Two of the three genes (alpha-2-macroglobin-like protein 1, or *a2ml1*, and *igfbp1*) that were significantly down-regulated in the oil_malf *v.* DC_malf comparison at ~4 FC; the highest of all comparisons, were not differentially expressed in any other pairings.

A select set of the over-represented gene set from ResNet database identified by GSEA can be found in Table 4, while the top-affected GSEA pathways within the four comparisons can be found in Table SB6. Only the oil_malf *v.* oil_norm comparison indicated significant changes within all major gene set collections measured.

The qPCR analysis of select DEGs successfully validated the microarray results (Table SB3b). Only *socs3b* showed a disparate response to the microarray for oil_malf fish when compared to non-dilbit exposed fish (NC_norm, DC_malf fish); however, its regulation appeared variable throughout.

4. DISCUSSION

The sublethal exposure of developing medaka to dilbit at TPH-F concentrations of about 180 µg/L (≅ 1 µg/L TPACs) caused an increased prevalence of BSD phenotype at hatch. The types and frequency of malformations observed in newly-hatched fish were comparable to those we previously reported for medaka embryos exposed by similar methods to the same parent stocks of AWB and CLB (Madison et al. 2015, 2017). A suite of previously-identified gene biomarkers associated with dilbit toxicity were assessed, as well as many other molecular targets identified by microarray analysis. Several novel genes and their associated regulatory pathways were identified as molecular biomarkers that could predict the effects of dilbit toxicity. However, a lack of change within individual DEG targets of fish without signs of BSD could also indicate low signal-to-noise ratios in genomic targets, or a temporal effect from exposure that was not captured at the time of sample. A time-course assessment of these types of DEG targets during developmental stages was beyond the scope of this survey of potential new dilbit-responsive genes.

4.1 Biomarkers of sublethal dilbit exposure

Transcriptomic responses of the genes proposed as bioindicators of dilbit exposure from our earlier work clearly indicated that cytochrome P450 transcripts remain the most consistent and responsive biomarkers for demonstrating and monitoring dilbit exposure in fishes from areas at risk of a spill. The induction of P450 family enzymes by diverse chlorinated and non-chlorinated compounds, however, suggested that additional genes that respond to dilbit exposure should be identified to increase the specificity of biomonitoring programs using molecular techniques.

Significant responses to dilbit treatments were observed for three genes (*cyp1a*, *ahr*, and *hsp70*) in the current and past studies. Dilbit exposure, even at low concentrations (< 100 µg/L TPH-F), resulted in significant up-regulation of *cyp1* homologs (e.g., *cyp1a1*, *cyp1b1*, *cyp1c1*) and several related P450 enzyme transcripts (e.g., *cyp2*, *cyp19*, *cyp51*). This was expected as CYP1A activity and gene expression of fish have been consistently characterized in response to a number of petroleum hydrocarbons and crude oils (Mu et al. 2016; Adeyemo et al. 2015; Holth et al. 2014; Kim et al. 2013), including dilbit

(McDonnell et al. 2019; Alsaadi et al. 2018b; Madison et al. 2017; Alderman et al. 2017a). Our most recent work suggests the pattern of concentration-responsive *cyp1a* induction may even act as a surrogate of chronic dilbit toxicity (Madison et al. 2017). A number of additional P450 enzyme transcripts have been reported as responsive to dilbit, including those related to xenobiotic activity (e.g., CYP2) and sex steroid biosynthesis (e.g., CYP19, aromatase), highlighting the endocrine-disrupting effects of oils and petroleum-related products (Truter et al. 2016; Wiseman et al. 2013). In addition, the current study also noted significant inhibition of upstream regulators of cholesterol biosynthesis as did Brown et al. (2019); for example, both CYP51 and SQLE gene expression were altered in dilbit-exposed fish.

The expression of *ahr* appears to be a functional biomarker for general oil exposure and effects as well as dilbit toxicity (e.g., Alderman et al. 2017a, Madison et al. 2015, 2017), although the relationship between exposure and response of *ahr* transcripts appears statistically variable (e.g., Madison 2015). A possible reason for variations in *ahr* transcript levels during dilbit exposure may be changes in expression of the AHR repressor (*ahrr*) which were observed in this study, but a consistent trend was not obvious. Similar effects have been reported for *ahrr* of polar cod (*Boreogadus saida*), exposed to crude oil at TPAC concentrations similar- to, or higher, than in the present study (Andersen et al. 2015). These results may be explained by PAC-AHR linked cardiotoxicity characterized in fish exposed to oil. AHR-dependent activation of the xenobiotic response element (XRE) and modulation of ARNT (aryl-hydrocarbon receptor nuclear translocator) by AHRR (Jenny et al. 2009) could affect the specificity and magnitude of *ahr* mRNA synthesis and its use as a biomarker for dilbit exposure.

Concentrations of dilbit > 200 µg/L TPH-F (Hi treatments) elevated *hsp70* expression, and represented a state of general cellular stress in malformed newly-hatched medaka. However, the expression of HSP genes may be influenced by sample methods and, by virtue of their function, reflect environmental conditions, adaptive traits, and exposure to a number of other chemicals (e.g., Andersen et al. 2015). These interactions could confound the interpretation of this biomarker when comparing effects in developing fishes to oil-exposure if responses to specific environmental conditions are not accounted

453 for. Yet, as a metric of a general state of cellular stress, the significant up-regulation of *hsp70* was
454 strongly linked to dilbit exposure when all other factors were held constant.
455
456 *4.2 DEGs and pathways associated with the BSD phenotype in dilbit-exposed fish*
457 Dilbit exposure led to the highest number of DEGs in exposed fish; however a number of unique
458 transcriptomic responses were noted in the oil_malf *v.* oil_norm comparison that were specific to fish
459 with visible malformations. Cross-group comparisons identified *fgf7* as a central transcript related to
460 malformations observed in exposed medaka. The role of *fgf7* and related homologs (e.g., *fgf10a*, *fgf24;*
461 Jung et al. 2017) as biomarkers for fish embryogenesis and development during oil exposure was
462 suggested by Andersen et al. (2015), and the present study extends this role to dilbit exposure. The
463 importance of *fgf7* in coordinating epithelial development was further supported by significant up-
464 regulation within the hedgehog (Hh) signaling pathway, Wnt planar cell polarity non-canonical signaling,
465 and tight junction (occludin) assembly pathways were also noted for malformed fish. The changes
466 observed within signalling pathways of Hh and Wnt could have occurred independently of dilbit effects
467 on abnormal epithelial and craniofacial development. However FGF, Hh, and Wnt have all previously
468 been linked with crucial roles in epithelial-mesenchymal development of the swim bladder in fishes (Yin
469 et al. 2012, 2011; Korzh et al. 2011; Winata et al. 2009).
470 Significant differences in *igfbp4* expression were also noted across all comparisons involving
471 oil_malf fish, however the 1.5-FC threshold was not met in any comparison. These transcripts were only
472 modestly down-regulated in oil_norm *v.* NC_norm fish. The specific role of *igfbp4* in fishes is unclear.
473 Garcia de la Serran and Macgueen (2018) proposed roles include action on growth and development via
474 IGF-2, and possible IGF ligand-independent inhibition of cardiogenesis. Similarly, significant expression
475 differences in angiogenic factor *angpt2*, a critical regulator of tumorigenesis (He et al. 2014), were
476 observed in comparisons involving oil_malf, but the differences were lower than the 1.5 FC threshold.
477 Together, changes in these genes critical to cardiac development appear consistent with the observed
478 frequency of pericardial malformations in the present study of dilbit, as well as cardiac remodeling

observed by Alderman et al. (2018; 2017a,b). A transmembrane anion channel, *tmem144*, was also upregulated in malformed medaka following dilbit exposure. Its suspected actions relate to innate immunity and tumorigenesis in mammals (Wrzesinki et al. 2015), but these actions in fish have yet to be examined.

Gene set analyses of malformed dilbit-exposed fish indicated that dilbit affected cell processes such as adipokine production, immune function (e.g., natural killer cell activation), inflammation- (e.g., eicosanoids), and metabolic pathways (e.g., amino sugars synthesis; tryptophan metabolism; regulation of the tricarboxylic acid cycle). Regulation of immune and inflammation pathways strengthens proposed linkages between oil exposure and the immuno-endocrine regulation of inflammation well characterized in fishes (reviewed by Khansari et al. 2017; Nardocci et al. 2014). Metabolic pathways regulated by dilbit-exposure may be similar to associated energetic changes following exposure to other crude oils (Incardona et al. 2015; Klinger et al. 2015; Mager et al. 2014). However, these responses may be ambiguous without direct isolation from additional physiological stresses associated with oil exposure.

Inhibition of *sqle* and *cyp 51* transcripts in malformed fish appeared in contrast to other monooxygenase and CYP proteins. However, as *sqle* is a rate-limiting step in biosynthesis of cholesterol (Brown et al. 2019), such results led to enrichment of this pathway from the ResNet database (Fig. SB7a), as well as the steroid biosynthesis from KEGG pathways (Fig. SB3b). While stimulatory effects of 48 h crude oil WAF exposure on SQLE mRNA have been reported in the Japanese flounder (*Paralichthys olivaceus*) by Zhu et al. (2016), we observed the opposite for malformed fish exposed to dilbit. Differences in SQLE expression levels may be due to the duration of exposure; i.e., 48 h by Zhu et al. compared to 15 d in the present study. However, both studies report significant inhibitory enrichment of circadian clock pathways, presenting a potential avenue for future biomarker work.

Cholesterol and steroid biosynthesis (e.g., sex steroids) were down-regulated in malformed fish, suggesting dilbit toxicity to steroidogenic pathways. In support of this, *soat-2*, a known element of the synthesis of intracellular cholesterol esters from acetyl COA during yolk-trafficking in embryogenesis (Chang et al. 2016), showed marked up-regulation in malformed fish compared to normal fish.

505 Cholesterol and steroid biosynthesis pathways could provide novel dilbit-responsive biomarkers to help
506 elucidate specific mechanisms of endocrine disruption.
507 Fish exposed to dilbit with malformations displayed significant changes in KEGG and metabolic
508 pathways associated with arachidonic acids (AA). Examples included up-regulation of lecithin pathways
509 and down-regulation of phase I conjugation products (dihydroxyeicosatrienoic-, DHET; midchain
510 hydroxyeicosatetraenoic acids, HETE) via *cyp2* isoforms, and associated prostaglandin pathways. The
511 metabolism of AA by P450 enzymes is well characterized in fish (Schleziner et al. 1998), and has wide-
512 ranging effects on a variety processes, including: ion balance regulation (Carrier et al. 2011); blood
513 platelet aggregation (Tavares-Dias and Oliveira 2009); temperature-induced changes to membrane
514 fluidity (Tian et al. 2017); metabolic fuel supply for growth and swimming performance (Norambuena et
515 al. 2015); regulation of development (Tocher et al. 2010); and hatching success (Pickova et al. 1997). It
516 was suggested that AA is metabolized by CYP1A enzymes induced by exposure to 2,3,7,8-
517 tetrachlorodibenzo-*p*-dioxin (Rifkind 2006). However, there is only limited information about the effects
518 of oil on the regulation of AA metabolism (e.g., Balk et al. 2011). Other biologically active compound
519 contaminants in watersheds (i.e., emerging organic contaminants from personal care products and
520 pharmaceuticals) are possible mediators of oxidative stress in fishes, with effects on the synthesis of
521 isoprostanes, prostaglandin-like molecules produced by AA peroxidation independent of the typical
522 action mediated by cyclooxygenases (reviewed by Gaw and Glover 2016).
523
524 *4.3 Dilbit, dispersant, and swim bladder development*
525 A high frequency of swim bladder abnormalities was observed in medaka embryos exposed to dilbit
526 (Madison et al. 2015, 2017), possibly linked to dispersants used in these experiments. The effect of dilbit
527 on the normal swim bladder development may be most important in physoclistic species (swim bladder
528 inflation using a gas gland) such as the medaka (Madison et al. 2015; 2017). Similar dilbit effects on
529 swim bladder development were not observed in either fathead minnow (Barron et al. 2018), or rainbow
530 trout (Philibert et al. 2016); however, both are physostomes (oral inflation of the swim bladder). In the

present and previous studies with medaka, dispersant control treatments (1% v/v Nujol CEWAF) also caused a higher prevalence of swim bladder malformation than any dilbit treatment, including those with dispersant. The dispersant, Corexit 9500A, contributed little to the sublethal or lethal toxicity of oil in larval fishes (Greer et al. 2019; Esbaugh et al. 2016; Adams et al. 2014a), except indirectly by enhancing the bioavailability of PAC from suspensions of finely dispersed oil droplets (Adams et al. 2014a). However, hypoxic conditions enhance the toxicity of dispersants combined with petrochemicals, likely due to interactions with the more environmentally-persistent components of Corexit such as dioctyl sulfosuccinate (Dasgupta et al. 2015). Whether Corexit alone or in combination with dilbit affects swim bladder development or function in physoclists and physostomes has not been assessed. The need for such research may become apparent in future studies of dilbit toxicity to more species with each type of swim bladder.

4.4 Dilbit exposure without developmental malformations

Major pathways altered in malformed fish included the metabolism of xenobiotics by cytochrome P450 (and related CYP-family proteins) and cell cycling via TGF-β. However, the transcriptomic responses of newly-hatched medaka without BSD (i.e., oil_norm) identified some genes and affected pathways that could be novel indicators of dilbit exposure and toxicity prior to obvious malformations. Aside from *cyp1* isoforms, *eif3d* was the lone DEG expressed in unaffected dilbit-exposed fish. The role of altered *eIF-3d* expression during oil toxicity has not yet been evaluated, but expression levels of *eif2* were altered in mahi-mahi (*Coryphaena hippurus*) embryos and larvae exposed to DWH crude oil (Xu et al. 2016). The activity of eIF protein complexes are rate-limiting in initiation of ribosomal translation events in mammals (Chen et al. 2012), and the over-expression of eIF isoforms is linked to the production of growth factors and oncogenic proteins associated with tumor malignancy. In accordance, the minor but significant down-regulation of translationally-controlled tumor protein 1 (TPT1) mRNA, a known element of tumor reversion (Venugopal 2005), was also noted in oil_norm fish.

Analysis of over-represented gene sets between normal fish from oil and water treatments revealed a number of effects on immunological and inflammatory pathways. These included inhibition of the adaptive immune response via down-regulation of CD8+ T-cell activation and other cell types (e.g., mast cells, lipoxins, neutrophils, and interleukins). The regulation of immune response by the TGF-β pathway is well characterized across taxa (e.g. Lucas et al. 2004), but it can also be traced back to regulation of tumorigenesis via associated PAC toxicity in fishes through tumor suppressing protein 53 (TP53, Williams and Hubberstey 2014; Wang et al. 2010).

Here we observed trends with dilbit exposure in a number of related receptor signaling pathways of critical developmental growth factors (e.g., EGF-R, FGF-R1, and several NF-κB signaling pathways, including GHR, as well as signal transduction pathways for TGF-β and TNF-α). Though no KEGG pathways were enriched significantly between analyses, the cell process pathway for the role of melatonin in both cell survival and antioxidant response was down-regulated in fish exposed to < 100 µg/L TPH-F (Lo dilbit; oil_norm). Immunoregulation of melanophore function and the endocrine stress response has been well documented in fishes (reviews, Harris and Bird 2000; Luger et al. 1998), including the role of melano-macrophage centers (MMCs) during oxidative stress (review, Agius and Roberts 2003). It appeared that the effects of exposure to low concentrations of dilbit centered on both the endocrine and cellular responses to the onset of oxidative stress, and receptor-level changes within signaling pathways of the immune response, particularly those associated with tumorigenesis.

4.5 The influence of Corexit 9500A dispersant on molecular responses to dilbit toxicity

A number of changing DEGs and pathways were observed when comparing the effects of dilbit exposure to those from dispersant toxicity in newly-hatched medaka. Four individual genes identified in oil_malf *v.* DC_malf comparisons: prostacyclin synthase (*ptgis*), *abcc4*, *a2ml1*, and *igfbp1*. Of these DEGs, only the organic anion transporter *abcc4* was altered, though variable, across other comparisons involving oil_malf fish, suggesting a general response to dilbit toxicity and not exclusively to dispersant toxicity. Up-

581 regulation of *abcc4* was also found in zebrafish exposed to organochlorine pesticides (e.g., DDT, lindane)
582 (Lu et al. 2014). Together these findings suggest *abcc4* transcript levels might indicate a general cellular
583 efflux and mitigation of toxicant accumulation and cytotoxicity. With respect to *a2ml1* and *igfbp1*, strong
584 down-regulation between oil_malf *v.* DC_malf fish might be related to abnormal skeletal development. In
585 developing fish, this effect is likely caused by alterations of mitogen-activated protein kinase (MAPK)
586 signaling pathways (review, Ahi 2016). The down-regulation (FC > 4) of *a2ml1, igfbp1*, and *pgits*, were
587 only observed in this comparison, so it is possible that these responses are specific to DC_malf fish.
588 Interestingly, *hsp90* was not identified as a target of toxicity within our dilbit exposures despite its role in
589 the PAC-AHR mechanism of oil toxicity, and the transcriptional response to chemically-dispersed oil by
590 Corexit 9500A in inland silverside (*Menidia beryllina*) embryos (Adeyemo et al. 2015). Together these
591 four DEGs may provide novel avenues for the further examination of the potential mechanisms of Corexit
592 9500A toxicity in embryonic fishes.
593 In oil_malf *v.* DC_malf comparisons, cardiac muscle contraction was the most up-regulated of the
594 KEGG pathways affected by dilbit, although not significant statistically ($p = 0.11$). Impaired cardiac
595 contractility is a primary mechanism of oil toxicity in fish embryos (Heuer et al. 2019; Xu et al. 2017;
596 Incardona et al. 2017, 2015; Jung et al. 2013), likely due to the modification of Ca^{2+}/K^{+} excitation-
597 contraction coupling in cardiomyocytes (e.g., Sørhus et al. 2016; Brette et al. 2014).The recent evidence
598 of cardiac remodeling in sockeye salmon exposed to dilbit (Alderman et al. 2017b) suggests a similar
599 effect with dilbit exposure. In the present study, dilbit exposure caused down-regulation of the expression
600 of the dihydropyridine receptor (DPHR; a component of Ca^{2+} channels) relative to the responses of
601 embryos with pericardial edemas caused by the dispersant control treatment. Concentrations of myosin,
602 tropomyosin (TPM), and Na^{+}/K^{+}/ATPase (i.e., NKA, solute transporter) mRNA in the KEGG cardiac
603 muscle contraction pathway were also elevated in dilbit-exposed fish. This effect was consistent with
604 higher concentrations of serum proteins in sockeye salmon during cardiac remodeling in response to
605 exercise following chronic dilbit exposure (Alderman et al. 2017b). Additionally, gene sets related to fatty
606 acid oxidation, cellular inflammation, interleukin signaling, and the metabolism of sex hormones were

down-regulated in oil_malf compared to DC_malf fish suggesting these pathways could be related to dilbit exposure. Of note, vascular endothelial cell activation by blood coagulation factors was down-regulated in oil_malf fish, which was reportedly related to the early development of swim bladder formation (Winata 2009). Interestingly, the Hh pathway, though identified in oil_malf *v.* NC_norm, was not differentially affected between malformed fish in dilbit treatments (including CEWAF) and dispersant controls, suggesting that the inflation of the swim bladder may be affected by similar mechanisms.

5. CONCLUSION

Distinct differences in transcriptomic responses were observed between fish exposed to dilbit that showed visible malformations and those that did not. Most notably, we demonstrated that the up- or down-regulation of several genes (e.g., *ahrr*, *socs3b*, *fgf7*, *sqle*, *soat2*, *tmem114*, and *abcc4*) were effective biomarkers of dilbit exposure and toxicity in embryonic fish before the onset of a BSD phenotype. In addition, a number of novel targets of dilbit exposure (e.g., key elements of cholesterol biosynthesis, developmental hedgehog pathways, and components of the circadian clock) were reported for future transcriptomic biomarker development. By implementing an ecotoxicogenomic approach, the current study identified effective biomarkers for monitoring the risks to aquatic ecosystems of dilbit spills to freshwater.

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Table 1a) Grouping characteristics of fish used for transcriptomic microarray analyses. These groups were subsampled from the chronic dilbit exposure experiment based on the prevalence of blue sac disease (BSD; malformations) (see Table SA1).

Group	Code	Oil Type	Treatment	Dispersant	BSD	No SB	*n* (pools of 3)	TPH-F* range (µg/L)	TPAC range (µg/L)
Water Control	NC_norm	None	Water	N	N	N	4	< 55	< 0.01 *lod*
Dispersant Control	DC_malf	Nujol	Dispersant	Y	Y	Y	3	820 to 896	0.05 to 0.09
Oil (all normal)	Oil_norm	AWB, CLB	Lo (WAF, CEWAF)	Mix	N	N	12	63 to 93	0.1 to 0.16
Oil (all malformed)	Oil_malf	AWB, CLB	Hi (WAF, CEWAF)	Mix	Y	Y (40%)	12	217 to 705	1.4 to 3.0

NC - water control, fish without malformations, (normal/"norm"); Nujol - mineral oil; DOR - dispersant-to-oil ratio. DC - Dispersant control (1% Nujol CEWAF, 1:10 DOR) *Note- measures for NC, DC treatments are estimated total fluorescent compounds in Corexit and TPAC ranges are estimated from total fluorescence values. Oils: AWB - Access Western Blend dilbit, CLB - Cold Lake Blend dilbit; WAF - water accommodated fraction, CEWAF - chemically enhanced WAF; Lo - combined dilbit treatments < 100 µg/L TPH-F (~1.0 µg/L TPAC); Hi -combined dilbit treatments > 100 µg/L TPH-F ; BSD – Blue-sac disease; No SB - no swim bladder at hatch; *n* - sample pools size (of 3 fish each).

Table 1b) The four central comparisons between individual groups from **1a)** compared via transcriptomic analyses.

No.	Comparison Coding	Description of terms	Exposure details of contrast
1.	Oil_malf *v.* NC_norm	Oil malformed *v.* Normal control	Hi dilbit concentration (all malformed) *v.* water control (all normal)
2.	Oil_malf *v.* Oil_norm	Oil malformed *v.* Oil normal	Hi dilbit concentration (all malformed) *v.* Lo dilbit concentration (all normal)
3.	Oil_norm *v.* NC_norm	Oil normal *v.* Normal control	Lo dilbit concentration (all normal) *v.* water control (all normal)
4.	Oil_malf *v.* DC_malf	Oil malformed *v.* Dispersant control	Hi dilbit concentration (all malformed) *v.* Nujol + Corexit 9500A (all malformed)

Table 2. The response of the twelve genes (+ housekeeping; via microarray) also examined in our previous work as dilbit biomarkers in newly-hatched Japanese medaka by Madison et al. (2015, 2017) across the four comparisons made in **Table 1b**.

	Gene Name	Gene *abbr.*	Biological Function	**1. Oil_malf *v.* NC_norm**		**2. Oil_malf *v.* Oil_norm**		**3. Oil_norm *v.* NC_norm**		**4. Oil_malf *v.* DC_malf**	
				FC	*p*	*FC*	*p*	*FC*	*p*	*FC*	*p*
HK	ribosomal protein L8	*rpl8*	housekeeping (HK)	1.01	0.84	-1.01	0.78	1.02	0.75	1.01	0.86
1	aryl hydrocarbon receptor	*ahr*	xenobiotic response	**1.76**	**9.9E-06**	1.47	2.4E-05	1.19	0.09	1.41	0.001
2	ahr nuclear translocator 2	*arnt2*	xenobiotic response	1.05	0.33	1.11	0.01	-1.05	0.33	1.16	0.02
3	catalase	*cat*	phase II	1.24	0.07	-1.04	0.68	1.28	0.03	-1.03	0.82
4	cytochrome P450 1A	*cyp1a*	phase I	**7.99**	**1.5E-11**	**5.22**	**1.9E-12**	1.53	0.03	**4.98**	**1.1E-07**
5	glucose-6-phosphate dehydrogenase	*g6pdh*	phase II	1.03	0.76	1.08	0.30	-1.05	0.63	-1.16	0.20
6	glutathione peroxidase 1	*gpx1*	phase II	1.10	0.31	1.05	0.48	1.05	0.59	1.03	0.80
7	glutathione reductase	*gsr*	phase II	1.20	0.01	1.18	0.001	1.02	0.75	-1.22	0.01
8	glutathione S-transferase	*gst*	phase II	1.19	0.06	1.16	0.03	1.03	0.77	1.44	0.03
9	heat shock protein (70 kDa)	*hsp70*	phase II	2.99	0.02	2.30	0.01	1.30	0.53	2.31	0.11
10	nuclear factor, erythroid 2	*nfe2*	transcription factor	1.05	0.40	1.02	0.55	1.02	0.66	1.03	0.63
11	tumor suppressing protein	*p53*	tumorigenesis	-1.17	0.03	-1.16	0.004	-1.00	0.96	-1.13	0.13
12	superoxide dismutase 1	*sod1*	phase II	-1.11	0.11	-1.14	0.01	1.02	0.74	-1.07	0.38

(FC) fold-change; *(p)* value. Bold values denote significant DEGs (FC ±1.5; $p \leq 0.001$). Underlined values denote significant values by one criterion (FC or *p*). Note: the responses of genes (±) are related to the direction of each comparison.

Table 3. The most responsive DEGs identified by each of the four treatment comparisons, organized by FC (fold change), *p* value and *p* rank (of 18487 total genes analyzed) for the identification of novel biomarkers of chronic dilbit exposure. Grey boxes denote the source of DEGs from original comparisons. Bold values represent significant changes within comparisons; FC ±1.5, or $p \leq 0.001$, and where responses meet thresholds across the comparisons for both fold-change and probability (final column): FC ±1.5 and $p \leq 0.001$, respectively. Genes noted in **Table 2** are not included below.

				1. Oil_malf *v.* NC_norm			**2. Oil_malf *v.* Oil_norm**			**3. Oil_norm *v.* NC_norm**			**4. Oil_malf *v.* DC_malf**			Across comparisons
	Gene Name	**Gene *abbr.***	**Accession No.**	*FC*	*p*	*p rank*	*FC*	*p*	*p rank*	*FC*	*p*	*p rank*	*FC*	*p*	*p rank*	*FC ±1.5; p ≤ 0.001*
1	fibroblast growth factor 7	*fgf7*	XM_004066893	**2.76**	**4.5E-07**	6	**2.6**	**2.9E-09**	8	1.06	0.70	12275	**2.23**	**1.5E-04**	31	1,2,4
2	aryl hydrocarbon receptor repressor	*ahrr*	XM_004080922	1.38	**3.2E-06**	10	1.37	**1.5E-08**	11	1.01	0.89	16353	1.41	**1.0E-05**	9	
3	suppressor of cytokine signaling 3b	*socs3b*	EF544580	1.35	**8.8E-06**	15	1.27	**1.6E-06**	59	1.06	0.29	4565	1.26	**0.001**	112	
4	squalene monooxygenase	*sqle*	XM_004077709	**-2.29**	**4.5E-04**	111	**-2.2**	**1.6E-05**	136	-1.04	0.83	14961	1.39	0.19	4444	1,2
5	sterol O-acyltransferase 2	*soat2*	XM_004068681	**2.01**	**1.7E-07**	5	**1.78**	**1.6E-08**	12	1.13	0.22	3260	-1.00	0.99	18336	1,2
6	transmembrane protein 144	*tmem144*	XM_004086472	**2.75**	**5.1E-05**	34	**3.1**	**4.4E-08**	15	-1.13	0.56	9646	1.01	0.97	18023	1,2
7	angiopoietin-2	*angpt2*	XM_004074025	-1.28	**2.3E-05**	24	-1.3	**4.9E-08**	17	1.01	0.8	14374	-1.15	0.021	744	
8	insulin-like growth factor-binding protein 4	*igfbp4*	XM_004065769	1.35	**7.2E-04**	145	1.40	**2.6E-06**	69	-1.04	0.65	11276	**1.58**	**2.9E-05**	15	4
9	abhydrolase domain-containing protein 3	*abhd3*	XM_004079493	-1.33	**5.1E-05**	35	1.05	0.28	8475	-1.39	**2.1E-06**	1	1.05	0.45	9373	
10	MHC class I A	*orla-uaa*	AB026977	1.32	**1.4E-04**	54	-1.03	0.47	11379	1.37	**1.3E-05**	2	1.05	0.52	10515	
11	eukaryotic translation initiation factor 3 sub. D	*eif3d*	XM_004071157	**1.53**	**0.001**	159	-1.00	0.99	18295	**1.53**	**4.4E-04**	5	1.07	0.61	12061	1,3
12	translationally-controlled tumor protein	*tpt1*	NM_001164864	-1.27	0.004	340	1.03	0.63	13603	-1.31	**8.6E-04**	7	-1.12	0.23	5262	
13	prostacyclin synthase	*ptgis*	XM_004068471	1.16	0.059	1939	1.13	0.045	3337	1.03	0.65	11349	**-1.69**	**2.0E-06**	5	4
14	multidrug resistance-associated protein 4	*abcc4*	XM_004085335	1.40	**8.3E-05**	41	1.27	**1.2E-04**	291	1.10	0.17	2415	**1.56**	**1.6E-05**	12	4
15	alpha-2-macroglobulin-like protein 1	*a2ml1*	NM_001201498	1.17	0.54	10682	1.03	0.87	16792	1.13	0.6	10461	**-4.21**	**3.2E-05**	17	4
16	insulin-like growth factor-binding protein 1	*igfbp1*	XM_004079272	1.06	0.83	15621	1.06	0.79	15759	1.01	0.98	18139	**-4.44**	**1.1E-04**	26	4

Table 4. Select gene sets enriched by GSEA test for each of the comparisons of dilbit exposure treatment groups. Complete gene sets for each of the four comparisons can be found in **Table SB7a-d**.

Comparison	Gene set	Name	Total Entities	Measured Entities	Median change	*Adjusted* *p* value
1. Oil_malf *v.* NC_norm	Cell Signaling	Atlas of Signaling	2022	363	1.01	0.007
		Apoptosis Regulation	319	62	1.04	0.012
		Hedgehog Pathway	128	30	1.04	0.021
	Inflammation Pathways	Eicosanoids in Inflammation	173	7	1.06	0.023
	Metabolic Pathways	Biosynthesis of cholesterol	158	15	-1.20	0.006
		Metabolism of estrogens and androgens	195	8	-1.01	0.011
		Tricarboxylic acid cycle	71	18	1.02	0.018
2. Oil_malf *v.* Oil_norm	Cell Process Pathways	Adipokines Production by Adipocyte	92	21	1.07	0.013
		Tight Junction Assembly (Occludin)	170	22	1.02	0.018
		Circadian Clock	54	9	-1.00	0.020
	Immunological Pathways	Natural Killer Cell Activation	130	29	1.05	0.004
	Metabolic Pathways	Amino sugars synthesis	71	12	1.10	0.002
		Tryptophan metabolism	292	46	-1.06	0.006
	Signal Transduction Pathways	WNT Planar Cell Polarity (PCP) Non-Canonical Signaling	73	10	1.02	0.015
3. Oil_norm *v.* NC_norm	Cell Process Pathways	Role of Melatonin in Cell Survival and Antioxidant Response	61	11	-1.07	0.024
	Immunological Pathways	CD8+ T-cell Activation	225	31	-1.02	0.046
	Receptor Signaling	TGFBR -> ATF/GADD/MAX/TP53 signaling	17	7	1.02	0.016
		EGFR -> AP-1/CREB/ELK-SRF/MYC signaling	85	27	-1.01	0.025
		FGFR1 -> STAT signaling	13	6	1.11	0.026
		GHR -> NF-kB signaling	22	9	-1.07	0.047
	Signal Transduction Pathways	TGF-beta Signaling	155	44	-1.01	0.006
		TNF-alpha/TNFRSF1A Signaling	90	22	-1.03	0.018
4. Oil_malf *v.* DC_malf	Cell Process Pathways	Male Sex Determination	70	11	-1.10	0.002
	Inflammation Pathways	Vascular Endothelial Cell Activation by Blood Coagulation Factors	166	41	-1.05	0.016
		Anti-Inflammatory Response of Hypothalamic-Pituitary-Adrenal Axis	92	16	-1.11	0.018
	Metabolic Pathways	Fatty acid oxidation	110	23	-1.09	0.002
		Omega-3-fatty acid metabolism	204	29	-1.05	0.016
		Metabolism of estrogens and androgens	195	8	-1.11	0.019
	Receptor Signaling	ProstaglandinIR -> ATF1/ELK-SRF/CREB signaling	71	18	-1.04	0.024

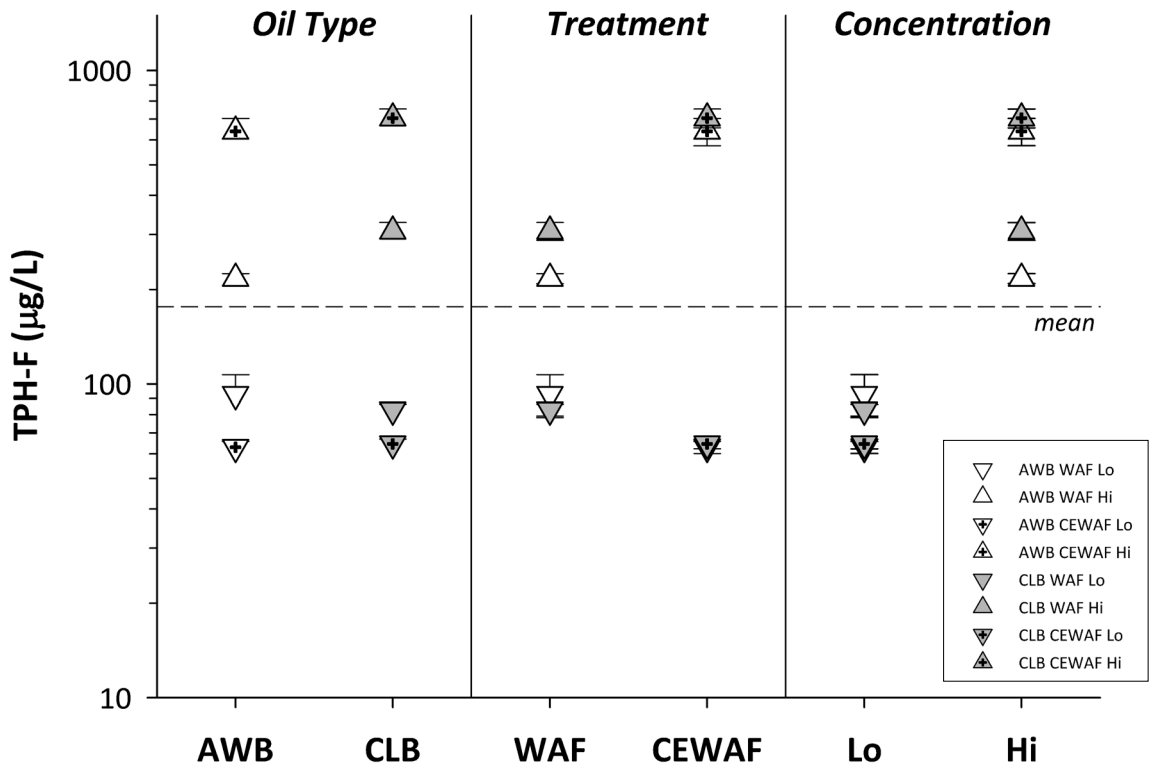
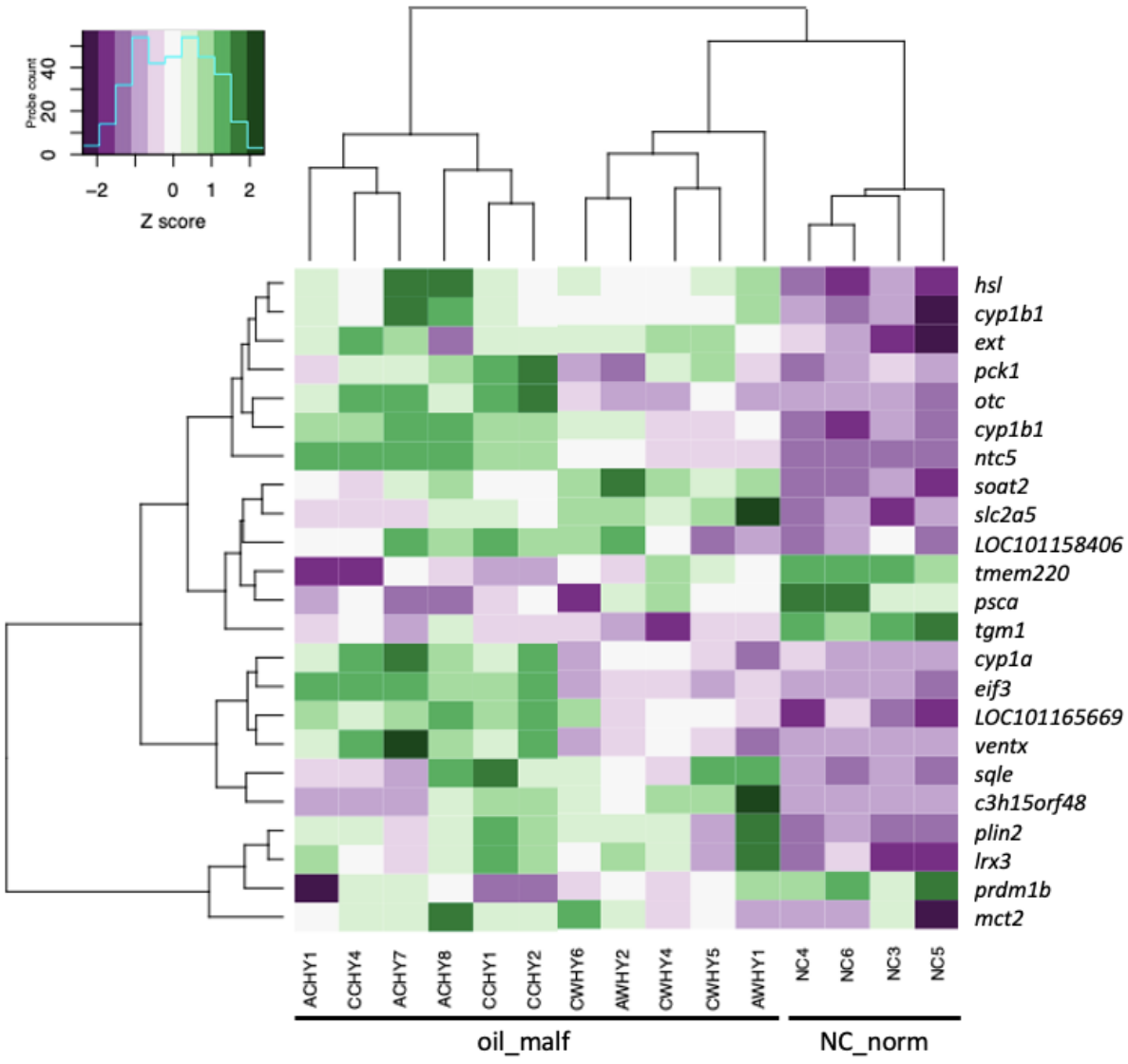
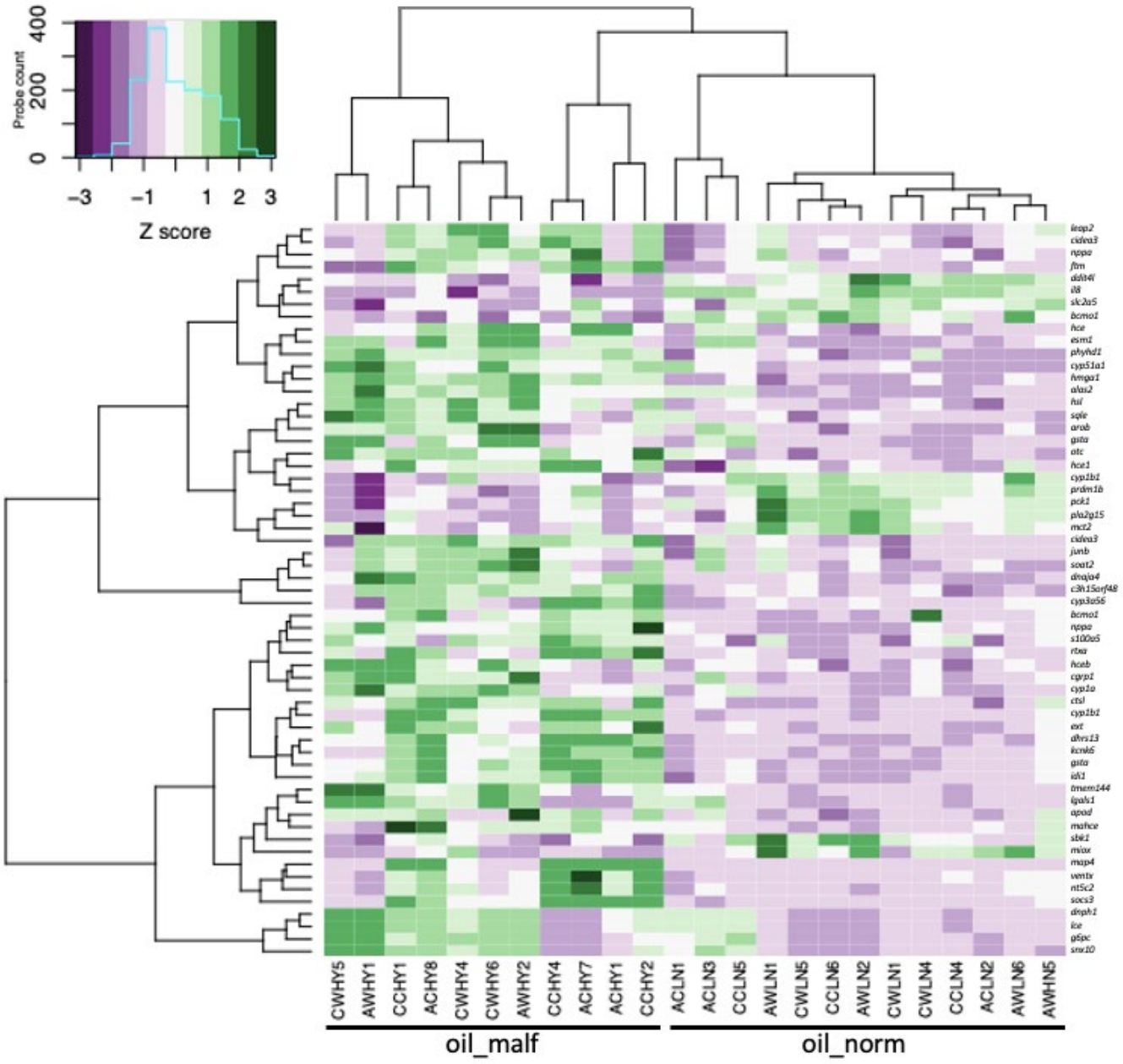


Figure 1. Total petroleum hydrocarbon concentration (by fluorescence), or TPH-F (µg/L), in the 16 d chronic sublethal dilbit exposure experiment by *oil type* (AWB - Access Western Blend dilbit, CLB - Cold Lake Blend dilbit), *treatment* (WAF - water accommodated fraction, CEWAF - chemically enhanced WAF) and *concentration* (Lo - combined dilbit treatments: 63-93 µg/L TPH-F (0.10-0.16 µg/L TPAC); Hi -combined dilbit treatments 217-705 µg/L TPH-F (1.4-3.0 µg/L TPAC). Dashed line is the estimated mean of all data from this study (Lo and Hi combined). Dispersant control is not shown (TPH-F: 858 ± 38 µg/L Total fluorescence).

Figure 2(a-c). Heat map visualizing supervised cluster analysis of the differentially expressed genes (DEGs) within pooled samples which make up the four comparison groups analyzed following chronic dilbit exposure. Green genes represent increasing and purple represent decreasing probe intensities within specific comparison groupings by Z score. No significant differences were noted within the oil_norm *v.* NC_norm comparison. Gene names can be found in Table SB5.

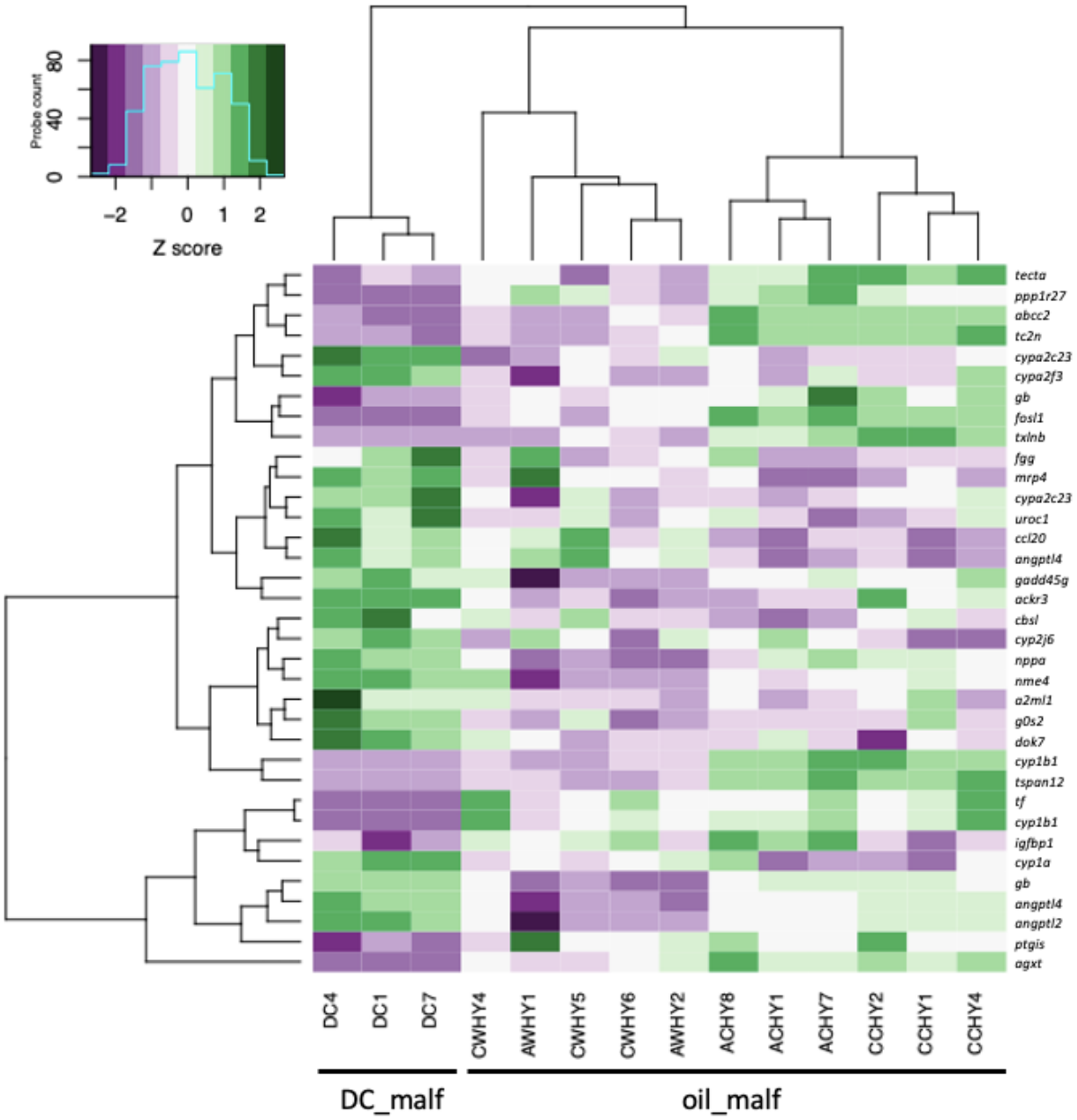


2a) oil_malf *v.* NC_norm



969

970 **2b) oil_malf *v.* oil_norm**



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972 **2c) oil_malf *v.* DC_malf**

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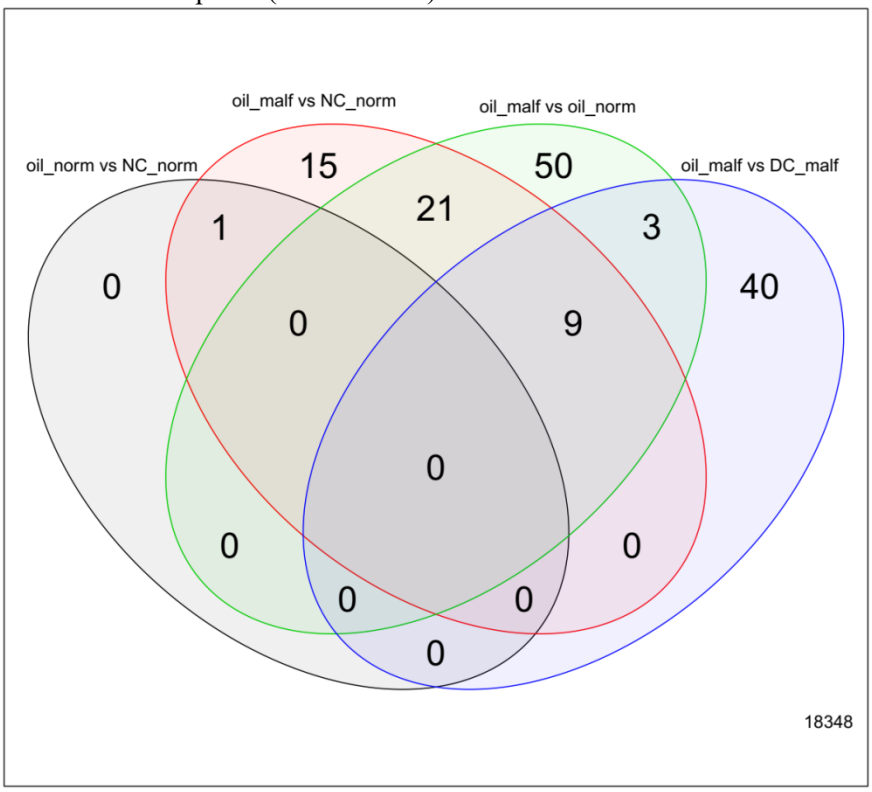
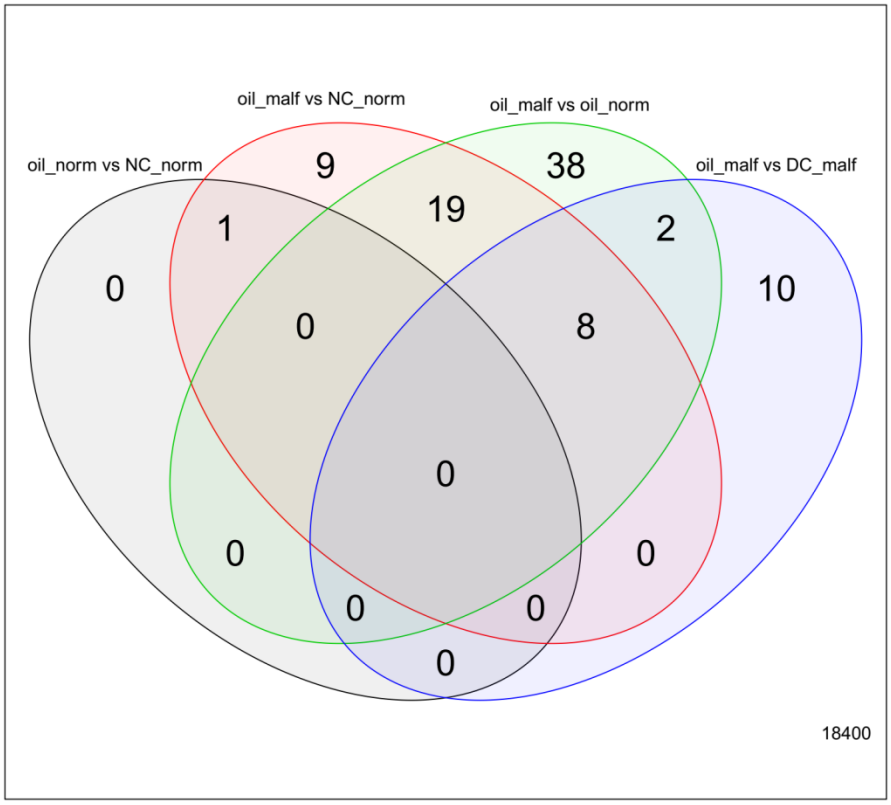
Figure 3(a-c). Venn diagrams showing overlapping and isolated a) total, b) up- and c) down-regulated differentially expressed genes (DEGs; FC ± 1.5; $p < 0.001$) between the four comparisons following chronic dilbit exposure. Total number of genes not included with comparison diagrams is included on the lower right hand section of each panel (out of 18487).



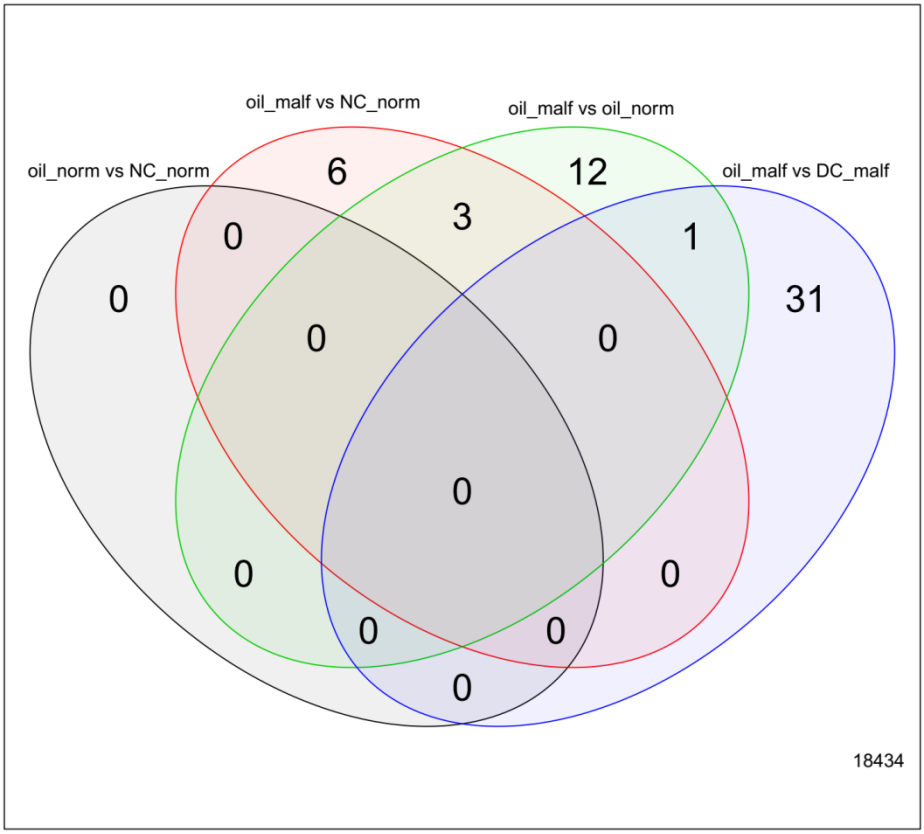
3a) Total DEGs = 139 (one DEG was uncharacterized)



983

984 **3b)** Up-regulated DEGs = 87

985



3c) Down-regulated DEGs = 53