Reproductive Toxicity of Crude oil-Dispersant Mixture in *Caenorhabditis elegans*

By

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DEPARTMENT OF BIOLOGY

As crude oil remains a vital natural resource for the energy need of the world, environmental crude oil spills continue to be a health risk to human beings and ecological systems. During clean-up efforts, surfactant-like dispersants are used to break down big oil slicks into small droplets. Therefore it is necessary to investigate the health impacts of dispersed oil as a mixture entity rather than based on the toxicological profile of individual chemicals. Since reproductive stages of organisms are generally being more sensitive to the effects of toxicants than other stages, investigation of crude oil/dispersant exposure effects on reproduction is critically important. However, studies on the reproductive effects of crude oil-dispersant mixture exposure and its mechanism remain insufficient. The nematode *Caenorhabditis elegans (C. elegans)* has been a useful tool for environmental toxicity studies, and it is a well-known animal model to study the reproduction system. Therefore in this study, we employed the nematode *C. elegans* to test impacts of crude oil/dispersant exposure on basic biological processes growth, reproduction, microRNAs and protein-coding gene expression and its underlying mechanisms.

In Chapter 1, growth and reproduction assays showed that in the model both the

crude oil and the dispersant significantly inhibited the reproduction of *C. elegans*. Dose-dependent inhibition of hatched larvae production was observed in worms exposed to both crude oil and dispersant. Importantly, the chemical dispersant Corexit 9500A potentiated crude oil effects; the dispersant-oil mixture induced more significant effects than oil or dispersant-alone exposures.

In Chapter 2, we showed that crude oil-dispersant mixture affected reproduction by inducing abnormal sperm during the process of spermatogenesis. Results showed that the abnormal immature sperm were significantly increased in the gonad arms of Dis-Oil mixture treated animals compared to controls (K-medium). In addition, Dis-Oil mixture induced more abnormal spermatids including irregular shape of the cell membrane and unexpected tails. Moreover we utilized *puf-8; lip-1* tumor sensitive strain to test the cell fate of immature sperm induced by Dis-Oil mixture treatment. We found increased tumor occurrence in dispersed oil treatments compared to control.

Based on the genome-wide investigation of microRNA profile, in Chapter 3, we found that the aberrant expression of miRNAs was induced. The KEGG pathway enrichment analyses indicated that those significantly changed miRNAs regulate many biological processes in *C. elegans*. Many affected pathways are related to environmental information processing and oil uptake and/or metabolism. Since the currently identified proteins and microRNAs in *C.elegans* show remarkable conservation with mammals including humans, the oil/dispersant may also induce similar change at physiological and molecular levels and affect many biological processes in mammals.

REPRODUCTIVE TOXICITY OF CRUDE OIL-DISPERSANT MIXTURE IN *CAENORHABDITIS ELEGANS*

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DEDICATION

I dedicate this dissertation to the memory of my beloved grandmother Yufang Hu. I love and miss her so much and I will carry her faith, encouragement and love with me always.

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Chapter 1: Chemical Dispersant Potentiates Crude Oil Impacts on Growth, Reproduction, and Gene Expression in *Caenorhabditis elegans*

Abstract

The economic, environmental, and human health impacts of the deepwater horizon (DWH) oil spill have been of significant concern in the general public and among scientists. This study employs parallel experiments to test the effects of crude oil from the DWH oil well, chemical dispersant Corexit 9500A, and dispersant-oil mixture on growth and reproduction in the model organism *Caenorhabditis elegans*. Both the crude oil and the dispersant significantly inhibited the reproduction of *C. elegans*. Dose-dependent inhibitions of hatched larvae production were observed in worms exposed to both crude oil and dispersant. Importantly, the chemical dispersant Corexit 9500A potentiated crude oil effects; dispersant-oil mixture induced more significant effects than oil or dispersant-alone exposures. While oil-alone exposure and dispersant-alone exposure have none to moderate inhibitory effects on hatched larvae production, respectively, the mixture of dispersant and oil induced much more significant inhibition of offspring production. The production of hatched larvae was almost completely inhibited by several high concentrations of the dispersant-oil mixture. This suggests a sensitive bioassay for future investigation of oil/ dispersant impacts on organisms. We also investigated the effects of crude oil/dispersant exposure at the molecular level by measuring the expressions of 31 functional genes. Results showed that the dispersant and the dispersant-oil mixture induced aberrant expressions of 12 protein-coding genes (cat-4, trxr-2, sdhb-1, lev-8, lin-39, unc-115, prdx-3, sod-1, acr-16, ric-3, unc-68, and

acr-8). These 12 genes are associated with a variety of biological processes, including egg-laying, oxidative stress, muscle contraction, and neurological functions. In summary, the toxicity potentiating effect of chemical dispersant must be taken into consideration in future crude oil cleanup applications.

Introduction

In the late April 2010, BP deepwater horizon (DWH) exploded and the oil spill released about 4.1 million barrels of crude oil (7.0 9 10^5 m^3) into the north-central Gulf of Mexico over a period from April 20, 2010 to July 15, 2010 (Allan et al. 2012). The economic, environmental, and human health impacts of the DWH oil spill have been of significant concern to the federal government, scientists, and the public. The class of polycyclic aromatic hydrocarbons (PAH) compounds in crude oil, such as benzo[a]pyrene and fluoranthene, have been well known as potent genotoxic and carcinogenic agents (Bispo et al. 1999; HHS 1995). PAHs present in oil also cause immunotoxicity in a variety of animals, including fish, birds, and mammals (Barron 2012). In response to the oil spill, approximately 2.1 million gallons of dispersants, detergent-like chemicals, were applied to the surface water and around the wellhead (Allan et al. 2012). The chemical dispersant was intended to break down the oil into smaller droplets to facilitate natural degradation and to prevent the oil from spreading and contaminating shorelines and wetlands. The scope dispersant use was unprecedented and prompted interest in understanding the consequence of using dispersants to treat oil spills. Two recent studies on the chemical dispersant Corexit 9500A, the primary dispersant applied during the DWH spill, suggest immunotoxicity and neurotoxicity in rodent models (Anderson et

al. 2011; Sriram et al. 2011). Reproductive stages of organisms are suspected of being more sensitive to the effects of environmental pollutants than other stages, so investigation of crude oil/dispersant exposure effects on reproduction is critically important. One recent report indicates that the Macondo crude oil causes a variety of significant defects in zebra fish embryogenesis and development (de Soysa et al. 2012). Another recent study reported toxicity of the crude oil-dispersant mixture to the ducks embryos when the mixture was applied to egg shells (Finch et al. 2012). However, studies on reproductive effects of crude oil/dispersant exposure and its mechanism remain insufficient. Most recent reports are studies of fish, birds, or rats and focus on the lethality, metabolic effects, and reproductive phenotypes (de Soysa et al. 2012; Van Scoy et al. 2012; Milinkovitch et al. 2012; Wise and Wise 2011; Roberts et al. 2011). Few studies have worked on *Caenorhabditis elegans* and the genetic response underlying reproductive phenotypes. In this study, we employed the nematode *C. elegans*, which has been shown to be a useful model for testing effects of environmental pollutants, to test impacts of crude oil/dispersant exposure on basic biological processes growth, reproduction, and gene expression.

Caenorhabditis elegans, a free-living nematode, lives mainly in the liquid phase of soil and feeds on soil microorganisms. It is one of the best-characterized animals at the genetic, physiological, molecular, and developmental levels. It is the first multicellular eukaryote to have its genome completely sequenced (Anonymous 1998), and the genome of *C. elegans* showed a high level of conservation with vertebrates (Cutter et al. 2009). In addition, *C. elegans* is an ideal animal model for the study of ecotoxicology due to its abundance in soil ecosystems and its properties of short life cycle, small size,

ease of handling in the laboratory and convenient to monitoring behavior under microscope (Brenner 1974; Riddle et al. 1997). *C. elegans* has been widely used in ecological risk assessment for many kinds of chemicals that exist in the environment, including metals (Ho¨ss et al. 2003; Wang and Xing 2008), persistentorganicpollutants (Sochova´etal.2007), pesticides (Cole et al. 2004), and nanomaterials (Roh et al. 2010). Nematodes are sensitive to many different kinds of stresses and can change their growth rate, reproductive speed, brood size, life cycle, and other properties (Dhawan et al. 1999; Anderson et al. 2001). Because of all these advantages, *C. elegans* is frequently used in ecotoxicological studies and ecological risk assessment in both water and soil medium.

Here, we exposed the *C. elegans* to varying concentrations of oil and dispersant solutions to determine the effects of crude oil and crude oil-dispersant mixtures on fundamental biological processes growth and reproduction. Three endpoints (body length, number of offspring beyond the eggs per worm, number of eggs inside body per worm) were used in assays to assess the growth and reproduction and to serve as a source of samples for the measurement of gene expression. We were interested to understand the effects of crude oil on organismal growth and reproduction because these are basic biological processes that may be differentially sensitive to crude oil/dispersant exposures. The toxicity test using *C. elegans* in this study provides a rapid and effective method to study the impact of the DWH oil spill and following usage of dispersant on the ecosystem. It will also be helpful for our further understanding the genetic mechanism underlying toxicity of oil/dispersant to nematodes and other organisms.

Materials and methods

C. elegans cultivation

The wild-type N2 strain of *C. elegans* used in this study was obtained from the *Caenorhabditis* Genetics Center, and worms were maintained at 20 °C on NGM (nematode growth medium)-agar plates seeded with E. coli strain OP50 as food, according to the standard method (Brenner 1974). L1 or L4 stage larvae from an age-synchronized culture were used in the experiments. To obtain age-synchronized cultures, eggs from 3-day-old mature adults plates were isolated via bleaching, followed by rinse with M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 l sterilized by autoclaving), and the eggs were hatched and allowed to grow to L1 larvae in M9 buffer without food. L1 larvae were allowed to grow to L4 larvae (36 h) on agar plates with E. coli OP50 as a food source at 20 °C.

Oil and dispersant preparation

Unweathered crude oil from the Macondo well was provided by BP and Corexit 9500A, the primary dispersant used was provided by NALCO. The stock solution of the dispersant-oil mixture (Dis-Oil) was made as follows: 20 volumes of crude oil and 1 volume of dispersant (according to the approximate proportion in commercial use) were vortex mixed, shaken overnight in a reciprocating shaker at 120 rpm and then diluted 10 times with K-medium (0.032 M KCl and 0.051 M NaCl). K-medium is a commonly used salt medium suitable for *C. elegans* aquatic toxicity testing (Ura et al. 2002; Khanna et al. 1997). While performing dilution, the parent solution was intensively mixed by inversing the conical tube for 25 times, and solutions were immediately taken from the middle of the mixed emulsion. To make the dispersant-alone (Dis) stock solution, 20

volumes of K-medium and 1 volume of dispersant were mixed, shaken overnight, and then intensively shaken, diluted 10 times with K-medium. Similarly, to make oil-alone (Oil) stock, 20 volumes of crude oil and 1 volume of K-medium were mixed overnight, shaken, and then diluted 10 times with K-medium. Different concentrations of working solutions of Dis-Oil, Dis, and Oil were made by dilution of corresponding stock solutions by 20, 50, 100, and 500 times with K-medium. K-medium was used as control.

Growth and reproduction tests

Three endpoints were assessed to test the effects of oil and dispersant on the growth and reproduction in *C. elegans*, which included the body length, number of eggs inside the worm body and number of offspring beyond the eggs per worm. We exposed two developmental stages (L1 and L4-larval) of the *C. elegans* to investigate toxicological effects associated with exposure initiated at different developmental stages. The L1-larval stage nematodes were treated for 5 days, and the L4-larval ones were treated for 3 days. The nematodes were exposed in three different kinds of treatments: oil-alone (Oil), dispersant-alone (Dis), and the mixture of oil and dispersant (Dis-Oil). Each test consisted of four concentrations (diluted 20X, 50X, 100X, 500X of corresponding stock solutions) and a K-medium control. Exposures were conducted in 24-well tissue culture plates (Corning Costar) in a 20 °C incubator. A 1.0 ml of test solution was added to each of the wells with E. coli OP50 as food. One individual worm was loaded into each well with the dosing solution or control. Six replicates were conducted in each of the tests. Growth was assessed by the body length of anaesthetized worms measured using a dissecting microscopy with a scaled lens. Reproduction was assessed by counting the eggs of inside the body per worm using an inversed compound microscope

and counting the offspring larvae in the medium under a dissecting microscope. Six replicates were conducted in each treatment.

Gene expression assay

The impact of oil and dispersant was also investigated at the molecular level. A total of thirty-one selected genes were assessed. Of the selected 31 genes, 20 are related to egg-laying, which are cat-4, egl-5, egl-10, egl-19, egl-44, egl-46, egl-47, hlh-14, lev-1, lev-8, lin39, unc-29, unc-38, unc-43, unc-63, unc-103, unc-115, pink-1, sdhb-1 and sod-1; 9 are stress-response-related genes that are pink-1, sdhb-1, sod-1, age-1, gcs-1, old-1, oxi-1, prdx-3 and trxr-2; 5 are muscle or neuronal function related genes, which are acr-8, acr-16, ric-3, unc-50 and unc-68. Some of the genes have multiple functions; some egg-laying or reproduction related genes also play a role in development and neurological functions. For example, pink-1, sdhb-1, and sod-1 have functions in reproduction as well in stress response. The detailed description and classification information of these genes is listed in Table 1-1.

Based on the growth and reproduction results, we found the L4 larvae were sensitive to dispersant and oil exposure. So we conducted the gene expression assays in worms collected after L4 larvae exposure to Dis and Dis-Oil solutions for 24 h. The objective was to use gene expression as early responsive endpoint for reproductive toxicity, as genetic response in general is more sensitive than classical reproduction phenotypes. Thereby, 24-h exposure instead of 3 day was performed to detect aberrant early expressions. L4 stage larvae were exposed for 24 h in three dosing solutions that were 20X, 50X, and 100X dilutions of the Dis stock solution or the Dis-Oil stock solution. We also use the K-medium as control. Three biological replicates were conducted for each

of the seven different groups: d20, d50, d100, do20, do50, do100, and K-medium control (d: dispersant-alone; do: dispersant-oil mixture). After exposure, worms were harvested and rinsed with K-medium, frozen in liquid nitrogen, and then stored at -80°C until RNA extraction and gene expression analysis.

Total RNA isolation and cDNA synthesis

Total RNA was extracted from the worms using mirVana^{TM} miRNA Isolation Kit (Ambion, Inc) according to the manufacturer's instructions. After adding the lysis buffer, worms were homogenized by using a sonic dismembrator (Model 100, Fisher Scientific) for 10 s at setting 3. RNA concentration and quality were measured with a NanoDrop ND-1000 Micro-Volume UV–Vis Spectrophotometer (NanoDrop Technologies). RNA purity was evaluated by absorbance ratios of 260/280 and 260/230. A total of 1 lg RNA was used for reverse transcription with TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA), using polyT as the reverse transcription primer.

Quantitative real-time PCR (qRT-PCR)

Thirty-one genes were selected to test the impact of oil and dispersant on the gene expression. These genes were selected based on their relationship to important aspects of physiology including reproduction (egg-laying) and stress response. The primer information is listed in Table 1-2. qRT-PCRs were carried out using 10 lL of Real-Time SYBR Green PCR master mix, 3 lL of diluted reverse transcription product, 2 lL of forward and reverse primer, and 5 lL of DNase/RNase free water in a total volume of 20 lL. Amplification was performed using a 7300 Real-Time PCR System (Applied Biosystems) with initial polymerase activation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s denaturation, 60 °C for 60 s for primer-specific annealing and

elongation. Each reaction has three replicates. For qRT-PCR, Y45F10D.4, a conserved iron-binding-related protein, was used to normalize the expression of mRNAs of interest; our previous study demonstrates that Y45F10D.4 was a reliable reference gene in *C. elegans* (Zhang et al. 2012). In this study, Y45F10D.4 also showed stable expression in different treatments. Relative miRNA expression data were analyzed using the DDCT method (Livak and Schmittgen 2001).

Data analysis

All the experiments were repeated for at least three times. The results were presented as mean ± standard error. IBM SPSS Statistics 19 for windows was used for statistical analyses. Analysis of variance (ANOVA) was used for comparing means of different treatment groups in growth, reproduction, and gene expression tests. If there was a significant difference among treatment groups at a 0.05 significant level, least significant difference (LSD) multiple comparisons were conducted to compare the mean of each group.

Results

Crude oil exposure impact C. elegans growth and reproduction

All the F0 worms survived during different exposure conditions tested in this study. Oil exposure affected the L1, but not the L4 stage *C. elegans* body length growth (Fig. 1-1 A1 and B1). After L1 worms were exposed to oil-alone for 5 days, the body length was significantly reduced in 50X ($p = 0.007$) and 500X ($p = 0.010$) oil dilution groups as compared to control. At 100X oil dilution group, the worm body length was marginally lower than control ($p = 0.074$). It is surprising that the highest concentration of oil (20X dilution group) did not affect body length growth. There is no significant difference in

body length between 20X dilution treatment group and control. It is unclear what mechanism caused this phenomenon. Oil is a mixture of many compounds; organism may develop adaption to the contaminated environment by inducing certain gene expression, which could affect growth and development. *C. elegans* may also adapt to the environment by utilizing some compounds in the oil as food resources. Another explanation is that *C. elegans* exposed to oil droplet suspended in the solution, but not the surface oil. It is possible that at high concentrations (20X dilution group), oil tends to be aggregated on the surface and less available to *C. elegans*. There was an obvious oil layer on the surface of the solution in the high concentration group (20X). Although oil affected the growth of L1-stage worms, no significant difference was observed when the exposure was initiated at L4-stage (Fig. 1-1 B1). More likely, this is because that L4-stage *C. elegans* are near mature, and body size growth is not significant.

In contrast, oil exposure affected reproduction when exposure was initiated at L4-stage of *C. elegans*, but not at L1 worms (Fig. 1-1 A2 and B2). After L4 larvae was treated with oil for 3 days, the number of offspring in all treated groups (20X, 50X, 100X, and 500X) were significantly lower than controls (Fig. 1-1 B2). In particular, the offspring number declined dramatically in the 50X dilution group which itself was significantly lower than other treated groups. The number of eggs inside the body was not significantly different among treatments after L4 worms were exposed to oil for 3 days ($p = 0.101$).

Dispersant potentiates the inhibitory effect of oil on C. elegans growth

Dispersant-alone (Dis) exposure did not inhibit the L1 *C. elegans* body length growth (Fig. 1-2 A1). In fact, after 5days of exposure began at L1 larvae, worms at 100X

dispersant dilution group even have 11.8 % longer body lengths (880 ± 48 lm) as compared to controls (787 ± 37 lm). It is possible that dispersant at this concentration produces optimal osmotic regulation in the solution which facilitates *C. elegans* growth and acquisition of nutrients (Matta et al. 2007). In contrast, after L1 worms were exposed to dispersant/oil mixture (Dis-Oil) for 5 days, the body length at low oil concentrations (100X and 500X dilutions) groups was significantly reduced by 8.7 and 9.9 %, respectively ($p < 0.05$) (Fig. 1-2 A1). However, no significant effect was observed when worms were exposed to the same concentrations of oil-alone (Fig. 1-1 A1) or dispersant-alone (Fig. 1-2 A1) treatments. This suggests dispersant potentiates the inhibitory effects of oil on *C. elegans* body length growth. Another interesting phenomenon is similar to what observed in oil-alone exposure described above; high concentrations of oil-dispersant mixture (20X and 50X dilutions) did not affect body length growth, suggesting L1 worms may develop adaption and be able to use some oil components as food source.

Similarly, dispersant-alone exposure did not inhibit the L4 *C. elegans* body length growth (Fig. 1-2 B1). However, when high concentration of oil (20X dilution) was mixed with dispersant, the body length growth of L4 worms was significantly inhibited by 19.6 % (Fig. 1-2 B1). This suggested that suspension of crude oil by dispersant potentiates the growth inhibitory effects even on L4 worms where body size growth rate is limited.

Dispersant potentiates the inhibitory effect of oil on C. elegans reproduction

The potentiating role of dispersant on oil toxicity was even more evident in assays that measure reproductive effects (Fig. 1-2). Under oil-alone exposure starting at L1 worms, we did not observe any impact on the number of offspring or the number of egg inside

the body per worm (Fig. 1-1 A2 and A3). However, when oil was mixed with dispersant, reductions in the number of offspring and also the number of egg inside the body per worm was observed (Fig. 1-2 A2 and A3). Strikingly, with dispersant, high concentrations (20X, 50X, and 100X dilutions) of oil almost completely inhibited the production of offspring larvae ($p < 0.01$) (Fig. 1-2 A2). Although treatment with dispersant-alone also caused a reduction in the production of offspring larvae, the dispersant-oil mixture led to even more significant reductions in the number of offspring larvae (compare Fig. 1-2 A2, dispersant treatment and dispersant-oil treatment at each concentration). This strongly suggests that dispersant potentiates the deleterious effects of oil.

Effects on offspring larvae production by worms exposed for 3 days starting at L4 stage were also measured, in comparison between dispersant-alone and dispersant-oil treatments (Fig. 1-2 B2). Notably, there was a dose-dependent inhibition of offspring production by dispersant-alone treatment. From low to high concentrations (500X, 100X, 50X, 20X dilutions), there are four distinct statistical groups defined (represented as ab, c, d, and e on the bar graph of Fig. 1-2 B2). Importantly, there was also a dose-dependent inhibition of offspring production by treatment with Dis-Oil mixture. Including control, there were four distinct statistical groups shown (represented as a, a, b, c, e on the bar graph of Fig. 1-2 B2). With dispersant, all tested oil concentrations significantly inhibited worms to produce offspring ($p < 0.01$); production of hatched offspring larvae was completely inhibited in treatments of 20X and 50X diluted dispersant-oil mixture. These findings also indicate that exposure starting at L4 larvae results in more sensitive response in offspring production than starting at L1 larvae. One explanation is that L1

larvae developed tolerance to the oil or dispersant when they developed into the mature stage; alternatively, it is possible that there are physiological and developmental differences between L1 and L4 larvae that predispose the L4 larvae to greater sensitivity. One study demonstrated that middle-aged and old *C. elegans* are more sensitive to oxidative stress than younger worms, due to the lower activity of superoxide dismutase (SOD) and catalase and deficits in reactive oxygen species (ROS) scavenger enzymes induction in older worms (Darr and Fridovich 1995).

As compared to offspring larvae production, the number of egg inside body per worm was not a sensitive endpoint. Although some significant inhibitory effects were found in highest concentrations (20X dilutions) of dispersant-alone and Dis-Oil mixture treatments of L4 larvae (Fig. 1-2 B3), the overall effects were less significant. This may be because the number of eggs inside the body per worm (no more than 10 eggs) was already low even at the control group, making the comparison among different toxicant treatments difficult.

Oil exposure impact gene expression in C. elegans

Both dispersant and oil exposure induced aberrant expression of genes. Of the 31 tested genes, the expression levels of 12 genes were significantly changed in *C. elegans* after 24-h exposure of L4 worms to dispersant or the mixture of oil and dispersant (Fig. 1-3). The change in gene expression varied from down-regulation by 1.92-fold (cat-4) to up-regulation by 7.82-fold (lev-8). Three genes (cat-4, trxr-2, sdhb-1) were down-regulated and seven genes (lev-8, lin-39, unc-115, prdx-3, sod-1, acr-16, and ric-3) were up-regulated. The expression of unc-68 was concentration specific; it was up-regulated at a high (20X dilution) and a low (100X dilution) dispersant treatment,

but was down-regulated by 50X dilution of dispersant. In addition, unc-68 was up-regulated under the treatment of a high concentration (20X dilution) of Dis-Oil mixture. The acr-8 was down-regulated when treated with 50X dilution of dispersant, but up-regulated when treated with 50X dilution of Dis-Oil mixture. For some genes, dispersant-alone did not induce aberrant expression, but the mixture of oil and dispersant resulted in significant changes in their expressions. This type of genes included cat-4, trxr-2, sdhb-1, acr-16, and ric-3. This suggests that the aberrant expression of these genes was more likely caused by oil or by dispersant-oil interaction.

Discussion

The initial purpose of using chemical dispersants is to break down the crude oil to smaller droplets and facilitate natural degradation by microorganisms. However, recent evidence suggests that dispersant use could pose additional health risks, including immunotoxicity (Anderson et al. 2011) and neurotoxicity (Sriram et al. 2011). In this study, parallel experiments were performed using the *C. elegans* model to test the effects of crude oil, dispersant, and dispersant-oil mixture on basic biological processes growth and reproduction. Findings indicated that reproduction effects were more sensitive than growth effects in general. Both the crude oil from the deepwater horizon oil well and the dispersant Corexit 9500A using during the cleanup significantly inhibited the reproduction of *C. elegans*. Across the dose range of control to 50X dilution of the stock solution, there was a dose-dependent inhibition of hatched larvae production by worms following 3-day exposure to oil-alone (Fig. 1-1 B2). For dispersant-alone exposure, there was also a dose-dependent inhibition of larvae production across all the tested concentrations (Fig. 1-2 B2). Importantly, results demonstrated that the chemical

dispersant potentiates crude oil effects; oil-dispersant mixture induced more significant effects than oil or dispersant-alone exposures in several growth and reproduction endpoints. For example, while oil-alone and dispersant-alone exposure have none to moderate inhibitory effects on hatched larvae production, respectively (Figs. 1-1 A2, 1-2 A2); the mixture of oil-dispersant induced much more significant inhibition of offspring production (Fig. 1-2 A2 and B2). The production of hatched larvae was almost completely inhibited by several high concentrations of the mixture (Fig. 1-2 A2 and B2). One potential reason is that the dispersant breaks down the crude oil into small oil droplets, so that toxic chemicals, such as PAHs in the crude oil, become more bioavailable. Thus, this potentiating effect must be taken into consideration in the future when using chemical dispersant to treat oil spill for a cleanup purpose.

Transcriptional responses offer insights into the potential biological consequences. Results showed that dispersant and the mixture of dispersant and oil induced aberrant expressions of 12 protein-coding genes. These 12 genes are related to many biological processes, including egg-laying, oxidative stress, muscle contraction, and neuronal functions. Among all aberrantly expressed genes, lev-8 was the most up-regulated (up to 7.82-fold) in response to the dispersant-oil mixture exposure. Lev-8, encoding a novel nicotinic acetylcholine receptor (nAChR) subunit, is expressed in diverse cell types including neurons, body wall and uterine muscle cells, and socket cells. Lev-8 activity is required for various physiological functions including foraging, egg-laying, and locomotion behaviors (Kim et al. 2001; Towers et al. 2005). The upregulation of lev-8 may reflect its roles in sensory and/or reproductive adaption under oil/dispersant exposure. Another example is the cat-4 gene. Cat-4, encoding an ortholog

of the human GTP cyclohydrolase I gene, is involved in serotonin and dopamine biosynthesis. Mutant strain deficient of cat-4 reduced the synthesis of serotonin and dopamine, which further affected movement, egg-laying, mating, and foraging behaviors (Sanyal et al. 2004; Hardaker et al. 2001). In this study, we found that oil-dispersant mixture caused significant decrease in the expression level of cat-4, which may result in disturbed dopaminergic neurotransmission implicated in observed reproduction defects. A recent study in rat also observed deficiency in dopaminergic signaling following exposure to the same dispersant Corexit 9500A. The expression of dopamine D2 receptor (Drd2) was upregulated in the midbrain, accompanied by corroborative changes of several synaptic proteins. Drd2 has been shown to reduce dopamine synthesis and release (Einhorn et al. 1991; Elmestikawy et al. 1986), which is in agreement with the similar overall effects of cat-4 downregulation.

The toxicity test using *C. elegans* in this study provided a rapid and effective method to evaluate the potential environmental health impact of the deepwater horizon oil spill and the dispersants used in the cleanup. *C. elegans*, along with the other thousands of free-living nematode species, are the most abundant soil-dwelling invertebrates that occupy a key position in the terrestrial ecosystem by influencing energy flow and nutrient cycling (Freckman 1988). Sustainability and functionality of terrestrial ecosystems are dependent on healthy biological processes of soil-dwelling organisms. Bacterivorous nematodes including *C. elegans* contribute to the decomposition of soil organic matters and serve as nutrients for soil fauna and microflora, thereby support functions of the terrestrial ecosystem (Neher 2001). Besides its ecological significance, the nematode *C. elegans* is a well-known genetic model used

in neurobiology and developmental biology. With the high conservation of gene pathways between *C. elegans* and vertebrates including humans, findings in *C. elegans* would provide useful assessment of physiological and genetic response of a board range of animal species in response to the DWH oil spill. From previous oil spill experience, it is expected that the oil and dispersant contamination in the environment will persist for many years to come. Further studies about transgenerational effects on *C. elegans*, as well in other species, will be helpful to understand the long-term impact of crude oil and dispersant exposure.

References

Allan SE, Smith BW, Anderson KA (2012) Impact of the deepwater horizon oil spill on bioavailable polycyclic aromatic hydrocarbons in Gulf of Mexico coastal waters. Environ Sci Technol 46(4):2033–2039

Anderson GL, Boyd WA, Williams PL (2001) Assessment of sublethal endpoints for toxicity testing with the nematode *Caenorhabditis elegans*. Environ Toxicol Chem 20(4):833–838

Anderson SE, Franko J, Lukomska E, Meade BJ (2011) Potential immunotoxicological health effects following exposure to corexit 9500a during cleanup of the deepwater horizon oil spill. J Toxicol Environ Health A Curr Issue 74(21):1419–1430

Anonymous (1998) Genome sequence of the nematode *C. elegans* : a platform for investigating biology. Science 282(5396):2012– 2018

Ayyadevara S, Tazearslan C, Bharill P, Alla R, Siegel E, Shmookler Reis RJ (2009) *Caenorhabditis elegans* PI3 K mutants reveal novel genes underlying exceptional stress resistance and lifespan. Aging Cell 8(6):706–725

Bany IA, Dong MQ, Koelle MR (2003) Genetic and cellular basis for acetylcholine inhibition of *Caenorhabditis elegans* egg-laying behavior. J Neurosci Off J Soc Neurosci 23(22):8060–8069

Barron MG (2012) Ecological impacts of the deepwater horizon oil spill: implications for immunotoxicity. Toxicologic pathology (in press)

Bispo A, Jourdain MJ, Jauzein M (1999) Toxicity and genotoxicity of industrial soils polluted by polycyclic aromatic hydrocarbons (PAHs). Org Geochem 30(8):947–952

Brenner S (1974) The genetics of *Caenorhabditis elegans*. Genetics 77(1):71–94

Camon E, Magrane M, Barrell D, Binns D, Fleischmann W, Kersey P et al (2003) The gene ontology annotation (GOA) project: implementation of GO in SWISS-PROT, TrEMBL, and InterPro. Genome Res 13(4):662–672

Chase DL, Pepper JS, Koelle MR (2004) Mechanism of extrasynaptic dopamine signaling in *Caenorhabditis elegans*. Nat Neurosci 7(10):1096–1103

Clark SG, Chisholm AD, Horvitz HR (1993) Control of cell fates in the central body region of *C. elegans* by the homeobox gene lin-39. Cell 74(1):43–55

Cole RD, Anderson GL, Williams PL (2004) The nematode *Caenorhabditis elegans* as a model of organophosphate-induced mammalian neurotoxicity. Toxicol Appl Pharmacol 194:248–256

Culetto E, Baylis HA, Richmond JE, Jones AK, Fleming JT, Squire MD et al (2004) The *Caenorhabditis elegans* unc-63 gene encodes a levamisole-sensitive nicotinic acetylcholine receptor alpha subunit. J Biol Chem 279(41):42476–42483

Cutter AD, Dey A, Murray RL (2009) Evolution of the *Caenorhabditis elegans* genome. Mol Biol Evol 26(6):1199–1234

Darr D, Fridovich I (1995) Adaptation to oxidative stress in young, but not in mature or old, *Caenorhabditis elegans*. Free Radic Biol Med 18(2):195–201

de Soysa TY, Ulrich A, Friedrich T, Pite D, Compton SL, Ok D et al (2012) Macondo crude oil from the deepwater horizon oil spill disrupts specific developmental processes during zebrafish embryogenesis. BMC Biol 10:40

Dhawan R, Dusenbery DB, Williams PL (1999) Comparison of lethality, reproduction, and behavior as toxicological endpoints in the nematode *Caenorhabditis elegans*. J Toxicol Environ Health A 58(7):451–462

Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P et al (2008) Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. Genes Dev 22(23):3236–3241

Eimer S, Gottschalk A, Hengartner M, Horvitz HR, Richmond J, Schafer WR et al (2007) Regulation of nicotinic receptor trafficking by the transmembrane golgi protein UNC-50. EMBO J 26(20):4313–4323

Einhorn LC, Gregerson KA, Oxford GS (1991) D2 dopamine receptor activation of potassium channels in identified rat lactotrophs— whole-cell and single-channel recording. J Neurosci 11(12): 3727–3737

Elmestikawy S, Glowinski J, Hamon M (1986) Presynaptic dopamine auto receptors control tyrosine-hydroxylase activation in depolarized striatal dopaminergic terminals. J Neurochem 46(1): 12–22

Finch BE, Wooten KJ, Faust DR, Smith PN (2012) Embryotoxicity of mixtures of weathered crude oil collected from the Gulf of Mexico and corexit 9500 in mallard ducks

(Anas platyrhynchos). Sci Total Environ 426:155–159

Fleming JT, Squire MD, Barnes TM, Tornoe C, Matsuda K, Ahnn J et al (1997) *Caenorhabditis elegans* levamisole resistance genes lev-1, unc-29, and unc-38 encode functional nicotinic acetylcholine receptor subunits. J Neurosci Off J Soc Neurosci 17(15): 5843–5857

Frank CA, Baum PD, Garriga G (2003) HLH-14 is a *C. elegans* achaete-scute protein that promotes neurogenesis through asymmetric cell division. Development 130(26):6507–6518

Freckman DW (1988) Bacterivorous nematodes and organic-matter decomposition. Agric Ecosys Environ 24(1–3):195–217

Frokjaer-Jensen C, Kindt KS, Kerr RA, Suzuki H, Melnik-Martinez K, Gerstbreih B et al (2006) Effects of voltage-gated calcium channel subunit genes on calcium influx in cultured *C. elegans* mechanosensor neurons. J Neurobiol 66(10):1125–1139

Garcia LR, Sternberg PW (2003) *Caenorhabditis elegans* UNC-103 ERG-like potassium channel regulates contractile behaviors of sex muscles in males before and during mating. J Neurosci Off J Soc Neurosci 23(7):2696–2705

Halevi S, McKay J, Palfreyman M, Yassin L, Eshel M, Jorgensen E et al (2002) The *C. elegans* ric-3 gene is required for maturation of nicotinic acetylcholine receptors. EMBO J 21(5):1012–1020

Hardaker LA, Singer E, Kerr R, Zhou GT, Schafer WR (2001) Serotonin modulates

locomotory behavior and coordinates egg-laying and movement in *Caenorhabditis elegans*. J Neurobiol 49(4):303–313

HHS (1995) Agency for toxic substances and disease registry. Toxicological profile for polycyclic aromatic hydrocarbons http://www. atsdr.cdc.gov/toxprofiles/tp69.pdf

Ho¨ss S, Traunspurger WBA, Markert AMB, Zechmeister HG (2003) Chapter 15 Nematodes. Trace Metals and other Contaminants in the Environment. Elsevier, pp 529–554

Huang J, Lemire BD (2009) Mutations in the *C. elegans* succinate dehydrogenase iron-sulfur subunit promote superoxide generation and premature aging. J Mol Biol 387(3):559–569

Inoue H, Hisamoto N, An JH, Oliveira RP, Nishida E, Blackwell TK et al (2005) The *C. elegans* p38 MAPK pathway regulates nuclear localization of the transcription factor SKN-1 in oxidative stress response. Gene Dev 19(19):2278–2283

Kalis AK, Murphy MW, Zarkower D (2010) EGL-5/ABD-B plays an instructive role in male cell fate determination in the *C. elegans* somatic gonad. Dev Biol 344(2):827–835

Khanna N, Cressman CP 3rd, Tatara CP, Williams PL (1997) Tolerance of the nematode *Caenorhabditis elegans* to pH, salinity, and hardness in aquatic media. Arch Environ Contam Toxicol 32(1):110–114

Kim J, Poole DS, Waggoner LE, Kempf A, Ramirez DS, Treschow PA et al (2001) Genes affecting the activity of nicotinic receptors involved in *Caenorhabditis elegans*

egg-laying behavior. Genetics 157(4):1599–1610

Liao VH, Yu CW (2005) *Caenorhabditis elegans* gcs-1 confers resistance to arsenic-induced oxidative stress. Biometals 18(5): 519–528

Liu Q, Chen B, Yankova M, Morest DK, Maryon E, Hand AR et al (2005) Presynaptic ryanodine receptors are required for normal quantal size at the *Caenorhabditis elegans* neuromuscular junction. J Neurosci 25(29):6745–6754

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25(4):402–408

Matta SG, Balfour DJ, Benowitz NL, Boyd RT, Buccafusco JJ, Caggiula AR et al (2007) Guidelines on nicotine dose selection for in vivo research. Psychopharmacol 190(3):269–319

Milinkovitch T, Lucas J, Le Floch S, Thomas-Guyon H, Lefrancois C (2012) Effect of dispersed crude oil exposure upon the aerobic metabolic scope in juvenile golden grey mullet (Liza aurata). Mar Pollut Bull 64(4):865–871

Moresco JJ, Koelle MR (2004) Activation of EGL-47, a Galpha(o)-coupled receptor, inhibits function of hermaphrodite-specific motor neurons to regulate *Caenorhabditis elegans* egg-laying behavior. J Neurosci 24(39):8522–8530

Murakami S, Johnson TE (2001) The OLD-1 positive regulator of longevity and stress resistance is under DAF-16 regulation in *Caenorhabditis elegans*. Curr Biol 11(19):1517–1523

Neher DA (2001) Role of nematodes in soil health and their use as indicators. J Nematol 33(4):161–168

Nicholas HR, Hodgkin J (2009) The *C. elegans* Hox gene egl-5 is required for correct development of the hermaphrodite hindgut and for the response to rectal infection by Microbacterium nematophilum. Dev Biol 329(1):16–24

Reiner DJ, Newton EM, Tian H, Thomas JH (1999) Diverse behavioural defects caused by mutations in *Caenorhabditis elegans* unc-43 CaM kinase II. Nature 402(6758):199–203

Riddle DL, Blumenthal T, Meyer BJ, Priess JR. 1997. Introduction to *C. elegans*. In: C elegans II, Riddle DL, Blumenthal T, Meyer BJ, Priess JR (Eds) Cold Spring Harbor: NY

Roberts JR, Reynolds JS, Thompson JA, Zaccone EJ, Shimko MJ, Goldsmith WT et al (2011) Pulmonary effects after acute inhalation of oil dispersant (COREXIT EC9500A) in rats. J Toxicol Environ Health A 74(21):1381–1396

Roh J-Y, Park Y-K, Park K, Choi J (2010) Ecotoxicological investigation of CeO_2 and TiO_2 nanoparticles on the soil nematode *Caenorhabditis elegans* using gene expression, growth, fertility, and survival as endpoints. Environ Toxicol Pharmacol 29:167–172

Samann J, Hegermann J, von Gromoff E, Eimer S, Baumeister R, Schmidt E (2009) Caenorhabditits elegans LRK-1 and PINK-1 act antagonistically in stress response and neurite outgrowth. J Biol Chem 284(24):16482–16491

Sanyal S, Wintle RF, Kindt KS, Nuttley WM, Arvan R, Fitzmaurice P et al (2004) Dopamine modulates the plasticity of mechanosensor responses in *Caenorhabditis elegans*. EMBO J 23(2): 473–482

Shibata Y, Branicky R, Landaverde IO, Hekimi S (2003) Redox regulation of germline and vulval development in *Caenorhabditis elegans*. Science 302(5651):1779–1782

Shteingauz A, Cohen E, Biala Y, Treinin M (2009) The BTB-MATH protein BATH-42 interacts with RIC-3 to regulate maturation of nicotinic acetylcholine receptors. J Cell Sci 122(Pt 6):807–812

Sochova´I,HofmanJ,HoloubekI(2007)Effectsofsevenorganic pollutants on soil nematode *Caenorhabditis elegans*. Environ Int 33:798–804

Sriram K, Lin GX, Jefferson AM, Goldsmith WT, Jackson M, McKinney W et al (2011) Neurotoxicity following acute inhalation exposure to the oil dispersant corexit EC9500A. J Toxicol Environ Health A Curr Issue 74(21):1405–1418

Struckhoff EC, Lundquist EA (2003) The actin-binding protein UNC-115 is an effector of Rac signaling during axon pathfinding in *C. elegans*. Development 130(4):693–704

Touroutine D, Fox RM, Von Stetina SE, Burdina A, Miller DM 3rd, Richmond JE (2005) acr-16 encodes an essential subunit of the levamisole-resistant nicotinic receptor at the *Caenorhabditis elegans* neuromuscular junction. J Biol Chem 280(29):27013– 27021

Towers PR, Edwards B, Richmond JE, Sattelle DB (2005) The *Caenorhabditis elegans* lev-8 gene encodes a novel type of nicotinic acetylcholine receptor alpha subunit. J

Neurochem 93(1):1–9

Ura K, Kai T, Sakata S, Iguchi T, Arizono K (2002) Aquatic acute toxicity testing using the nematode *Caenorhabditis elegans*. J Health Sci 48(6):583–586

van der Linden AM, Simmer F, Cuppen E, Plasterk RH (2001) The G-protein beta-subunit GPB-2 in *Caenorhabditis elegans* regulates the G(o)alpha-G(q)alpha signaling network through interactions with the regulator of G-protein signaling proteins EGL-10 and EAT-16. Genetics 158(1):221–235

Van Scoy AR, Anderson BS, Philips BM, Voorhees J, McCann M, De Haro H et al (2012) NMR-based characterization of the acute metabolic effects of weathered crude and dispersed oil in spawning top smelt and their embryos. Ecotoxicol Environ Saf 78:99–109

Wang D, Xing X (2008) Assessment of locomotion behavioral defects induced by acute toxicity from heavy metal exposure in nematode *Caenorhabditis elegans*. J Environ Sci 20:1132–1137

Wise J, Wise JP Sr (2011) A review of the toxicity of chemical dispersants. Rev Environ Health 26(4):281–300

Wu J, Duggan A, Chalfie M (2001) Inhibition of touch cell fate by egl-44 and egl-46 in *C. elegans*. Genes Dev 15(6):789–802 Yanase S, Ishi N (1999) Cloning of the oxidative stress-responsive

genes in *Caenorhabditis elegans*. J Radiat Res (Tokyo) 40(1):

39–47 Yu H, Pretot RF, Burglin TR, Sternberg PW (2003) Distinct roles of

transcription factors EGL-46 and DAF-19 in specifying the functionality of a polycystin-expressing sensory neuron necessary for *C. elegans* male vulva location behavior. Development 130(21):5217–5227

Zhang Y, Chen D, Smith MA, Zhang B, Pan X (2012) Selection of reliable reference genes in *Caenorhabditis elegans* for analysis of nanotoxicity. PLoS ONE 7(3):e31849

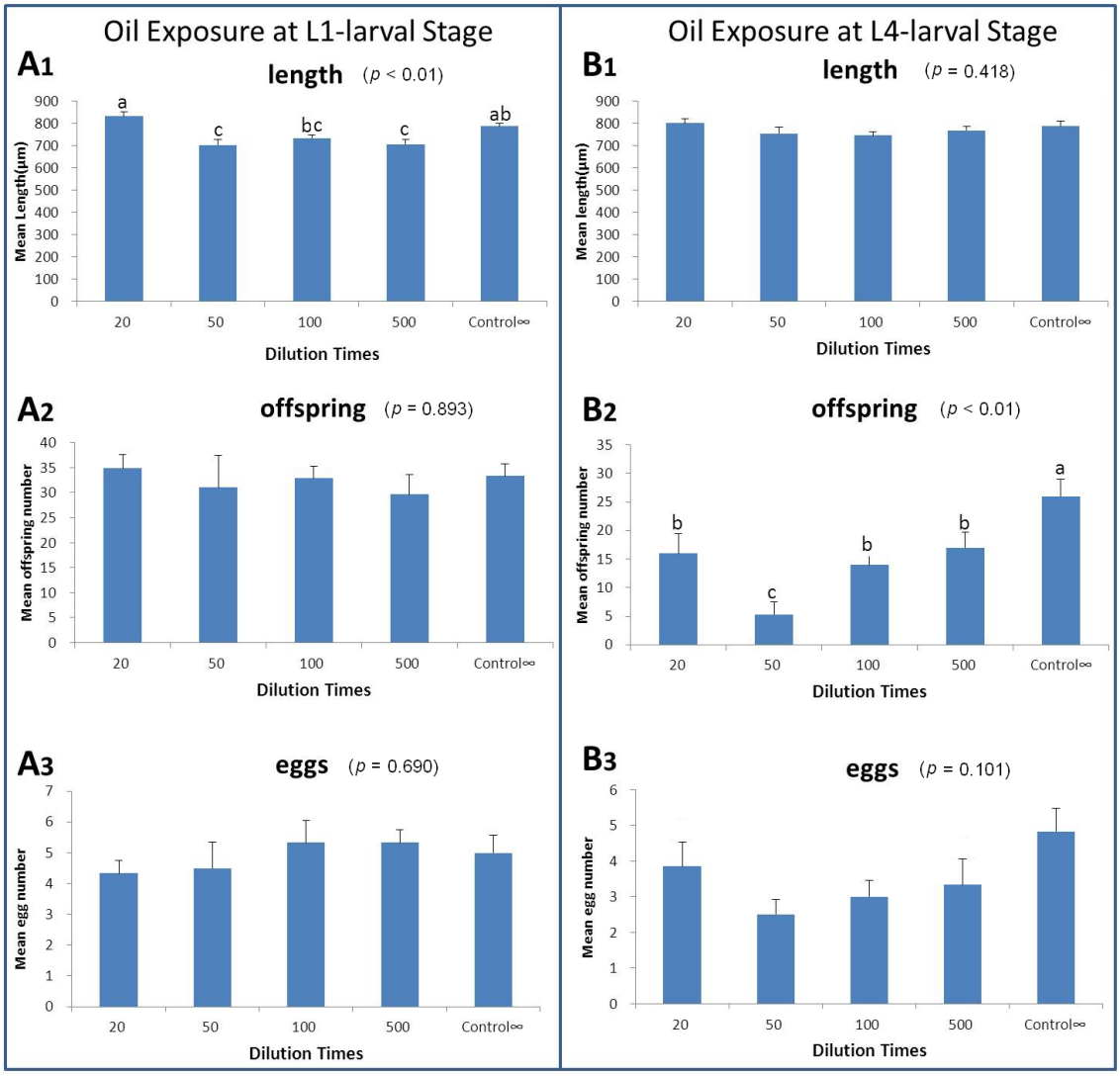


Fig. 1-1

Growth and reproduction effects examined in the L1 or L4 stage larvae of *C. elegans* exposed to five concentrations of oil-alone solutions. Growth was assessed by measuring the body length of the worms after exposure. Reproduction was assessed by counting the number of offspring beyond the eggs and the number of eggs inside the body per worm. A1 through A3: treated from L1-larval stage for five days; B1 through B3: treated from L4-larval stage for three days. Dilution: diluted 20, 50, 100, and 500 times of oil stock solution (oil stock solution was made of 10 times dilution of 20 volume crude oil:1 volume K-medium). Control was K-medium solution. Six replicates were conducted in each treatment. Means sharing a same letter above the error bar are not significantly different at the 0.05 level.

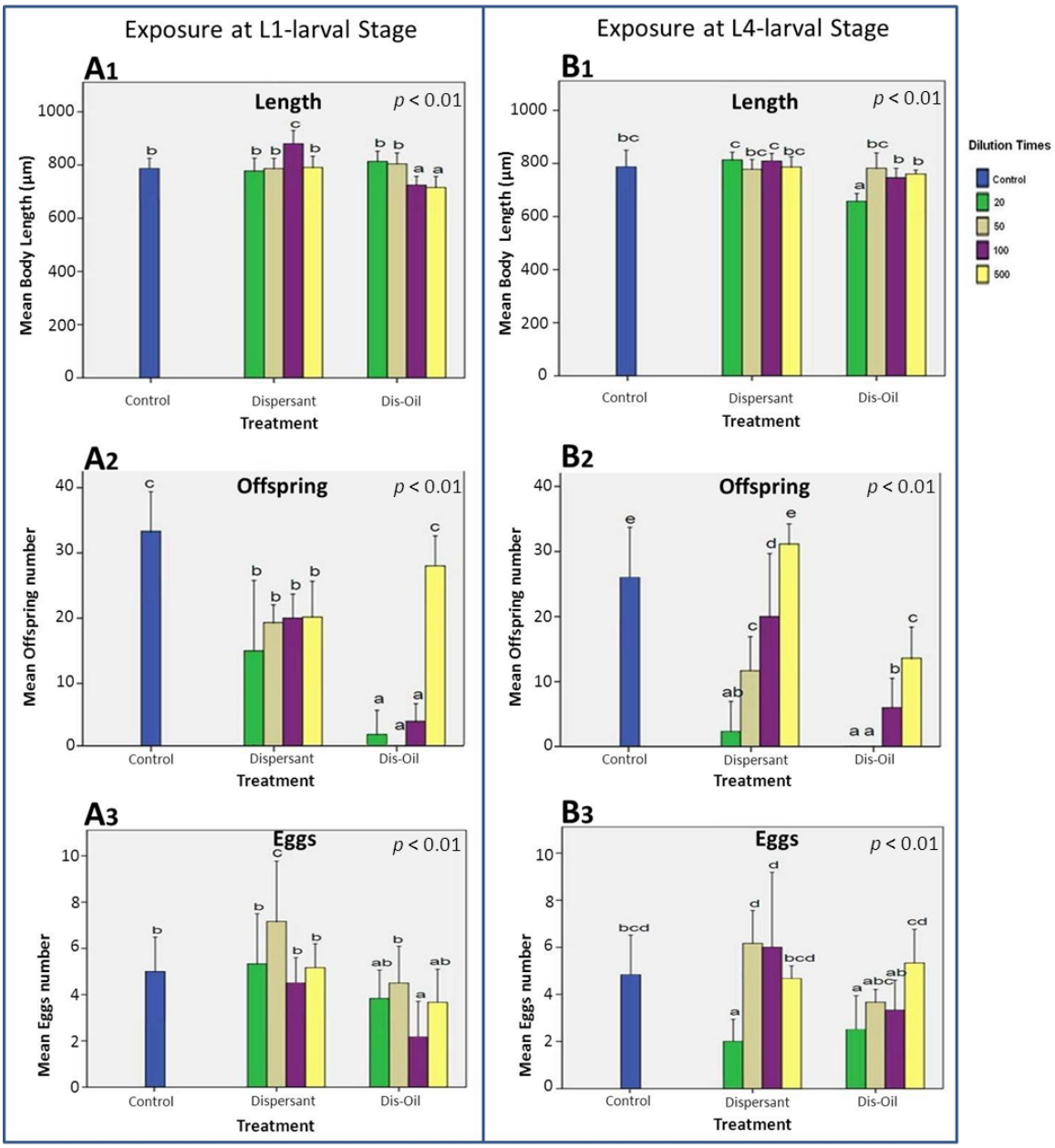
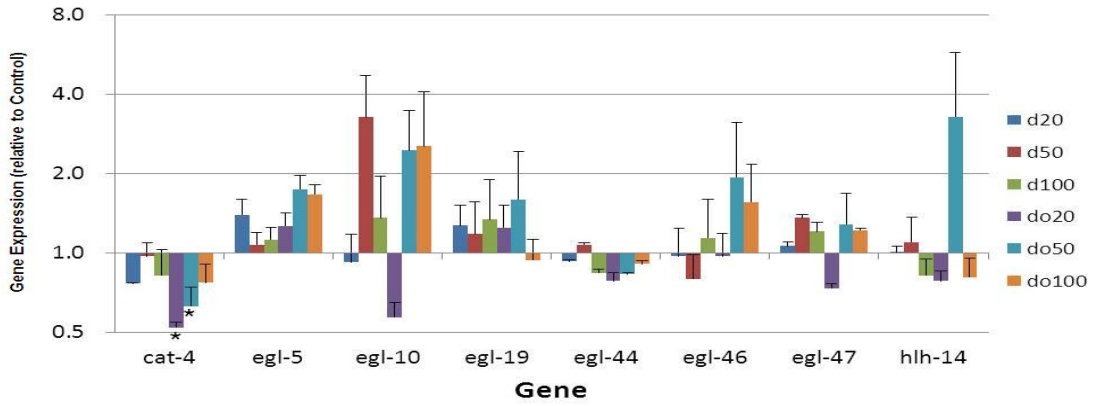
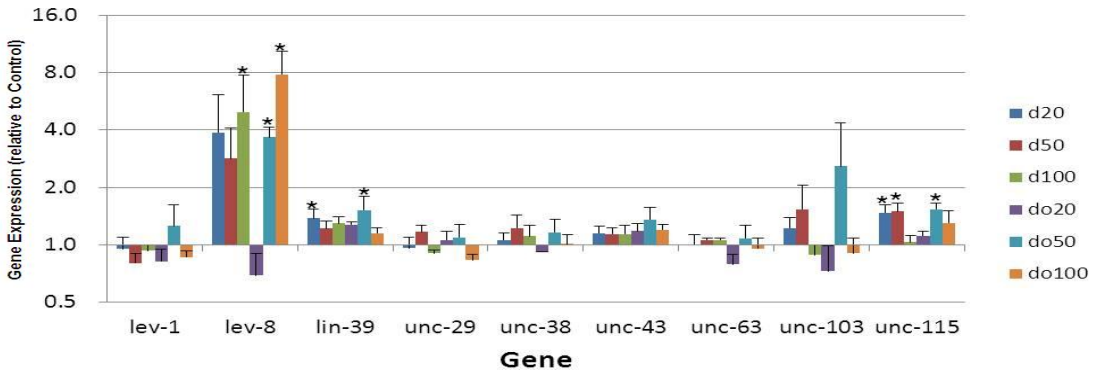


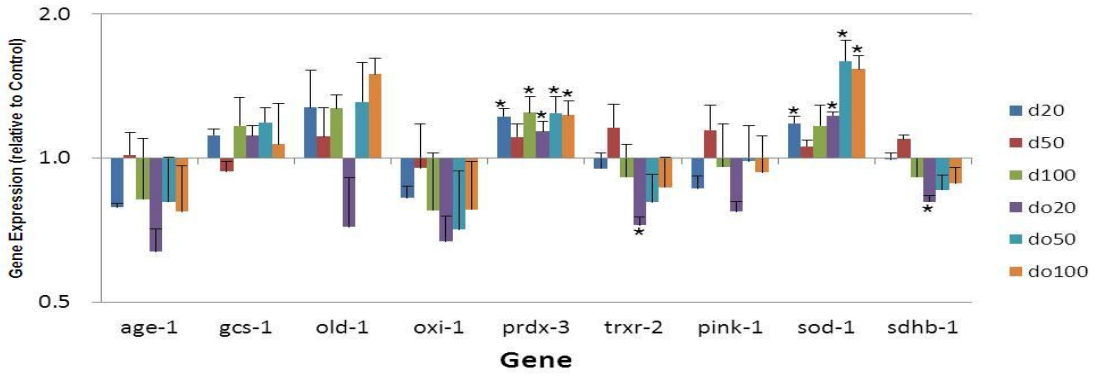
Fig. 1-2

Effects of dispersant-alone and Dis-Oil mixture treatments on growth and reproduction in *C. elegans*. Growth was assessed by measuring the body length of the worms after exposure. Reproduction was assessed by counting the number of offspring beyond the eggs and the number of eggs inside the body per worm. A1 through A3: treated from L1-larval stage for 5 days; B1 through B3: treated from L4-larval stage for 3 days.

Dilution: diluted 20, 50, 100 and 500 times of stock solution (Dis stock solution was made of 10 times dilution of 20 volume K-medium:1 volume dispersant; Dis-Oil mixture stock solution was made of 10 times dilution of 20 volume crude oil:1 volume dispersant). Control was K-medium solution. Six replicates were conducted in each treatment. Means sharing a same letter above the error bar are not significantly different at the 0.05 level.


Gene Expression (relative to Control)
8.0
4.0
2.0
1.0
0.5
cat-4
egl-5
egl-10
egl-19
egl-44
egl-46
egl-47
hlh-14
Gene
d20
d50
d100
do20
do50
do100


Gene Expression (relative to Control)
16.0
8.0
4.0
2.0
1.0
0.5
lev-1
lev-8
lin-39
unc-29
unc-38
unc-43
unc-63
unc-103
unc-115
Gene
d20
d50
d100
do20
do50
do100


Gene Expression (relative to Control)
2.0
1.0
0.5
age-1
gcs-1
old-1
oxi-1
prdx-3
trxr-2
pink-1
sod-1
sdhb-1
Gene
d20
d50
d100
do20
do50
do100

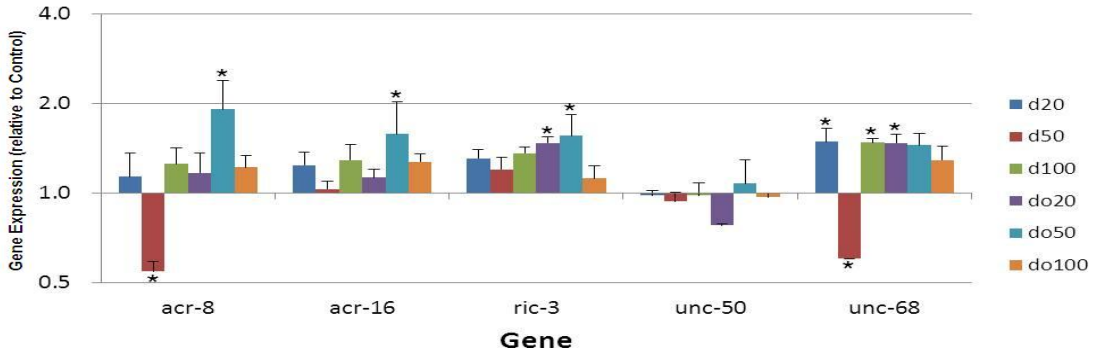

Gene Expression (relative to Control)
4.0
2.0
1.0
0.5
acr-8
acr-16
ric-3
unc-50
unc-68
Gene
d20
d50
d100
do20
do50
do100

Fig. 1-3

The fold change of gene expression (relative to control = 1, above 1 mean up-regulated, below 1 means down-regulated) in L4 larvae of *C. elegans* exposed to dispersant and Dis-Oil solutions for 24 h. Y-axis uses logarithmic scale and the base is 2 (log2). d20, d50, and d100 represent the nematodes dosed in the dispersant-alone solutions diluted 20, 50, and 100 times from the stock solution, respectively. do20, do50, and do100 represent the nematodes dosed in the Dis-Oil mixture solutions which were also diluted 20, 50, or 100 times from the Dis-Oil mixture stock solution. The fold changes of genes normalized using Y45F10D.4 mRNA are presented as relative units compared to control (control = 1, dosed in K-medium; n = 3; mean ± standard error of the mean; *p < 0.05)

Table 1-1

The description and classification of thirty-one tested genes

Gene symbol	Gene description	Egg-laying/ reproduction	Stress/ life span	Development/ Cell fate	muscle & neuron	Reference
cat-4	abnormal CATecholamine distribution	√			√	(Hardaker et al. 2001)
egl-5	Egg Laying defective	√		√		(Kalis et al. 2010; Nicholas and Hodgkin 2009)
egl-10	Egg Laying defective	√			√	(Chase et al. 2004; van der Linden et al. 2001)
egl-19	Egg Laying defective	√			√	(Frokjaer-Jensen et al. 2006)
egl-44	Egg Laying defective	√		√		(Wu et al. 2001)
egl-46	Egg Laying defective	√		√		(Wu et al. 2001; Yu et al. 2003)
egl-47	Egg Laying	√			√	(Moresco and Koelle

	defective					2004)
hlh-14	helix loop helix	√		√		(Frank et al. 2003)
lev-1	Levamisole resistant	√			√	(Kim et al. 2001)
lev-8	Levamisole resistant	√			√	(Towers et al. 2005)
lin-39	abnormal cell LINeage	√		√		(Clark et al. 1993)
unc-29	UNCoordinated	√			√	(Kim et al. 2001; Fleming et al. 1997)
unc-38	UNCoordinated	√			√	(Kim et al. 2001)
unc-43	UNCoordinated	√		√	√	(Reiner et al. 1999)
unc-63	UNCoordinated	√			√	(Culetto et al. 2004)
unc-103	UNCoordinated	√			√	(Garcia and Sternberg 2003)
unc-115	UNCoordinated	√				(Bany et al. 2003; Struckhoff and Lundquist 2003)
pink-1	PINK (PTEN-INduced Kinase) homolog	√	√			(Samann et al. 2009)
sdhb-1	Succinate Dehydrogenase complex subunit B	√	√	√		(Huang and Lemire 2009)
sod-1	SOD (superoxide dismutase)	√	√			(Doonan et al. 2008; Shibata et al. 2003)
age-1	AGEing alteration		√	√		(Ayyadevara et al. 2009)
gcs-1	gamma GlutamylCysteine Synthetase		√	√		(Inoue et al. 2005; Liao and Yu 2005)
old-1	Overexpression Longevity Determinant		√			(Murakami and Johnson 2001)
oxi-1	Oxidative stress Induced		√			(Camon et al. 2003; Yanase and Ishi 1999)
prdx-3	PeRoxireDoXin		√			(Camon et al. 2003)
trxr-2	ThioRedoXin Reductase		√			(Camon et al. 2003)
acr-8	AcetylCholine Receptor				√	(Camon et al. 2003)
acr-16	AcetylCholine Receptor				√	(Touroutine et al. 2005)
ric-3	Resistance to				√	(Halevi et al. 2002;

	Inhibitors of Cholinesterase					Shteingauz et al. 2009)
unc-50	UNCoordinated				√	(Eimer et al. 2007)
unc-68	UNCoordinated				√	(Liu et al. 2005)

Table 1-2

The primer information of the thirty-one tested genes

Gene symbol	Locus tag	Forward primer	Reverse primer	Aplicans size
cat-4	F32G8.6	CGAGTGATCGACCTGCGAGTGC	GACTGGAATTCGCGAGTTGTGGA	105
egl-5	C08C3.1	ACCCAGGTATCTCGGCTGCG	ATCCGGGAAACGTGGCAGGG	96
egl-10	F28C1.2	CATGCGAGCGCCTCTCTCGG	AGATTCTGGCTCGACGCCGC	95
egl-19	C48A7.1	GACGGCCCCAGGTATCGGGA	ACAATCCGAGCGTCGGCGTA	98
egl-44	F28B12.2	ACGACAACGCCCACGACGAC	TCGGGAGCCATTGAGCCAGC	95
egl-46	K11G9.4	GGCTCTCCACCCGCACAATCC	ACGTCGACGAGTGCAGCTTGA	97
egl-47	C50H2.2	GTCCCAACACCTGCTCCAGGAT	CGGGGATGGAAGACCTGATTGGG	100
hlh-14	C18A3.8	CCCGCCCATCAGATTGTCCCG	AGAGGGAAGACGGTGGTGGC	99
lev-1	F09E8.7	TGCAGGAGGAACATGGCTCGG	ACCATCAGGCGATCCTCGGC	99
lev-8	C35C5.5	CGGCAAGATGATGGCGTGGA	GCGCGATGAAGTGGCACTGAG	105
lin-39	C07H6.7	GCGTCTGCTGCACTTTCTGCTC	TGAGGACCTCCCAGTCCTTGAC	104
unc-29	T08G11.5	CGGATGCCACAATGGGTTCGAG	ACGGACAGCGCTTCGCTCAG	99
unc-38	F21F3.5	ACCTGGGAGCCACCAGCAATC	CAGGAGCCGAACTTCAAATGGCA	98
unc-43	K11E8.1	GCCCCAAGAACCACACACCGC	TTGTGTTGCCCGTTGACGCT	95
unc-63	Y110A7A.3	TGCGAACAGAGATGCGAATCGG	CGTCACCACGAGTGTCTCTCCG	100
unc-103	C30D11.1	CGTCCAGCACCAATCATACCGGC	TGCGAAGCGACCGCATTTCC	100
unc-115	F09B9.2	AGGAGGCGAGAGAGACCAGGG	CGTCGGAAGTGTGGCGGCTC	97
pink-1	EEED8.9	GAGGAAATGCCAAGACAAAAGCACC	AGGCCTCCAGCTGCCCATGT	103
sdhb-1	F42A8.2	TGGCTGAACGTGATCGTCTTGATGG	TCCACCAGTAGGATGGGCATGACG	84
sod-1	C15F1.7	CGGACAAGACGACCTCGGCG	GCAATGACACCGCAGGCAGC	96
age-1	B0334.8	GGCAGCCGCGTTGACTTTGC	TGACCGGGCTCAGCTGCTCAT	95
gcs-1	F37B12.2	CGAAGAGCAGGTGAATGCGATGC	GCAAGCGATGAGACCTCCGTAAGG	118
old-1	C08H9.5	TCCGGGCAAACAGATCAAGAGGT	AGGGAGTTGGTTCTGGTGGAACT	105
oxi-1	Y39A1C.2	GCTCACGTGTCCGGCTCTCC	TGGCGCGAAGCCAGTGCAAC	101
prdx-3	R07E5.2	CATCTGGAACTGTCCGTCACACTAC	CAATCAGCTGGGCAAACCTCTCC	127
trxr-2	ZK637.10	TTGTTCAGGATCGCCAAGAGCTC	TGTAGTGGCAACTCCATCGAATCG	116
acr-8	ZC504.2	GTGGGATCCTGGTCGGTGGG	TGGGGTTCTCCGTCAGCATTGT	105
acr-16	F25G6.3	GCCATGGTTGTGGACCGCCT	AGGCGACAAGATACGGTGCTGA	97
ric-3	T14A8.1	TGGGTCGTTCACCACCATCAAGT	GCACCACCACCTGGATGACGTT	103
unc-50	T07A5.2	TTGTGGCGACACTGACCGGC	AAAGACCGCCGTGTTGGGGC	105
unc-68	K11C4.5	CGCCATTCTCCACACGACGGA	GCGGAAACACGCCACAACGG	103

Chapter 2: Crude Oil/Dispersant Induced Spermatogenesis Defects in *Caenorhabditis elegans*: Implications for Reproductive Toxicity

Abstract

As crude oil remains a vital natural resource for the energy needs of the world, environmental crude oil spills continue to be a health risk to human beings and ecological systems. During clean-up efforts, surfactant-like dispersants have been used to break down big oil slicks into small droplets. Thereby crude oil mixed with dispersant represents a well-defined mixture entity that warrants investigations of its health risks. Since reproductive stages of organisms are generally more sensitive to the effects of toxicants than other stages, investigation of crude oil-dispersant exposure effects on reproduction is critically important. However, studies on reproductive effects of crude oil-dispersant mixture exposure and its mechanism remain insufficient. Our previous study showed that exposure to crude oil and dispersant induced reproduction defects including decreased brood size and increased germ cell apoptosis in *Caenorhabditis elegans* (*C. elegans*). Here, we show that crude oil-dispersant mixture also affected reproduction by inducing abnormal sperm during the process of spermatogenesis. After L4 larvae of wild type N2 hermaphrodites were exposed to several different dilutions of oil/dispersant mixture (20:1) for 24h, young adults were dissected and subjected to DAPI staining. Results showed that the abnormal immature sperm were significantly increased in the gonad arms of treated animals compared to controls (K-medium). We further explored the oil-dispersant mixture toxicity effects on spermatogenesis by using a male *C. elegans* strain. After 48h exposure to oil- dispersant mixture, spermatids appeared with abnormal morphology including irregular shapes of the spermatid membrane and unexpected tails induced by dispersed oil. Moreover we utilized *puf-8;*

lip-1 tumor sensitive strain to test the cell fate of immature sperm induced by Dis-Oil mixture treatment. Our study suggests that oil-dispersant mixture induce toxic effects on reproduction by not only affecting oogenesis but also affecting spermatogenesis.

Introduction

Crude oil or petroleum is a complex liquid chemical mixture containing hundreds of chemicals including different types of hydrocarbons and other organic compounds. Crude oil has complicated components and can be changed during weathering. The dispersant is also a mixture of dozens of chemicals which makes more oil components dissolve in water, working like detergent during the oil spill clean up. Therefore it is necessary to investigate the health impacts of dispersed oil as a mixture entity rather than based on the toxicological profile of individual chemicals.

Crude oil and dispersants potentially can induce various toxic effects due to their complicated components (Jernelov 2010, Peterson et al. 2003). They may induce acute toxicity like lethality or sub-lethal effects like developmental or behavioral defects (Cohen, McCormick, and Burkhardt 2014, Lari et al. 2015, Miljeteig et al. 2013) depending on the exposure scenario.

Of all the toxic effects caused by chemical dispersed oil, the reproductive system is among the most vulnerable systems that has sensitive responses to many stressors. Research on marine organisms has found the crude oil causes a variety of significant defects in reproduction and development (Almeda et al. 2014, Couillard et al. 2005, Goodbody-Gringley et al. 2013, Rico-Martinez, Snell, and Shearer 2013, Studivan, Hatch, and Mitchelmore 2015). Hansen et al. (Hansen et al. 2015) found that the

reproduction dynamics were significantly affected by the chemically dispersed (CD) or mechanically dispersed (MD) oil in copepods. The two highest concentrations caused a lower initial production of eggs/nauplii for both MD and CD exposures. However, copepods exposed to MD oil exhibited compensatory reproduction during the last 10 days of the recovery period, reaching control level of cumulative egg and nauplii production whereas individuals exposed to CD oil did not. Han and coworkers (Han et al. 2014) also showed that crude oil exposure resulted in oxidative stress-mediated dysfunctional development and reproduction in the copepod. Other researchers also found the embryogenesis defects and other reproductive toxicities in birds (Finch et al. 2012) and mammals (Afonne, Onyiaorah, and Orisakwe 2013, Ebokaiwe et al. 2015). Afonne *et al.* (Afonne, Onyiaorah, and Orisakwe 2013) showed the Chevron Escravos crude oil and Emulsol L.W. oil dispersant caused acute testicular toxicity in guinea pigs. They suggested that stimulation of hormone production from the adrenal cortex, causing a negative feedback on gonadotropin-releasing hormone in the pituitary gland, suppresses spermatogenesis. However most of the researches require collecting field samples and/or tedious work to dose organisms with a long life cycle such as fish, which are inconvenient for collecting data and time consuming.

The nematode *Caenorhabditis elegans* (*C. elegans*) has been used as a powerful alternative model organism in toxicology studies for several decades. Worms are very small (1mm in length for adults) and can be maintained in petri dishes at relatively low costs. The exposure of worms with a variety of chemicals can be performed on either solid agar or liquid media. It can grow to a fertile adult from an egg within approximate 72h whereas the time is much longer to reach mature age from

several months to years in fish, birds or mammalian models. During reproduction, a single worm will lay between 250 and 350 embryo eggs through self-fertilization. Because of these large brood size and short life cycle, researchers generally use the brood size and the growth rate to evaluate reproductive and developmental toxicities (Boyd et al. 2010). *C. elegans* has become an excellent model to investigate the mechanism underlying reproductive toxicity also due to its large special germline gonads. The reproductive system of the *C. elegans* hermaphrodite has two U-shape gonads. From its distal end (close to distal tip cell) to its proximal end (close to the vulva), the gonad consists of mitotic cells, transition cells and meiosis cells, oocytes and sperm (Fig. 2-1). Therefore the processes of meiotic differentiation, ovulation, and fertilization are spatially and temporally coupled in the gonad, which allow researchers to observe all stages of germline cells in a single worm. In addition, because the worm body is transparent, all different stages of meiosis can be identified through the canonical changes in nuclear architecture and morphology and are easily visualized by DAPI nuclear staining. Furthermore, the worm genome has been completely sequenced and mapped so it is easily manipulated by mutation, RNAi treatment or editing through CRISPR-Cas9 system. Additionally, thousands of transgenic and mutant worm strains are available from the *Caenorhabditis* Genetics Center (CGC) at low cost. Due to all these advantages *C. elegans* has been an ideal alternative model organism of vertebrates for studying various issues in the development and reproduction including the toxicity effects of crude oil and dispersants on reproduction system.

Our previous study showed that crude oil and the dispersant induced reproduction defects including decreased brood size (Zhang et al. 2013) and increased

germ cell apoptosis (Polli, Zhang, and Pan 2014) in *C. elegan*s. In the present study, we are interested in investigating whether crude oil-dispersant mixture also affects reproduction by inducing abnormal sperm or other defects during spermatogenesis.

Materials and Methods

C. elegans cultivation

The wild-type N2 and the male DR466 (*him-5, e1490, V*) strains of *C. elegans* were obtained from the *Caenorhabditis* Genetics Center. *puf-8; lip-1* double mutant strain and *mpk-1(gal11)* was generously provided by the collaborator Dr. Myon-Hee Lee's lab at Brody School of Medicine, East Carolina University. Worms were maintained at 20 ºC on NGM (Nematode-Growth-Medium)-agar-plates seeded with *E. coli* strain OP50 as food, according to the standard method previously described by Brenner (Brenner 1974). Age-synchronous populations of L4 stage larvae were used. To obtain age-synchronized cultures, eggs from 3-day-old mature adults plates were isolated via bleaching, followed by rinse with M9 buffer (3 g KH2PO4, 6 g Na2HPO4, 5 g NaCl, 1 ml 1 M MgSO4, H2O to 1 liter. Sterilized by autoclaving), and the eggs were hatched and arrested at L1-stage larvae in M9 buffer without food. L1-stage larvae were allowed to grow to L4-stage larvae (~36 hours) on agar plates with *E. coli* OP50 as a food source at 20ºC.

Oil and dispersant preparation

Unweathered crude oil from the Macondo well was provided by BP and Corexit 9500A, the primary dispersant used, was provided by NALCO. The dispersant-oil mixture (Dis-Oil) was made as follows: 20 volumes of crude oil and 1 volume of dispersant (according to the approximate proportion in commercial use) were vortex mixed, shaken

overnight in a reciprocating shaker at 120 rpm. The Dis-Oil mixture was then diluted with K-medium (0.032 M KCl and 0.051 M NaCl) to make dosing solutions. K-medium is a commonly used salt medium suitable for *C.elegans* aquatic toxicity testing (Ura et al. 2002, Khanna et al. 1997). While performing dilution, the parent solution was intensively mixed by inverting the conical tube for 25 times and solutions were immediate taken from the middle of the mixed emulsion.

Oil and dispersant exposure treatments

Based on our previous study (Zhang et al. 2013), we found the L4-stage larvae is more sensitive to oil and dispersant compared to that of L1-larvae. Therefore in this study, we treated the wild type N2 L4-stage larvae with Dis-Oil mixture in 15ml centrifuge tubes for 24hr and then rinsed with K-medium twice and collected the worms for the following assays. We chose 250X, 500X and 1000X dilution solutions of Dis-Oil as the treatment groups and K-medium alone was used as control. *E.coli* OP50 was added for feeding the worms. For each treatment we have performed at least three biological replicates.

Morphology of spermatids in him-5 mutant

The *him-5* (DR466) male mutant was used to investigate effects on the size and shape of spermatids. Since the *him-5* culture has approximately 33% males, after synchronization L1 larvae were grow to L4 on NGM and then L4 larvae of males were picked out for dosing. After 48 h exposure, a male *him-5* mutant was put into a small drop (~15µl) of sperm medium solution (50mM Hepes, 1mM $MgSO_4$, 25mM KCl, 45mM NaCl and 5mM $CaCl_2$, pH7.0) on the microscope slide. By cutting the male's tail, spermatids were released in sperm medium solution. Randomly five different visual fields for each male sample were captured under a DIC microscope (Zeiss Observer

Z1) and images were acquired with a CCD camera (Nikon, DS-Fi2).

Morphology of activated sperm in vitro

A spermatid can be activated *in vitro* to become a spermatozoa with pseudopod by adding various activating compounds including Pronase E (Nelson and Ward 1980). In this study, we used Pronase E to trigger spermatid activation due to its proteolytic activity. After exposing L4-larval *him-5* males for 48h, spermatids released from dissected males in a small drop of sperm medium. Then a drop of 200μg ml^{-1} Pronase E was added to the sperm medium and wait for 5 min. The sample sperm were observed under a DIC microscope (Zeiss Observer Z1) and images were acquired with a CCD camera (Nikon, DS-Fi2).

Immunohistochemistry

For immunohistochemistry, gonads were dissected from the worm body and then fixed in 3% paraformaldehyde with 100 mM K_2HPO_4 (pH 7.2) for 10-60 min at room temperature, and post-fixed in 100% cold methanol for at least 5 min at -20 °C (Francis et al. 1995). Antibody incubations and washes were performed as following. After blocking for 1 h with 0.5% BSA in 1 × PBS (+ 0.1% Tween 20), fixed gonads were incubated for 2 h at room temperature with primary antibodies followed by 1 h at room temperature with secondary antibodies. DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) staining followed standard methods. Fixed samples were incubated with 100 ng/ml DAPI in PTW (PBS containing 0.1% Tween 20) for 5-10 min. After staining rinse with PTW twice and mount in VECTASHIELD® mounting medium.

EdU staining

M9 buffer added with Tween-20 (1 microliter of Tween-20 for 10ml of M9) was

used for rinsing worms between different solutions and washing worms off the NGM plate. Briefly, around fifty worms were collected and incubated with 200 times dilution of 10mM EdU for 30 min at room temperature. Then the worms were placed on NGM plate with OP50 for 15 min to remove excess EdU and then subjected to dissection and fixation in 3% PFA (Paraformaldehyde) for 10 min and in cold methanol (-20°C) for 10 min. Blocking the dissected worms with extrude gonads in 0.5% BSA at room temperature for 30 min. EdU incorporation was revealed with Click-iT EdU Alexa 488 Imaging Kit (Invitrogen). DAPI staining was applied just before making slides.

mpk-1b RNAi in puf-8; lip-1 mutants

RNAi experiments were performed by feeding bacteria expressing double strand RNAs corresponding to the gene of interest (Kamath et al. 2001). The *mpk-1b* isoform-specific RNAi bacteria were from Dr. Myon-Hee Lee's lab. In my experiment, synchronized *puf-8; lip-1* L1-stage larvae were plated onto *mpk-1b* RNAi plates and allowed to grow two days to become L4-stage larvae. The L4 larvae were washed off the plates and separated into two groups. Worms for the Dis-Oil mixture treatment group were plated onto *mpk-1b* RNAi plates containing 100 µL 500X dilution of Dis-Oil mixture on the NGM agar. For the control group, worms were plated onto *mpk-1b* RNAi plates with 100 µL K-medium on the NGM agar. The L4 *puf-8; lip-1* mutants were continued to grow on the *mpk-1b* RNAi plates for several days. We sampled around 30 *puf-8; lip-1* mutants from each treatment after two days, three days and four days from L4 larvae. All experiments were performed at 20 °C. Germline phenotypes were determined by staining dissected gonads with specific markers and/or DAPI.

Real-time Quantitative PCR

Total RNA of each group was isolated according to the manufacturer's protocol using the mirVanaTM miRNA Isolation Kit (Ambion, Austin, USA). The extracted RNAs were evaluated and quantified using the NanoDrop ND-1000 Micro-Volume UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, USA). One microgram of total RNA were used for reverse transcription to synthesize cDNA using the TaqMan microRNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). PolyT were used as the reverse transcription primer. In brief, the reactions were run using a thermal cycler for 16°C for 30 min followed by 42°C for 30 min, 85°C for 5 min and finally held at 4°C. The cDNAs were then diluted by adding 80 µL DNase/RNase-free water for qRT-PCR.

Real-time quantitative PCR amplifications were carried out using 10 µL of Real-Time SYBR Green PCR master mix, 2 µL of diluted reverse transcription product, 2 µL of forward and reverse primer and 6 µL of DNase/RNase free water in a total volume of 20 µL. Amplification was carried out on a 384-well-plate using the ViiA™ Real-Time PCR System (Applied Biosystem). The reaction program include initial polymerase activation at 95°C for 10 min, followed by 40 cycles of: 95°C for 15 sec denaturation, 60°C for 60 sec for primer-specific annealing and elongation. After 40 cycles, a melting curve analysis was carried out (60°C to 95°C) to verify the specificity of amplicons. Y45F10D.4 was used as reference genes based on our previous studies (Zhang et al. 2012, Zhang et al. 2013).

Statistical Analysis

Minitab 17 software was used for the statistical analysis. One-way ANOVA was used to

determine the significance of group differences. The rate of abnormal immature sperm and the rate of tumor formation were analyzed using the χ^2 test. The probability level of 0.05 was considered statistically significant.

Results

Chemical dispersed oil induced diploid immature sperm during the spermatogenesis

Wild-type N2 *C. elegans* are hermaphrodite nematodes, which make sperm starting at L4 larval stage and switch to make oocytes in adulthood. Therefore for each adult wild-type animal, all the sperm in spermatheca are supposed to be mature haploid sperm (Fig. 2-1 A). However when we used DAPI staining to investigate the germline phenotype after exposed to Dis-Oil mixture, abnormal immature spermatocytes were observed in the spermatheca (Fig. 2-1 B and Fig. 2-2) of the adult N2 animals. The abnormal immature sperm were bigger than the normal mature sperm and appeared brighter following DAPI staining (see the arrows in Fig. 2-2). The sp56 (sperm marker) antibody staining further confirmed they are immature sperm occurred at the spermatheca region (see the Fig. 2-3).

In order to analyze the toxic effect of Dis-Oil mixture on spermatogenesis in *C. elegans*, we generated three test concentrations of Dis-Oil mixture based on our previous brood size assessment, which are 250X, 500X and 1000X times diluted from the original Dis-Oil mixture (1:20, V/V). K-medium only was used as the vehicle control solution. We collected wild-type N2 nematode at the L4 larval stage and exposed them in the above three treatment solutions as well as in control K-medium solution with OP50 as food for worms. After exposed for 24h, worms were picked out into a small

glass dish and then the worms were dissected under the microscope by cutting off the heads, and gonad(s) were extruded out of the worm body. Then the dissected gonads were applied fixing and DAPI staining. The proportion of “abnormal” gonads with immature spermatocytes (Fig. 2-2 & Fig. 2-4 A) and the average number of immature spermatocytes in one gonad (Fig. 2-2 & Fig. 2-4 B) were counted and calculated. The percentage of “abnormal” gonad in 1000X, 500X and 250X dilutions of Dis-Oil mixture treatment groups were 56%, 83% and 97% respectively while that in control group was 11%. All the Dis-Oil mixtures groups had significant higher proportions of “abnormal” gonads with immature sperm than those of in control group (Fig. 2-4 A) in a dose-dependent manner.

Dis-Oil mixture not only induced more “abnormal” gonads in the populations of N2 animals, but also induced significant larger number of immature spermatocytes in each gonadal arm in a dose-dependent manner (Fig. 2-4 A&B). As increase of Dis-Oil mixture concentration, more abnormal immature sperm were induced. The average number of immature sperm in one gonad in control group was 0.26 while that in 1000X, 500X and 250X dilutions of Dis-Oil mixture exposure groups were 1.47, 4.61 and 7.25 respectively. The results provide a mechanism explanation of our previous report (Zhang et al. 2013), which showed that the Dis-Oil mixture caused the decrease in offspring production in a dose-dependent manner.

Chemical dispersed oil induced abnormal spermatids in him-5

In order to further explore the toxic effect of Dis-Oil mixture on sperm properties, we utilized the male *C. elegans him-5* (DR466) mutant stain to observe the spermatid morphology. The male animals could accumulate a large number of spermatids without

mating. In addition, because the male sperm are larger than the hermaphrodite animals, it is easier to observe and compare by using the DIC microscope. We chose 500X dilution of the original Dis-Oil mixture (1:20, V/V) to perform the experiments. After we exposed L4 male larvae to Dis-Oil mixture (500X) for 48hr, the morphology of the spermatids were observed under the microscope (Fig. 2-5). The normal spermatids in control group (Fig. 2-5 A) were almost round with smooth edges; however, Dis-Oil mixture exposure induced the formation of spermatids with abnormal shapes (see white arrows in Fig. 2-5 B) compared to the control group. Moreover some of the Dis-Oil mixture treated males had the spermatids with unexpected extended projections or tails (see yellow arrows in Fig 2-5 C).

For the sperm activation assay, the *him-5* males were exposed from L4-larval stage for 48h, and then males were dissected by cutting the tail. The released spermatids in sperm medium were exposed to Pronase E for 5 min, and then observe under the microscope (Fig. 2-6). We observed that in control group the sperm had longer pseudopods (see white arrow in Fig. 2-6 A) than those in Dis-Oil mixture treatment group (see yellow arrows in Fig. 2-6 B). That means the Dis-Oil mixture might affect the spermatids properties and further affect the activation of sperm and eventually affect the fecundity of *C. elegans* due to at least partially the abnormal sperm formation.

Dis-Oil mixture induced aberrant expressions of spermatogenesis related genes

Based on observed abnormal sperm in Dis-Oil-exposed wild-type N2 and him-5 mutant, we selected five genes related to spermatogenesis to investigate their expression by qRT-PCR, which are *fer-1, spe-10, spe-11, spe-17* and *cnb-1*. The primers used are listed in Table 1. We use him-5 mutant strain that has lots of sperm for the gene

expression assays. After exposure to 500X dilution of Dis-Oil mixture from L4 for 48hr, of the five tested genes, expression levels of four (*fer-1, spe-10, spe-11* and *cnb*-1) genes were significantly decreased when comparing to control (Fig. 2-7). The down regulation in gene expression were 1.58-fold for *spe-10*, 1.62-fold for *spe-11*, 1.90-fold for *fer-1* and 2.26-fold for *cnb-1,* respectively (Fig. 2-7). The expression level of *spe-17* was not significantly changed compared with control, but still had the trend of down-regulation (Fig. 2-7).

Chemical dispersed oil induced proximal tumorigenesis in puf-8; lip-1 mutant

The *puf-8; lip-1* mutant, a sensitive strain to study the germ line "tumor", was utilized. The *puf-8; lip-1* mutants are Mog (Masculinization of Germline) at 20ºC, which produce excess sperm and will not switch to oogenesis. At 25ºC, they are Tum (Tumor phenotype); mitotic cells arise from dedifferentiation of immature primary and/or secondary spermatocytes at the proximal end of gonad (Cha et al. 2012). Therefore they are temperature sensitive and produce tumor-like (mitotic) cells at 25ºC. Here we investigated whether the Dis-Oil mixture can induce a tumor phenotype more frequently in the mutant than the control at 20ºC.

We exposed the L4 larvae of the *puf-8; lip-1* mutants in different dilutions of Dis-Oil mixtures for longer time (from 2 days to 4 days) because we tried to investigate the fate of induced immature sperm which may take longer time to undergo dedifferentiation to become tumor-like cells from immature sperm. As shown in Fig. 2-8, in control group there were excess mature sperm at the proximal end of the gonad (Fig. 2-8 A, DAPI staining) at 20ºC, but in Dis-Oil mixture treatments, at the proximal end, gonads had the mitotic cell morphology, which was only observed at the distal end of the gonad in

control at 20ºC (Fig. 2-8 B, DAPI staining). The appearance of mitotic cells at the proximal end is considered tumor-like phenotype (Subramaniam and Seydoux 2003, Datla et al. 2014). We further confirmed those tumor-like cells or stem cell-like cells had actual active mitotic properties by using EdU (newly synthesized DNA marker) staining (See Fig. 2-9). Results showed EdU staining positive for the proximal mitotic cells, indicating the tumor-like (active proliferating) property of these dedifferentiated cells.

For statistical analysis, we counted and calculated the percentage of gonads with "tumor-like" cells. We called it "tumor formation rate" (Fig. 2-10). After exposure to Dis-Oil mixture, the tumor formation rate increased in all treatment groups compared with control in day 3 and day 4 (Fig. 2-10). It showed that after 3 days of exposure both 1000X and 500X dilution groups had significant ($p < .05$) increased "tumor-like" phenotype gonads (11.2% and 6.3% respectively) compared to control (1.5%). After 4 days of exposure both 1000X and 500X dilution groups had significant ($p < .05$) more "tumor-like" phenotype gonads (14.2% and 17.7% respectively) compared to control (2.1%). For the control group, in days 2, 3, and 4 the changes in tumor formation rate was not significant. In the 1000X dilution group, the tumor formation rates in days 3 and 4 were significantly greater than that in day 2 ($p < .05$, Fig. 2-10). Similarly the tumor formation rate in 500X dilution group also showed a time-dependent increase. The tumor formation rate in day 4 was significantly higher than that in days 2 or 3. The results indicated that the Dis-Oil mixture has the potential to induce tumorigenesis in the germline.

Dis-Oil mixture may induce germline immature sperm through MAPK-independent pathway

The mitogen-activated protein kinase (MAPK) cascade is a highly conserved module

involved in controlling different developmental processes in *C. elegans*, including vulval development, sex myoblast migration and germline meiosis, among other pathways. In order to explore the underlying molecular mechanism of how Dis-Oil mixture can induce the germline immature sperm and how the diploid sperm undergo dedifferentiation to form tumor cells, we hypothesize that the chemically dispersed oil may induce abnormal spermatogenesis and cause dedifferentiation through the activation of MAPK signaling pathway (Fig. 2-11).

To test our hypotheses, first of all we used RNAi approach to knock down the *mpk-1* gene in the *puf-8; lip-1* mutant background and recorded the tumor rate and sterile rate of gonads after exposure to 500X dilution Dis-Oil mixture. The results showed that with the down-regulation of *mpk-1,* the tumor phenotype was no longer seen in the germline of *puf-8; lip-1* mutants both with and without Dis-Oil exposure (Table 2). The *mpk-1* gene is critical for transition to oogenesis from spermatogenesis and *puf-8; lip-1*; *mpk-1RNAi* mutants can produce oocytes normally and thus no more tumor phenotype was seen. It suggests that the highly expressed *mpk-1* in *puf-8; lip-1* mutants may induce tumorigenesis by undergoing dedifferentiation of diploid immature sperm. In other words, the Dis-Oil induced germline tumor in *puf-8; lip-1* mutants may be derived from diploid immature sperm and not from distal mitotic cells or other type of cells.

Previous studies showed that higher temperature of 25ºC may induce germline tumors by activation of RAS/MAPK signaling in *puf-8; lip-1* mutants. We proposed Dis-Oil might also induce tumorigenesis by increasing the activities of RAS/MAPK signaling pathway. Therefore, we tested the MAPK expression level by using antibody staining with Anti-MAPK(YT) Antibody to show diphosphorylated MAPK expression and with

Anti-mpk-1 antibody to show total MAPK expression in wild type N2, *him-5* (both males and hermaphrodites) and *puf-8; lip-1* mutants. All the worms were dissected after 24hr exposure to Dis-Oil mixture. For the total MAPK expression, we didn't see any differences in terms of fluorescence intensity between the Dis-Oil treated worms and the control ones in all three different strains (data not shown). Similarly, we didn't see any differences of diphosphorylated MAPK expression in both wild type N2 and *him-5* strains (data not shown). However, we did find diphosphorylated MAPK expression differences in *puf-8; lip-1* mutants exposure to Dis-Oil mixture treatment (see Fig. 2-12). The findings suggest that the Dis-Oil might only cause the up-regulation of diphosphorylated MAPK in *puf-8; lip-1* strain, which accumulates more immature spermatocytes. We didn't see the increased diphosphorylated MAPK in wild type N2 or *him-5* males might because there were just a few immature spermatocytes induced by Dis-Oil and was below the minimal level detected by antibody staining. However increased activity of diphosphorylated MAPK was observed for worms exposed by Dis-Oil in *puf-8; lip-1* mutants which had enlarged germline meiotic pachytene regions.

In addition to the antibody staining assays, we also used qRT-PCR to examine the mRNA level of several key regulators involved in the RAS/MAPK signaling pathway in wild type N2 strain (Fig. 2-11, Fig. 2-13 & Table 2-1). As shown in Fig. 2-13, the *let-60 (Ras)* was up regulated significantly in Dis-Oil mixture exposed worms than in control ones, while expression of *rskn-1*, the downstream target of mpk-1 (MAPK), was reduced significantly. However, the mRNA levels of *lin-45 (Raf), mek-2 (mek)* and *mpk-1 (MAPK)* were not significantly changed. The Dis-Oil didn't cause significantly changed *mpk-1* expression at the mRNA level in the N2 strain. Coupled with the results of antibody

staining with both total MAPK and diphosphorylated MAPK, it suggests that the Dis-Oil mixture may induce diploid immature spermatocytes in a MAPK-independent pathway. Some other cell cycle regulators that account for meiosis progression might be affected by Dis-Oil mixture exposure, which in turn cause delayed spermatogenesis during germline meiosis progression.

In order to further explore whether the Dis-Oil mixture can induce abnormal *mpk-1* expression, we utilized the *mpk-1* (temperature sensitive) mutant strain to do the Dis-Oil exposure experiment. *Mpk-1* will totally be knocked down when the temperature rises to 25ºC and worms will become sterile since the *mpk-1* is critical for spermatogenesis and meiosis will be arrested in the pachytene stage. Therefore we conducted the experiments at 20ºC and 22.5ºC to record the sterile rate of gonads. As shown in Fig. 2-14, the Dis-Oil mixture exposure didn't induce up- or down- regulation of *mpk-1* significantly based on the sterile rate phenotype. It suggests again the Dis-Oil mixture might not directly affect the expression and its activity of *mpk-1*. However, we found again more immature spermatocytes in Dis-Oil mixture treatments in *mpk-1* mutants. The observed diploid immature spermatocytes in both N2 and *mpk-1* mutants when exposed to Dis-Oil mixture might not directly caused by up- or down- regulation of *mpk-1* but by other cell cycle regulators. Therefore the up-regulation of diphosphorylated MAPK observed in *puf-8; lip-1* mutants might just happen because this specific strain tends to accumulate more immature spermatocytes, which are induced by affecting the cell cycle regulators in meiosis progression.

The accumulated diploid immature spermatocytes in *puf-8; lip-1* mutants, tumor sensitive stain, possess more opportunity to undergo dedifferentiation and become

tumor-like cells. The immature spermatocytes in wild type N2 may not have a chance to form tumor because the percentage of immature spermatocytes was low and the immature sperm might be pushed out of the gonad during the ovulation and egg laying stages.

Discussion

In previous work, we found both the crude oil and the dispersant had negative effects on reproduction (Zhang et al. 2013) Notably, the chemical dispersant potentiated crude oil effects, evidenced by Dis-Oil mixture induced more significant reduction of reproduction than oil or dispersant-alone exposures (Zhang et al. 2013). It has also been reported that the chemical dispersed crude oil exhibited more inhibition on reproduction in copepods than the mechanically dispersed crude oil (Hansen et al. 2015). Notably, our finding that chemical dispersant could induce reproduction inhibition significantly in a dose-dependent manner which is consistent with what later observed fertotoxicity in the mammalian *in vitro* follicle growth (IVFG) model system (Xu et al. 2015). By using the IVFG assay for female reproductive toxicity screening, Xu and colleagues (Xu et al. 2015) found that the dispersant Corexit EC 9500 A(CE) affected follicle survival and altered follicle morphology and cellular differentiation. They indicated that the CE exposure affected follicle hormone production and caused meiotic defects in the oocyte. They also argued that the cell line or organotypic test system from reproduction organ was more sensitive than those from somatic or cancer cell lines. It again suggests that reproduction system is more sensitive to environmental chemical exposure. Therefore the nematode *C. elegans* is an ideal animal model to investigate the reproductive toxicity, which had been shown to give results consistent with more complex animals,

including mammals.

The cause of reproductive toxicity is complex involving many factors. It would be developmental growth defects or the overall abnormal homeostasis that eventually leads to oogenesis, spermatogenesis, embryogenesis and/or other defects. In order to explore the underlying mechanism of reproduction reduction caused by Dis-Oil mixture, I worked with other colleagues to conduct experiments for assessing germline apoptosis during oogenesis and found that chemical dispersed crude oil amplified germ cell apoptosis in *C. elegans* and it followed a *cep-1*/p53 dependent pathway (Polli, Zhang, and Pan 2014). Genes involved in the apoptosis pathway were dysregulated. Many aberrant expressed genes encode for core components in apoptosis machinery (cep-1/p53, ced-13/BH3, ced-9/Bcl-2, ced-4/Apaf-1, and ced-3/caspase) displayed consistent expression patterns across all exposure levels. Significantly *ced-3*/caspase was upregulated at all dispersed oil-treated groups, consistent with the observed apoptosis phenotype. It suggests that the Dis-Oil mixture might cause DNA damage that leads to increased programmed cell death during oogenesis.

In the present study, we found that Dis-Oil mixture exposure also caused abnormal sperm during spermatogenesis. First, by DAPI staining, we found that germ cells in Dis-Oil mixture treatment groups contain immature sperm in the spermatheca region at the proximal end. Other cell types in treatment groups looked normal as in controls, which include mitotic cells at the distal end and followed by the transition zone, meiotic cells and oocytes (Fig. 2-1, Fig. 2-2). It suggests that Dis-Oil mixture caused some of the spermacytes failing to divide into haploid mature sperm, which consequently may affect sperm production during the spermatogenesis. Significantly, the percentage of gonad

with immature sperm and the average number of immature sperm in one gonad increased in a dose-dependent manner. The results were consistent with our previous studies (Zhang et al. 2013), which showed that the Dis-Oil mixture cause the decrease in offspring production in a dose-dependent manner.

Although increasing abnormal sperm had been found in high concentration of Dis-Oil mixture, it only represented a small percentage of immature sperm in total sperm in the spermatheca (1-7/250), which should not cause significant reduction of offspring. However sperm that appeared as normal mature haploid ones may have functional defects that cannot be detected based on DAPI staining. Therefore we utilized the male strain *him-5* to further test sperm properties and functions. Because the sperm in *him-5* male are bigger than that in hermaphrodites, it is easier to observe the size and morphology of spermatids of males under the DIC images. Significantly the Dis-Oil mixture treated *him-5* males produced a large number of irregular shaped spermatids than that of control which are smoothing spherical spermatids (Fig. 2-5). Some of spermatids in Dis-Oil treatment showed abnormal extended projections or tails, which was not observed in controls (Fig. 2-5). A spermatid needs to develop into spermatozoa to acquire its fertility function. The process of formation of spermatozoa from a spermatid through post-meiotic differentiation is called sperm activation or spermiogenesis. The spermatids be activated to become spermatozoa *in vitro* by adding Pronase E, therefore we continued to explore whether the Dis-Oil mixture can affect the sperm activation *in vitro*. We found that the Dis-Oil mixture exposure adversely affects sperm activation, thereby may result in malfunctional sperm (Fig. 2-6). It was shown that sperm functions were affected due to abnormal spermatid morphology and/or failure in

sperm activation (Ruan et al. 2012). In summary, our findings suggest that the Dis-Oil mixture caused the sperm defects via various mechanisms that may eventually affected fecundity.

To further investigate the molecular mechanisms underlying spermatogenesis defects, we selected five genes that related to spermatogenesis and sperm activation to measure the expression levels. Of the five selected genes, four of them (*fer-1, spe-10, spe-11* and *cnb*-1) showed significant down-regulation in Dis-Oil mixture treatment group. *spe-10* is required for the proper biogenesis and functioning of specialized Golgi-derived fibrous body-membranous organelle (FB-MO) complexes during spermatid formation (Gleason et al. 2006). *cnb-1* is required for maintenance of sperm morphology and brood size (Bandyopadhyay et al. 2002). Our findings suggest that Dis-Oil mixture regulates sperm development related genes, which may result in abnormal spermatids and sperm activation failure. *fer-1* is a regulator of membranous organelle fusion and is required during spermatogenesis for calcium-mediated fusion of membranous vesicles with the spermatid plasma membrane, a process which promotes development of spermatids into motile spermatozoa (Washington and Ward 2006). *spe-11* is required for early embryonic development and for regulating the dynamic morphology of sperm pseudopods (Royal et al. 1997). The findings suggest that the significant down regulation of *fer-1* and *spe-11* by Dis-oil treatment may affects sperm activation. It also suggests that the Dis-Oil mixture might affect the spermatid properties by preventing the proper biogenesis of FB-MO complexes and affect the sperm activation by inhibiting the pseudopod formation.

In mammals, it also reported that the crude oil induced alteration in testicular stress

response proteins and caspase-3 dependent apoptosis in albino Wistar rats (Ebokaiwe et al. 2015). Another study conducted by Afonne *et al* (Afonne, Onyiaorah, and Orisakwe 2013) found a dose-dependent decrease in the number of epididymal sperm following crude oil and chemical dispersant treatment. In addition, sperm motility and morphology were altered in the treated animals. For humans, it was suggested that crude oil may be a contributing factor for the ~ 12 million infertile population in the oil-rich Nigeria (Cates, Farley, and Rowe 1985, Orisakwe et al. 2004). Our studies supported that the crude oil and chemical dispersant can induce the spermatogenesis defects.

We also asked what's the fate of those immature diploid sperm? Is it a signal for tumorigenesis? The immature sperm might undergo dedifferentiation and reprogramming to mitotic cells that have the ability to proliferate themselves and lead to tumors or cancer (Subramaniam and Seydoux 2003, Datla et al. 2014, Cha et al. 2012). If this is the case, the Dis-Oil mixture may not only affect brood size, but also may induce gonadal tumorigenesis in the F0 generation. There have been lines of evidence that PAHs (poly aromatic hydrocarbons) components are carcinogenic (Mollerup, Ovrebo, and Haugen 2001, Shugart, Holland, and Rahn 1983, Zhou, Dai, and Gu 2003) and the crude oil containing various PAHs that can be uptake and accumulate in a variety of different organisms (Perez-Cadahia et al. 2004, Ramachandran et al. 2004, Ramachandran et al. 2006, Zeigler and Robbat 2012). It is possible that the PAHs in crude oil are also pose carcinogenic risks on the reproduction system and cause gonadal tumors in *C. elegans*.

In order to reveal that whether those diploid sperm cells will continue to

proliferate and become tumor-like or stem cell-like cells or just will degrade in germ line, we utilized a tumor formation sensitive strain *puf-8; lip-1* that has been used as a model system to study carcinogenesis in *C. elegans* (Datla et al. 2014). PUF-8 (Pumilio and FBF family of RNA-binding protein) and LIP-1 (lateral-signal-induced phosphatase) proteins suppress the dedifferentiation of spermatocytes into germline tumors. Most *puf-8;lip-1* double mutants are Mog (Masculinization of Germline) phenotype (Bachorik and Kimble 2005) at 20ºC, which produce excess sperm and will not switch to oogenesis. When the temperature is increased to 25ºC, *puf-8;lip-1* double mutants display the tumor phenotype, in which tumor cells derived from unsuccessful meiosis completion and the immature sperm underwent dedifferentiation and become mitotic cells. We utilized *puf-8; lip-1* tumor sensitive strain to investigate the fate of Dis-oil induced immature diploid spermatocytes. It showed that diploid spermatocytes might have the potential to undergoing dedifferentiation to become tumor-like cells or stem cell-like cells which can self-proliferate.

We further confirmed those tumor-like cells or stem cell-like cells have actual active mitotic properties by using EdU (newly synthesized DNA marker) staining (See Fig. 2-9). The results showed EdU staining positive in 500X dilution of Dis-Oil mixture treatment. This demonstrates that these dedifferentiated cells in the proximal end of gonad are tumor-like, and have the ability to proliferate and cause tumors in the *C. elegans* gonad. Combined with findings in N2 strain, which demonstrated that Dis-oil induced immature diploid sperm, it suggested that the tumorous cells in *puf-8; lip-1* may be derived from the diploid sperm dedifferentiation. It suggests that Dis-Oil mixture might have the potential carcinogenic effects on the reproduction system of the

nematode. Previous studies also showed that it contains the carcinogenic chemicals in the components of the crude oil (such as PAHs) (Collins et al. 1998, Olson, Meyer, and Portier 2016, Wickliffe et al. 2014). A recent study showed that there was a threefold increase in the kidney cancer risk for exposure to hydrocarbons in crude oil (Anttila et al. 2015). In addition, 2-Butoxy Ethanol, present in dispersant, may be a carcinogen that can induce liver damage and liver tumors based on the studies in mice and rats (National Toxicology 2000, Park, Kamendulis, and Klaunig 2002).

In summary, our study suggests that the oil-dispersant mixture induced toxic effects on reproduction by not only affecting oogenesis but also affecting spermatogenesis. Furthermore abnormal sperm induced by dispersed oil may become tumorous and produce gonadal tumors.

Since *C. elegans* has conserved genetic and biochemical pathways involved in germline gametogenesis with many organisms including humans, the Dis-Oil mixture may also induce immature sperm and those immature sperm have the potential risk to become tumor-like cells in human and poses carcinogenesis risk.

References

Afonne, O. J., I. V. Onyiaorah, and O. E. Orisakwe. 2013. "Toxicity of Chevron Escravos crude oil and chemical dispersant on guinea pig testicular function." *J Basic Clin Physiol Pharmacol* 24 (4):321-9. doi: 10.1515/jbcpp-2012-0060.

Almeda, R., S. Baca, C. Hyatt, and E. J. Buskey. 2014. "Ingestion and sublethal effects of physically and chemically dispersed crude oil on marine planktonic copepods." *Ecotoxicology* 23 (6):988-1003. doi: 10.1007/s10646-014-1242-6.

Anttila, A., A. Pokhrel, P. Heikkila, R. Viinanen, and E. Pukkala. 2015. "Kidney cancer risk in oil refining in Finland: a nested case-referent study." *J Occup Environ Med* 57 (1):68-72. doi: 10.1097/JOM.0000000000000301.

Bachorik, J. L., and J. Kimble. 2005. "Redundant control of the Caenorhabditis elegans sperm/oocyte switch by PUF-8 and FBF-1, two distinct PUF RNA-binding proteins." *Proc Natl Acad Sci U S A* 102 (31):10893-7. doi: 10.1073/pnas.0504593102.

Bandyopadhyay, J., J. Lee, J. Lee, J. I. Lee, J. R. Yu, C. Jee, J. H. Cho, S. Jung, M. H. Lee, S. Zannoni, A. Singson, D. H. Kim, H. S. Koo, and J. Ahnn. 2002. "Calcineurin, a calcium/calmodulin-dependent protein phosphatase, is involved in movement, fertility, egg laying, and growth in Caenorhabditis elegans." *Mol Biol Cell* 13 (9):3281-93. doi: 10.1091/mbc.E02-01-0005.

Boyd, W. A., S. J. McBride, J. R. Rice, D. W. Snyder, and J. H. Freedman. 2010. "A high-throughput method for assessing chemical toxicity using a Caenorhabditis elegans reproduction assay." *Toxicol Appl Pharmacol* 245 (2):153-9. doi: 10.1016/j.taap.2010.02.014.

Brenner, S. 1974. "The genetics of Caenorhabditis elegans." *Genetics* 77 (1):71-94.

Cates, W., T. M. Farley, and P. J. Rowe. 1985. "Worldwide patterns of infertility: is Africa different?" *Lancet* 2 (8455):596-8.

Cha, D. S., U. S. Datla, S. E. Hollis, J. Kimble, and M. H. Lee. 2012. "The Ras-ERK MAPK regulatory network controls dedifferentiation in Caenorhabditis elegans germline." *Biochim Biophys Acta* 1823 (10):1847-55. doi: 10.1016/j.bbamcr.2012.07.006.

Cohen, J. H., L. R. McCormick, and S. M. Burkhardt. 2014. "Effects of dispersant and oil on survival and swimming activity in a marine copepod." *Bull Environ Contam Toxicol* 92 (4):381-7. doi: 10.1007/s00128-013-1191-4.

Collins, J. F., J. P. Brown, G. V. Alexeeff, and A. G. Salmon. 1998. "Potency equivalency factors for some polycyclic aromatic hydrocarbons and polycyclic aromatic hydrocarbon derivatives." *Regul Toxicol Pharmacol* 28 (1):45-54. doi: 10.1006/rtph.1998.1235.

Couillard, C. M., K. Lee, B. Legare, and T. L. King. 2005. "Effect of dispersant on the composition of the water-accommodated fraction of crude oil and its toxicity to larval marine fish." *Environ Toxicol Chem* 24 (6):1496-504.

Datla, U. S., N. C. Scovill, A. J. Brokamp, E. Kim, A. S. Asch, and M. H. Lee. 2014. "Role of PUF-8/PUF protein in stem cell control, sperm-oocyte decision and cell fate reprogramming." *J Cell Physiol* 229 (10):1306-11. doi: 10.1002/jcp.24618.

Ebokaiwe, A. P., C. S. D'Cruz, R. Jubendradass, J. S. Amala Rani, P. P. Mathur, and E. O. Farombi. 2015. "Nigerian bonny-light crude oil induces alteration in testicular

stress response proteins and caspase-3 dependent apoptosis in albino wistar rats." *Environ Toxicol* 30 (2):242-52. doi: 10.1002/tox.21902.

Finch, B. E., K. J. Wooten, D. R. Faust, and P. N. Smith. 2012. "Embryotoxicity of mixtures of weathered crude oil collected from the Gulf of Mexico and Corexit 9500 in mallard ducks (Anas platyrhynchos)." *Sci Total Environ* 426:155-9. doi: 10.1016/j.scitotenv.2012.03.070.

Francis, R., M. K. Barton, J. Kimble, and T. Schedl. 1995. "gld-1, a tumor suppressor gene required for oocyte development in Caenorhabditis elegans." *Genetics* 139 (2):579-606.

Gleason, E. J., W. C. Lindsey, T. L. Kroft, A. W. Singson, and W. L'Hernault S. 2006. "spe-10 encodes a DHHC-CRD zinc-finger membrane protein required for endoplasmic reticulum/Golgi membrane morphogenesis during Caenorhabditis elegans spermatogenesis." *Genetics* 172 (1):145-58. doi: 10.1534/genetics.105.047340.

Goodbody-Gringley, G., D. L. Wetzel, D. Gillon, E. Pulster, A. Miller, and K. B. Ritchie. 2013. "Toxicity of Deepwater Horizon source oil and the chemical dispersant, Corexit(R) 9500, to coral larvae." *PLoS One* 8 (1):e45574. doi: 10.1371/journal.pone.0045574.

Han, J., E. J. Won, D. S. Hwang, K. H. Shin, Y. S. Lee, K. M. Leung, S. J. Lee, and J. S. Lee. 2014. "Crude oil exposure results in oxidative stress-mediated dysfunctional development and reproduction in the copepod Tigriopus japonicus and modulates expression of cytochrome P450 (CYP) genes." *Aquat Toxicol* 152:308-17. doi: 10.1016/j.aquatox.2014.04.027.

Hansen, B. H., I. Salaberria, A. J. Olsen, K. E. Read, I. B. Overjordet, K. M. Hammer, D. Altin, and T. Nordtug. 2015. "Reproduction dynamics in copepods following exposure to chemically and mechanically dispersed crude oil." *Environ Sci Technol* 49 (6):3822-9. doi: 10.1021/es504903k.

Jernelov, A. 2010. "The threats from oil spills: now, then, and in the future." *Ambio* 39 (5-6):353-66.

Kamath, R. S., M. Martinez-Campos, P. Zipperlen, A. G. Fraser, and J. Ahringer. 2001. "Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans." *Genome Biol* 2 (1):RESEARCH0002. doi: 10.1186/gb-2000-2-1-research0002.

Khanna, N., C. P. Cressman, 3rd, C. P. Tatara, and P. L. Williams. 1997. "Tolerance of the nematode Caenorhabditis elegans to pH, salinity, and hardness in aquatic media." *Arch Environ Contam Toxicol* 32 (1):110-4.

Lari, E., B. Abtahi, M. S. Hashtroudi, E. Mohaddes, and K. B. Doving. 2015. "The effect of sublethal concentrations of the water-soluble fraction of crude oil on the chemosensory function of Caspian roach, Rutilus caspicus (YAKOVLEV, 1870)." *Environ Toxicol Chem* 34 (8):1826-32. doi: 10.1002/etc.2994.

Miljeteig, C., A. J. Olsen, T. Nordtug, D. Altin, and B. M. Jenssen. 2013. "Sublethal exposure to crude oil enhances positive phototaxis in the calanoid copepod Calanus finmarchicus." *Environ Sci Technol* 47 (24):14426-33. doi: 10.1021/es4037447.

Mollerup, S., S. Ovrebo, and A. Haugen. 2001. "Lung carcinogenesis: resveratrol modulates the expression of genes involved in the metabolism of PAH in human bronchial epithelial cells." *Int J Cancer* 92 (1):18-25.

National Toxicology, Program. 2000. "NTP Toxicology and Carcinogenesis Studies 2-Butoxyethanol (CAS NO. 111-76-2) in F344/N Rats and B6C3F1 Mice (Inhalation Studies)." *Natl Toxicol Program Tech Rep Ser* 484:1-290.

Nelson, G. A., and S. Ward. 1980. "Vesicle fusion, pseudopod extension and amoeboid motility are induced in nematode spermatids by the ionophore monensin." *Cell* 19 (2):457-64.

Olson, G. M., B. M. Meyer, and R. J. Portier. 2016. "Assessment of the toxic potential of polycyclic aromatic hydrocarbons (PAHs) affecting Gulf menhaden (Brevoortia patronus) harvested from waters impacted by the BP Deepwater Horizon Spill." *Chemosphere* 145:322-8. doi: 10.1016/j.chemosphere.2015.11.087.

Orisakwe, O. E., D. D. Akumka, A. A. Njan, and O. J. Afonne. 2004. "Testicular toxicity of Nigerian bonny light crude oil in male albino rats." *Reprod Toxicol* 18 (3):439-42. doi: 10.1016/j.reprotox.2004.02.002.

Park, J., L. M. Kamendulis, and J. E. Klaunig. 2002. "Effects of 2-butoxyethanol on hepatic oxidative damage." *Toxicol Lett* 126 (1):19-29.

Perez-Cadahia, B., B. Laffon, E. Pasaro, and J. Mendez. 2004. "Evaluation of PAH bioaccumulation and DNA damage in mussels (Mytilus galloprovincialis) exposed to spilled Prestige crude oil." *Comp Biochem Physiol C Toxicol Pharmacol* 138 (4):453-60. doi: 10.1016/j.cca.2004.08.001.

Peterson, C. H., S. D. Rice, J. W. Short, D. Esler, J. L. Bodkin, B. E. Ballachey, and D. B. Irons. 2003. "Long-term ecosystem response to the Exxon Valdez oil spill." *Science* 302 (5653):2082-6. doi: 10.1126/science.1084282.

Polli, J. R., Y. Zhang, and X. Pan. 2014. "Dispersed crude oil amplifies germ cell apoptosis in Caenorhabditis elegans, followed a CEP-1-dependent pathway." *Arch Toxicol* 88 (3):543-51. doi: 10.1007/s00204-014-1198-6.

Ramachandran, S. D., P. V. Hodson, C. W. Khan, and K. Lee. 2004. "Oil dispersant increases PAH uptake by fish exposed to crude oil." *Ecotoxicol Environ Saf* 59 (3):300-8. doi: 10.1016/j.ecoenv.2003.08.018.

Ramachandran, S. D., M. J. Sweezey, P. V. Hodson, M. Boudreau, S. C. Courtenay, K. Lee, T. King, and J. A. Dixon. 2006. "Influence of salinity and fish species on PAH uptake from dispersed crude oil." *Mar Pollut Bull* 52 (10):1182-9. doi: 10.1016/j.marpolbul.2006.02.009.

Rico-Martinez, R., T. W. Snell, and T. L. Shearer. 2013. "Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A((R)) to the Brachionus plicatilis species complex (Rotifera)." *Environ Pollut* 173:5-10. doi: 10.1016/j.envpol.2012.09.024.

Royal, D. C., M. A. Royal, D. Wessels, S. L'Hernault, and D. R. Soll. 1997. "Quantitative analysis of Caenorhabditis elegans sperm motility and how it is affected by mutants spe11 and unc54." *Cell Motil Cytoskeleton* 37 (2):98-110. doi: 10.1002/(SICI)1097-0169(1997)37:2<98::AID-CM2>3.0.CO;2-D.

Ruan, Q. L., J. J. Ju, Y. H. Li, X. B. Li, R. Liu, G. Y. Liang, J. Zhang, Y. P. Pu, D. Y. Wang, and L. H. Yin. 2012. "Chlorpyrifos exposure reduces reproductive capacity

owing to a damaging effect on gametogenesis in the nematode Caenorhabditis elegans." *J Appl Toxicol* 32 (7):527-35. doi: 10.1002/jat.1783.

Shugart, L., J. M. Holland, and R. O. Rahn. 1983. "Dosimetry of PAH skin carcinogenesis: covalent binding of benzo[a]pyrene to mouse epidermal DNA." *Carcinogenesis* 4 (2):195-8.

Studivan, M. S., W. I. Hatch, and C. L. Mitchelmore. 2015. "Responses of the soft coral Xenia elongata following acute exposure to a chemical dispersant." *Springerplus* 4:80. doi: 10.1186/s40064-015-0844-7.

Subramaniam, K., and G. Seydoux. 2003. "Dedifferentiation of primary spermatocytes into germ cell tumors in C. elegans lacking the pumilio-like protein PUF-8." *Curr Biol* 13 (2):134-9.

Ura, Kazuhiro, Toshinori Kai, Sachiko Sakata, Taisen Iguchi, and Koji Arizono. 2002. "Aquatic acute toxicity testing using the nematode Caenorhabditis elegans." *Journal of health science* 48 (6):583-586.

Washington, N. L., and S. Ward. 2006. "FER-1 regulates Ca2+ -mediated membrane fusion during C. elegans spermatogenesis." *J Cell Sci* 119 (Pt 12):2552-62. doi: 10.1242/jcs.02980.

Wickliffe, J., E. Overton, S. Frickel, J. Howard, M. Wilson, B. Simon, S. Echsner, D. Nguyen, D. Gauthe, D. Blake, C. Miller, C. Elferink, S. Ansari, H. Fernando, E. Trapido, and A. Kane. 2014. "Evaluation of polycyclic aromatic hydrocarbons using analytical methods, toxicology, and risk assessment research: seafood safety after a petroleum spill as an example." *Environ Health Perspect* 122 (1):6-9. doi: 10.1289/ehp.1306724.

Xu, Yuanming, Francesca E Duncan, Min Xu, and Teresa K Woodruff. 2015. "Use of an organotypic mammalian in vitro follicle growth assay to facilitate female reproductive toxicity screening." *Reproduction, Fertility and Development*.

Zeigler, C. D., and A. Robbat, Jr. 2012. "Comprehensive profiling of coal tar and crude oil to obtain mass spectra and retention indices for alkylated PAH shows why current methods err." *Environ Sci Technol* 46 (7):3935-42. doi: 10.1021/es2030824.

Zhang, Y., D. Chen, A. C. Ennis, J. R. Polli, P. Xiao, B. Zhang, E. J. Stellwag, A. Overton, and X. Pan. 2013. "Chemical dispersant potentiates crude oil impacts on growth, reproduction, and gene expression in Caenorhabditis elegans." *Arch Toxicol* 87 (2):371-82. doi: 10.1007/s00204-012-0936-x.

Zhang, Y., D. Chen, M. A. Smith, B. Zhang, and X. Pan. 2012. "Selection of reliable reference genes in Caenorhabditis elegans for analysis of nanotoxicity." *PLoS One* 7 (3):e31849. doi: 10.1371/journal.pone.0031849.

Zhou, Z., Q. Dai, and T. Gu. 2003. "A QSAR model of PAHs carcinogenesis based on thermodynamic stabilities of biactive sites." *J Chem Inf Comput Sci* 43 (2):615-21. doi: 10.1021/ci0256135.

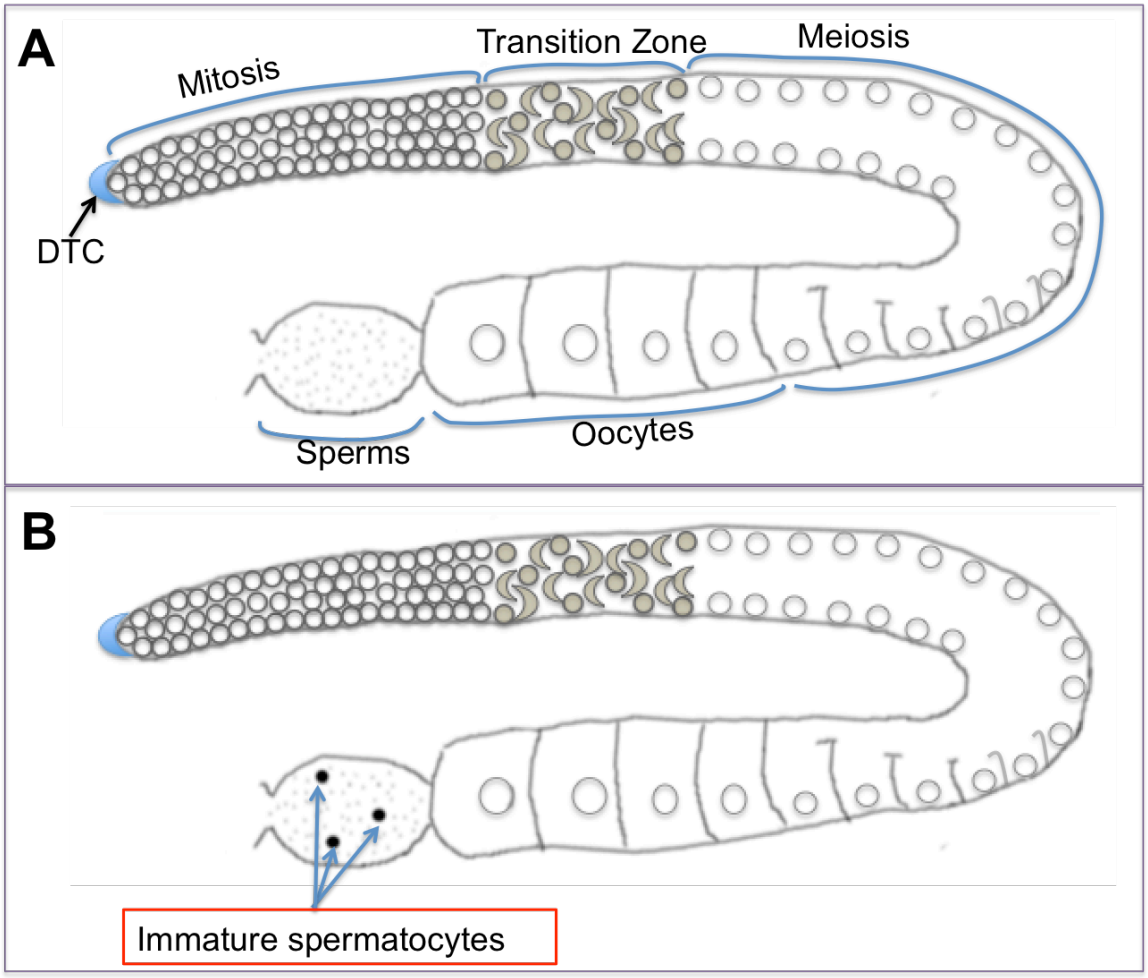


Fig. 2-1

Scheme of adult hermaphrodite germline. A: Normal adult hermaphrodite germline. This organization is typical of wild-type animals. DTC (distal tip cell) is a single somatic cell, which provides a niche for maintaining germline stem cell. In the mitosis region, the germ cells are undergoing proliferation. Germ cells switch from the mitotic cell cycle into the meiotic cell cycle as they enter into the “transition zone” (TZ). Mature sperm are made in larvae while oocytes are made in adults. B: Scheme of abnormal immature spermatocytes in sperm region (spermatheca). Based on DAPI staining, the immature spermatocytes had bigger size and brighter fluorescent signals.

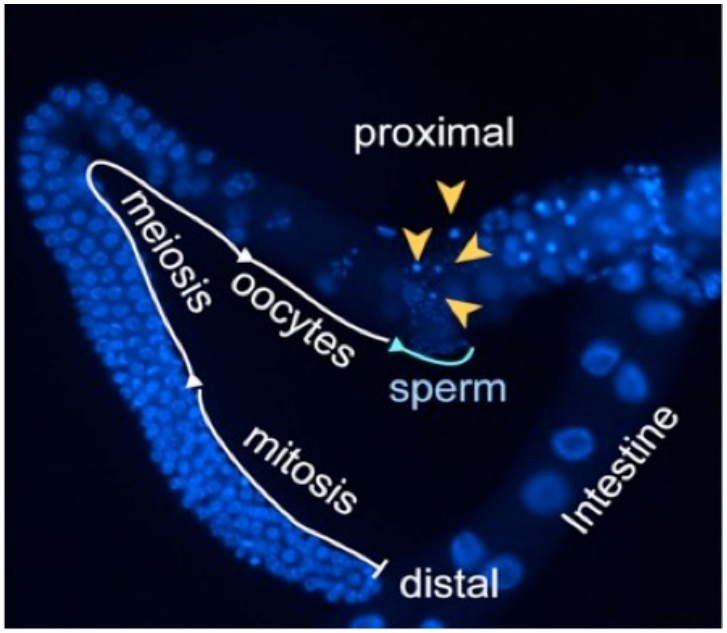


Fig. 2-2

Oil and dispersant mixture affect the spermatogenesis progression (L4 larvae of N2 hermaphrodites were exposed to 500x dilution of Dis-Oil mixture solution for 24hr). Dis-Oil mixture induced diploid sperm (DAPI staining, arrows point to immature diploid sperm).

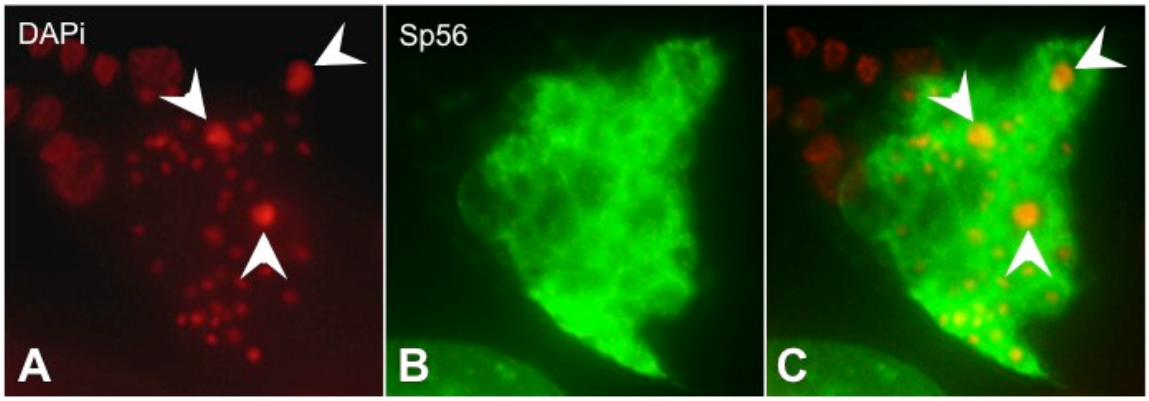


Fig. 2-3

sp56 antibody staining. N2 worms were treated by 500X dilution of Dis-Oil mixture from L4 stage for 24h. Here showed the sperm region of dissected gonads. A: The red color is DAPI (showed the DNA); B: The green color showed expression of sp56 (sperm marker); C: The merged image of A and B. Arrows point to immature diploid sperm.

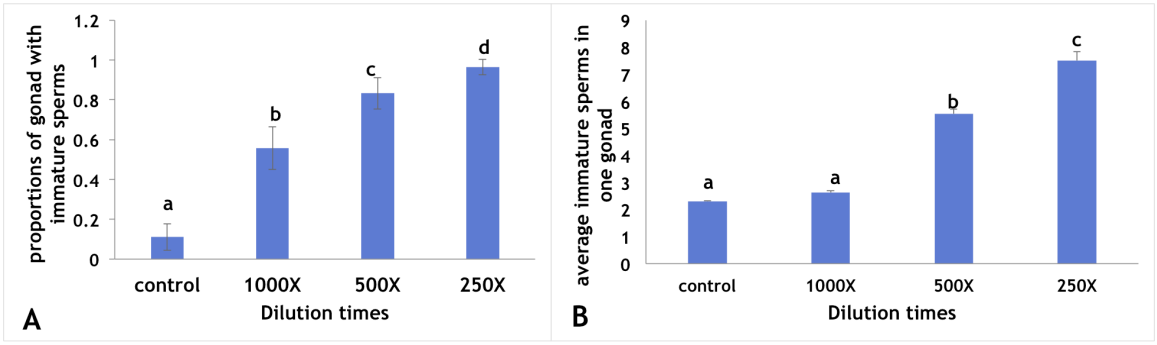


Fig. 2-4

Effects of different dilutions of Dis-Oil mixture on *C. elegans* germline development. A. Proportions of gonad with immature diploid sperm. B. Average number of immature sperm in one gonad for those abnormal gonads with immature sperm. Germline immature sperm were determined by cellular morphology and DAPI staining of dissected gonads. (n=90 in control, n=86 in 1000X, n=90 in 500X and n=87 in 250X

groups). Error bars represent standard deviations. Different letters above the error bars indicate statistically significant different means at $p < 0.05$ level.

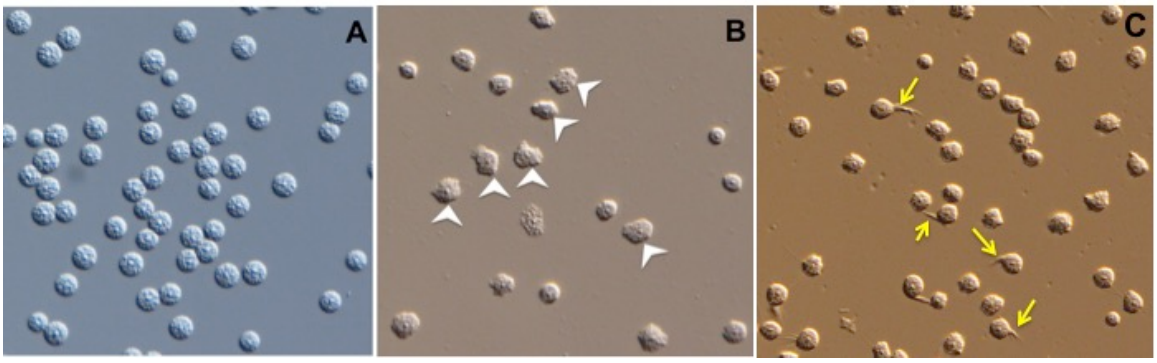


Fig. 2-5

The morphology of spermatids of L4-larval *him-5* males cultured in the control K-medium (A) and in 500X dilution of Dis-Oil mixture (B) for 48hr. Spermatids were dissected by cutting the tail of males in a drop of sperm medium solution on a microscope slide. Images were captured under a DIC microscope (Zeiss Observer Z1) with a CCD camera. White arrows point to the irregular shape of spermatids compared with those smooth circle ones in control. Yellow arrows point to the abnormal tail of the spermatids.

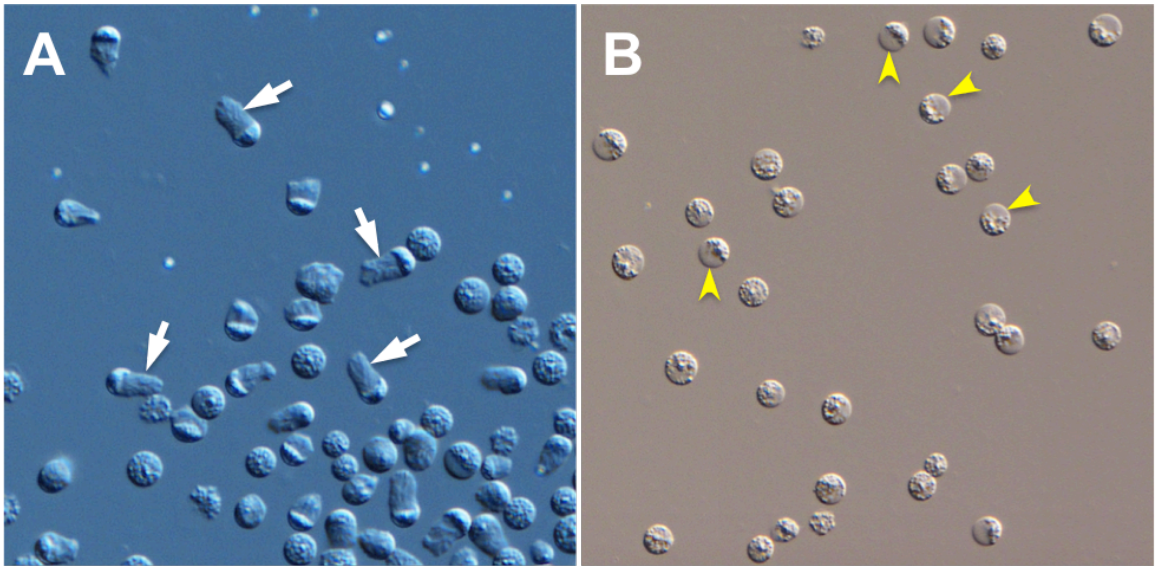


Fig. 2-6

The morphology of sperm of L4-larval him-5 males in the control (A) and in 500X dilution of Dis-Oil mixture exposed group (B) (X 400). Adding pronase E for 5 min after dissection male tail in sperm medium solution activates sperm. White arrows point to some normal activated sperm and yellow arrows point to some abnormal sperm.

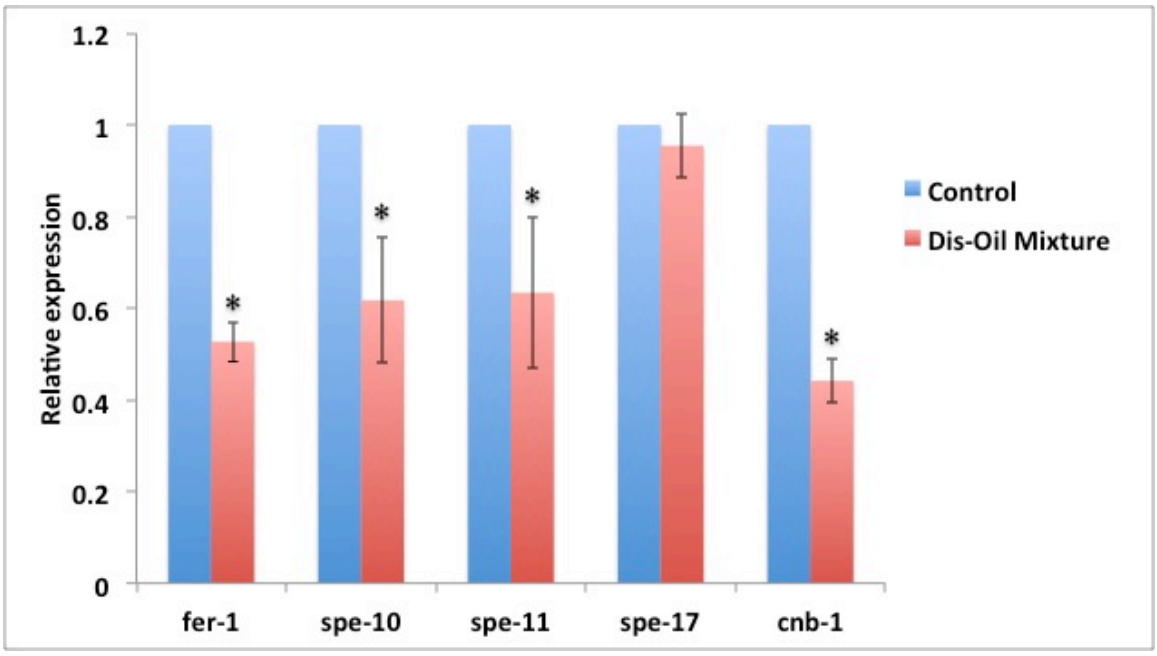


Fig. 2-7

Relative mRNA expression levels of genes involved in spermatogenesis progression.

Bars represent means ± SD. *$p < 0.05$ vs the control group.

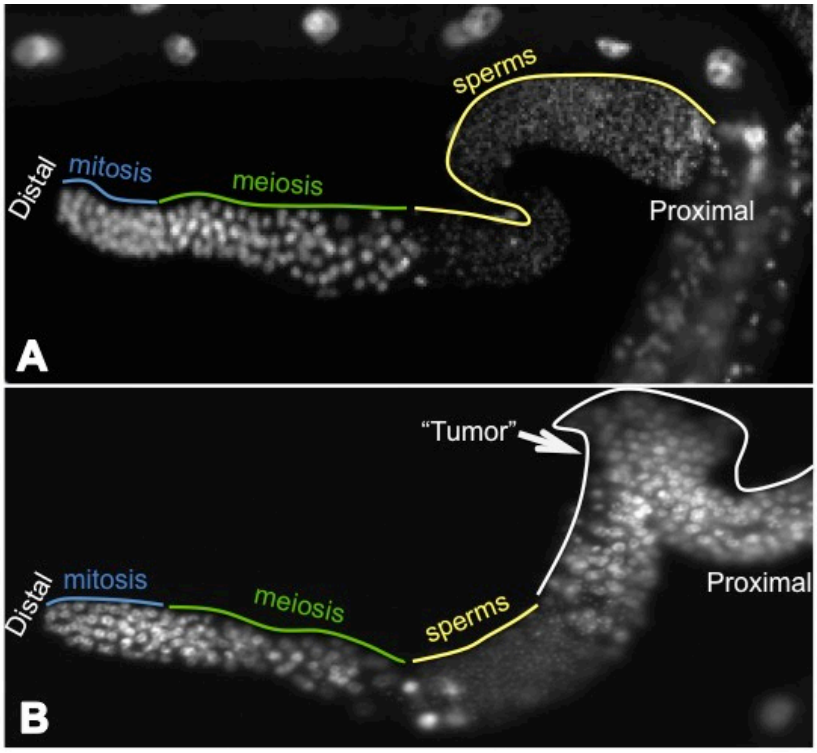


Fig. 2-8

Oil and dispersant mixture caused proximal tumorigenesis in *puf-8; lip-1* mutant. A: control (K-medium); B: 500X dilution of oil-dis mixture treatment for 3 days. White arrow showed the proximal "tumor-like" cells of the germline. The blue line showed mitosis region; the green line showed the meiosis region; the yellow color showed the sperm region and the white color showed the proximal "tumor-like" cells undergoing mitosis.

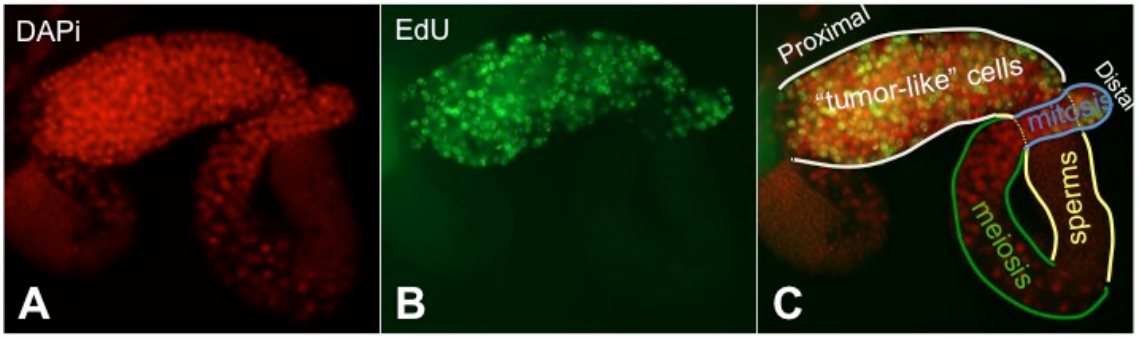


Fig. 2-9

EdU staining of Dis-Oil mixture treated puf-8; lip-1 mutant. Worms are exposed to 500X Dis-Oil mixture for 3 days from early L4 stage. A: The red color showed DAPI; B: The green color showed the newly synthesized DNA (EdU). The "tumor-like" cells at the proximal end of gonad had the green signal (EdU positive) as those cells at the distal mitotic region. The blue line showed mitosis region; the green line showed the meiosis region; the yellow color showed the sperm region and the white color showed the proximal "tumor-like" cells undergoing mitosis. The gonad shown here had its distal mitosis region cross over the sperm region.

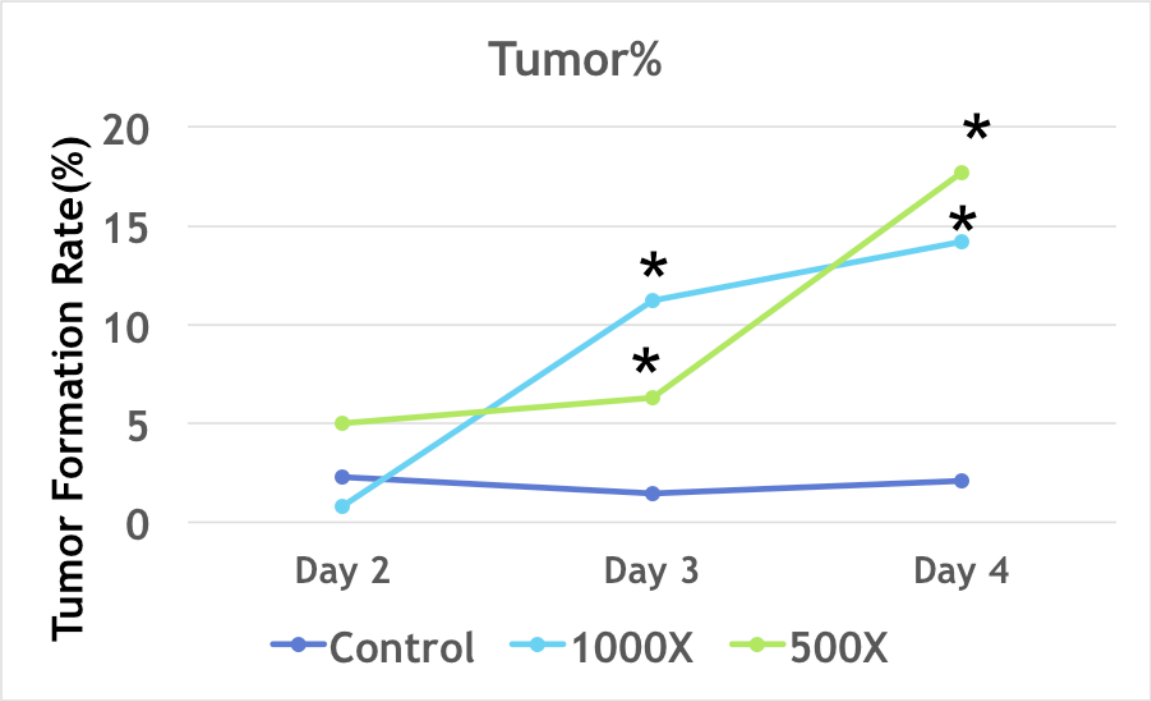


Fig. 2-10

Percentage of tumor-like cells found in *puf-8; lip-1* mutant strain. L4 larvae were exposed to control (K-media), 1000X, 500X dilution of Dis-Oil mixture for 2 days, 3 days and 4 days. (Day2: n=132, 129 and 120 in control, 1000X and 500X dilution of Dis-Oil mixture respectively; Day3: n=137, 143 and 159 in control, 1000X and 500X dilution of Dis-Oil mixture respectively; Day4: n=144, 169 and 175 in control, 1000X and 500X dilution of Dis-Oil mixture respectively. n is the total number of observed gonads.) Chi-square test was used to compare each treatment groups. * indicate the Dis-Oil mixture treatments have significant higher tumor rate than the control group $p < 0.05$.

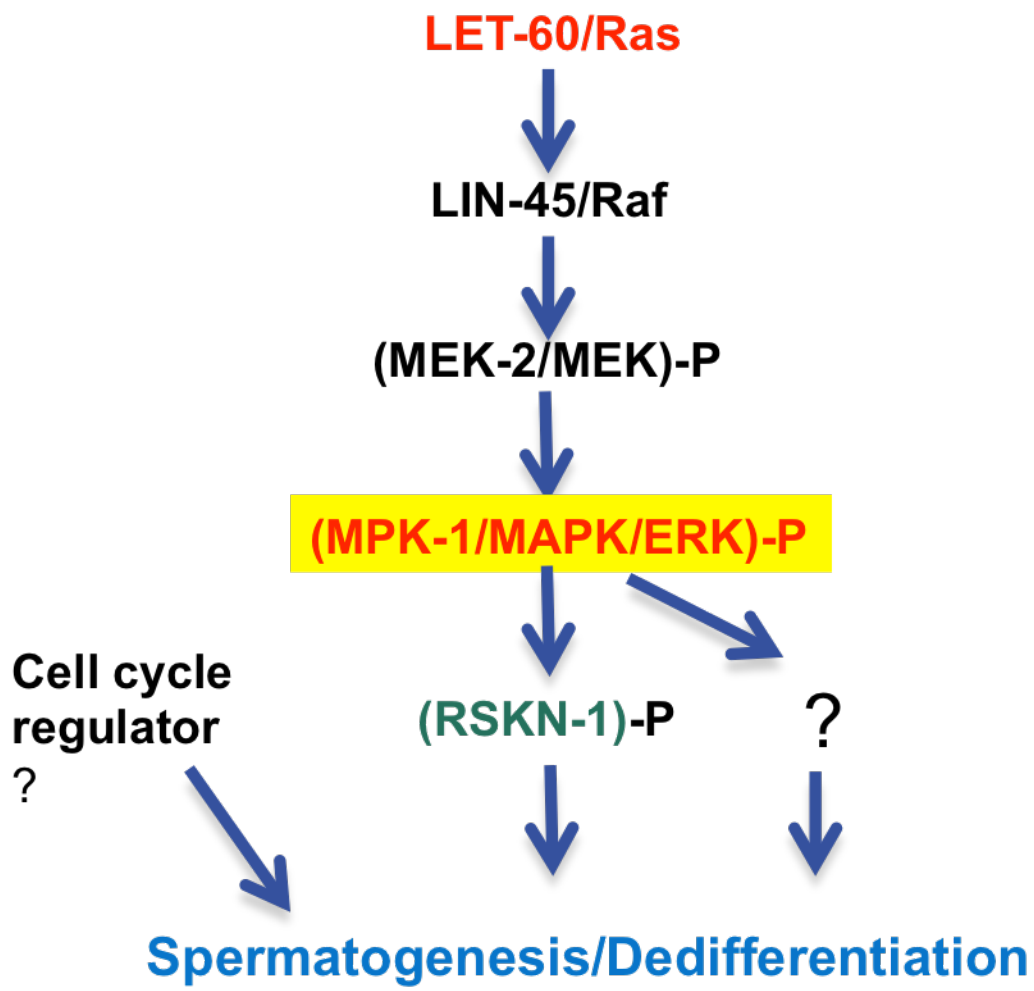


Fig. 2-11

MAPK pathway and spermatogenesis/dedifferentiation. In general, MPK-1/MAPK/ERK is activated by phosphorylation of specific tyrosine and threonine residues of dual-specificity protein kinase MEK-2/MEK (MAPKK). MEK-2/MEK is in turn activated by phosphorylation of serine and serine/threonine residues by upstream LIN-45/RAF (MAPKKK). The activated Ras (Ras-GTP) activates the protein kinase activity of RAF kinase. In *C. elegans*, MAPK/MPK-1 may activate its downstream regulator RSKN-1 or other MAPK targets to induce dedifferentiation. Other MAPK-independent regulators

such as cell cycle regulators may contribute to the abnormal spermatogenesis and raise the potential risk of tumorigenesis through dedifferentiation.

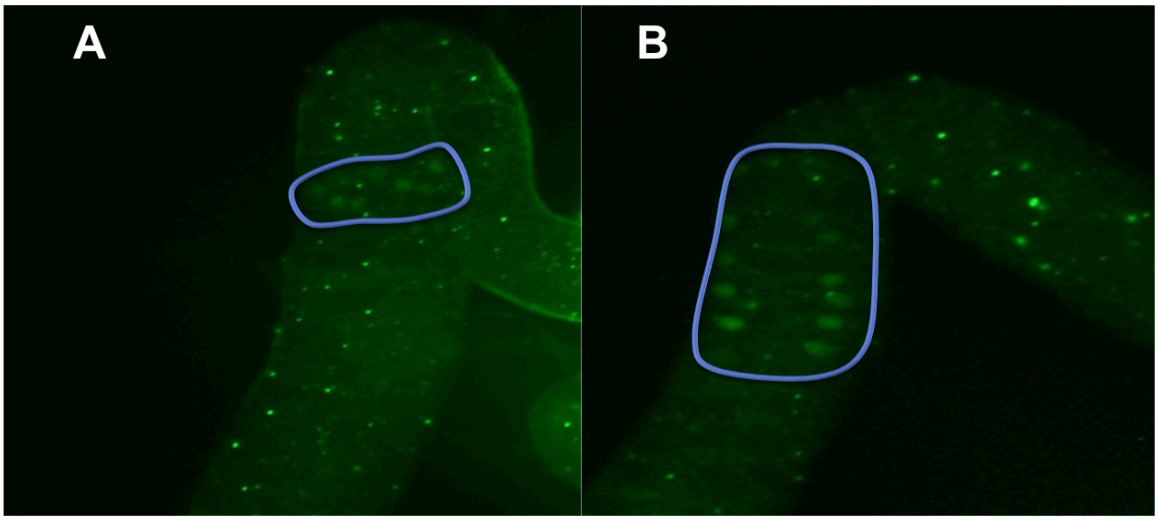


Fig. 2-12

Anti-MAPK(YT) Antibody staining. *Puf-8; lip-1* mutants were treated with Dis-Oil mixture at L4 stage for 24 h. A: Control; B: 500X Dis-Oil Mixture. Diphosphorylated-MAPK are shown as green dots in blue region.

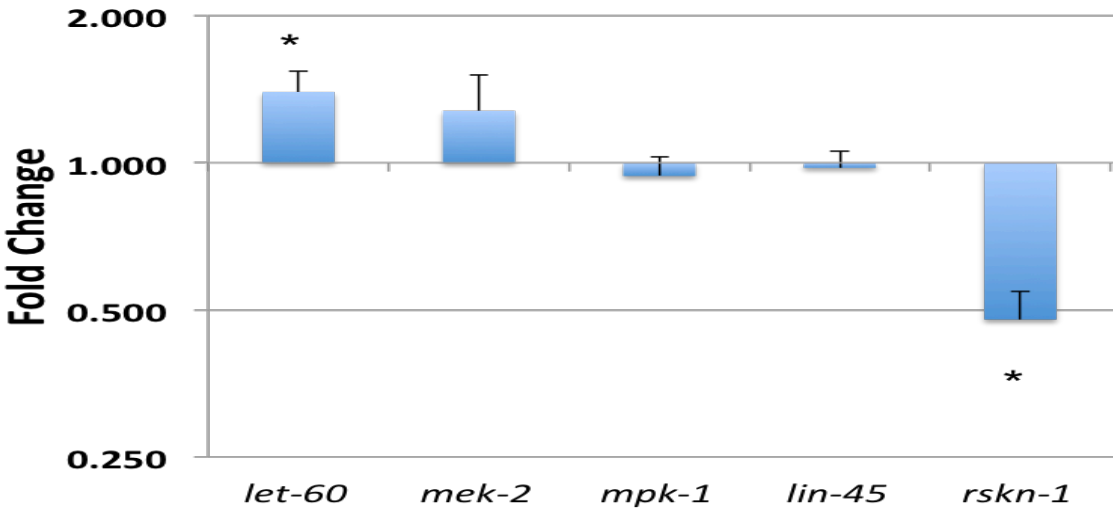


Fig. 2-13

Gene expression fold changes of the genes involved in MAPK pathway. Fold changes are relative to a control value of one. Values greater than one are classified as up-regulation; values less than one are classified as down-regulation. The fold changes on the Y-axis are based on a logarithmic scale with a base value of 2. Bars represent means ± SD. Notations above the expression bars indicate statistical significance at $p < 0.05$ (*)

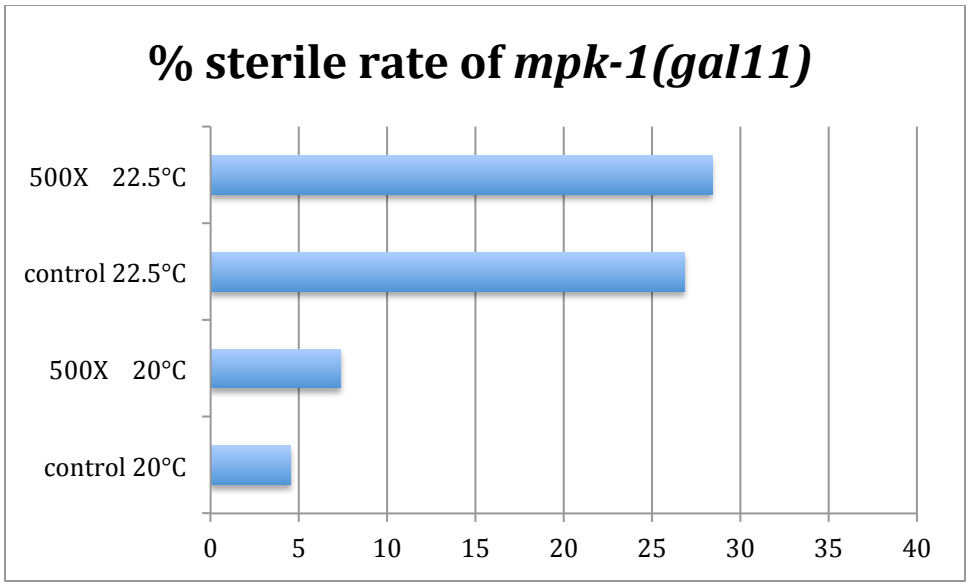


Fig. 2-14

Sterile rate of mpk-1(gal11) temperature sensitive stain. The *mpk-1(gal11)* mutants were exposed with 500X Dis-Oil mixture at L4 stage for 24h at two temperatures: 20ºC and 22.5ºC. This mutant strain is fertile at 20ºC but sterile at 25ºC.

Table 2-1. Information of selected spermatogenesis related genes and MAPK pathway involved genes

Gene	Forward primer	Reverse primer	Length
fer-1	ACCACGGAAACCCATCAACT	GCACAAGCTTCACAAGGGTC	193
spe-10	CACCCAGGATGGTCTGCATT	TTGCGCACGTTTTGTAGTGC	82
spe-11	CATCGGTGTTCCAATGGCTC	TCGCTCCTGCTTCCTGATAC	163
spe-17	TATGGAGTTGGCTCGTTGCG	ACGTTCTCAACTTCCGAGCC	163
cnb-1	TTGCATTCCGCATCTACGAC	TGTGCTCAACGACATCACAA	198
let-60	CATGAGGACAGGCGAAGGAT	CTCGCGGTAGTTAGCGACAT	82
mek-2	TAATGGACCTCCGCCAACAC	GGGTTGCTCGTTCACTTGGA	101
mpk-1	CAGTTGTTGGATCCCCGTCA	CGAGCCCATGGTTGTTTTGG	106
lin-45	GCTGTTTATGGGATGGGTTCG	CAAATTCGACGCGTGGCTC	110
rskn-1	TTCGCGATGGGTCCAAATGA	TGCTTGTTTTGAAGATCCTTTGCT	125

Table 2-2 The tumor rate and fertile rate of *puf-8; lip-1; mpk-1*RNAi

puf-8;lip-1	day	tumor%	mog%	fertile%	Total n
vector no Dis-Oil	2	12.12	87.88	0.00	33
vector with Dis-Oil	2	9.09	90.91	0.00	44
mpk-1 RNAi no Dis-Oil	2	0.00	0.00	100.00	25
mpk-1 RNAi with Dis-Oil	2	0.00	4.35	95.65	23
vector no Dis-Oil	3	9.09	87.88	3.03	33
vector with Dis-Oil	3	23.33	76.67	0.00	30
mpk-1 RNAi no Dis-Oil	3	0.00	0.00	100.00	34
mpk-1 RNAi with Dis-Oil	3	0.00	0.00	100.00	25
vector no Dis-Oil	4	26.47	73.53	0.00	34
vector with Dis-Oil	4	42.31	53.85	3.85	26
mpk-1 RNAi no Dis-Oil	4	0.00	0.00	100.00	21
mpk-1 RNAi with Dis-Oil	4	7.41	0.00	92.59	27

Chapter 3: Oil/dispersant induced the aberrant expression of miRNAs in *C. elegans*: implications for reproductive toxicity

Abstract

The BP oil spill is among the most severe environmental disasters in US history. The nematode *Caenorhabditis elegans (C. elegans)* has been a useful tool for environmental toxicity studies. In a previous study, we found the crude oil and/or dispersant induce growth inhibition and offspring reduction in *C.elegans.* At the molecular level, microRNA-mediated gene silencing has emerged as a fundamental regulatory mechanism of gene expression in many biological processes. However, miRNAs-mediated gene regulation in response to major pollution events is poorly understood. Here we systematically investigated a total of 231 microRNA expression by using *qRT-PCR* and analyzed the expression profile of miRNAs in *C. elegans* in response to oil-alone, dispersant-alone and the mixture of oil and dispersant. TargetScan6.2 and Miranda were applied to predict the targets of differentially expressed miRNA genes in each treatment individually. Then, a further target function analysis was performed based on the KEGG pathway database. The aberrant expression of miRNAs was induced and KEGG pathway enrichment analyses indicated that those significantly changed miRNAs affect many biological processes in *C. elegans*. Many affected pathways are related to environmental information processing, such as ABC transporters, MAPK signaling pathway, Erbb signaling pathway, JAK-STAT signaling pathway, MTOR signaling pathway and calcium-signaling pathway. Some pathways are related to oil uptake and metabolism, such as endocytosis, fatty acid biosynthesis and the phosphatidylinositol signaling system. Based on the genome-wide investigation of microRNA profile, it suggests that *C.*

elegans may respond to environmental stimuli, like oil, dispersant and oil-dis mixture and activate many pathways. These pathways are related to many important biological processes, such as cell cycle, cell proliferation, differentiation and apoptosis. Since the currently identified proteins and microRNAs in *C.elegans* show remarkable conservation with mammals including humans, the oil/dispersant may also induce similar change in microRNA expression and affect many biological processes.

Introduction

The Deepwater Horizon (DWH) oil spill (also known as the BP oil spill or the Gulf of Mexico oil spill) released 4.9 million barrels of crude oil into the north-central Gulf of Mexico over a period from 20 April 2010 through 15 July 2010 (Torres et al. 2003), resulting in large-scale environmental pollution with significant economic, environmental and human health impacts. The chemical dispersants, mixtures of solvents and surface-active agents, have been widely used to rapidly disperse spilled crude oil. By reducing the interfacial tension, dispersants enhance the breakdown of large oil slicks or oil sheen into smaller droplets that stabilize in water. Dispersant facilitates natural degradation and prevents the oil from spreading and contaminating shorelines and wetlands. In response to the DWH oil spill, approximately 2.1 million gallons of dispersants (primarily Corexit® 9500A) were applied to the surface water and around the wellhead (Allan et al. 2012). The scope of DWH oil spill and the additional dispersant use were unprecedented and prompted interests in understanding the toxicity of crude oil and the consequences of using dispersants to clean up oil spills.

Recently, several field and laboratory studies showed the crude oil might cause toxic effects on different animals. The class of polycyclic aromatic hydrocarbons (PAH)

compounds in crude oil, such as benzo[a]pyrene and fluoranthene, have been well known as potent genotoxic and carcinogenic agents (Bispo et al. 1999; HHS 1995). PAHs present in oil also causes immunotoxicity in a variety of animals, including fish, birds, and mammals (Barron 2012). In history, chemical dispersants were used on 18% of oil spills between 1995 and 2005 based on the report from Chapman et al. (Chapman et al. 2007). For the toxic effects of dispersant, two recent studies on the chemical dispersant Corexit 9500A, the primary dispersant applied during the DWH spill, suggested immunotoxicity and neurotoxicity in rodent models (Anderson et al. 2011; Sriram et al. 2011).

Reproductive stages of organisms are suspected of being more sensitive to the effects of environmental pollutants than other stages, investigation of crude oil/dispersant exposure effects on reproduction is critically important. Our previous study showed both the crude oil and the dispersant had the negative effects on the growth and reproduction in *Caenorhabditis elegans* (*C. elegans*). Also we found that dispersant potentiated the toxicity of crude oil on *C. elegans* growth and reproduction. Others studies also showed that dispersants enhance the toxic effects of crude oil (Goodbody-Gringley et al. 2013; Lee et al. 2013; Rico-Martinez et al. 2013). However, the underlying molecular mechanisms of how oil and dispersant can cause reproduction toxicity still remain unknown. In this study, we employed the model organism *C. elegans* to investigate impacts of crude oil/dispersant exposure on reproduction and underlying microRNA-mediated molecular mechanisms.

C. elegans is the first multicellular eukaryote to have its genome completely sequenced (1998). The genome of *C. elegans* showed a high level of conservation with

those of vertebrates (Cutter et al. 2009); approximately 60%–80% of *C. elegans* genes are conserved with humans (Vella 2005). Currently the 447 (updated 2016-3-22) identified microRNAs in *C.elegans* also show remarkable conservation with those of mammalians, including humans(Vella and Slack 2005). All these advantages greatly facilitate the dissection of miRNA-mediated genetic mechanisms underlying reproductive defects after environmental crude oil/dispersant exposure. However, miRNAs-mediated gene regulation in response to major pollution events is poorly understood.

MicroRNA-mediated gene silencing has emerged as a fundamental regulatory mechanism of gene expression in many biological processes. miRNAs inhibit gene expression by binding to specific sites located in the 3' untranslated region (UTR) of their target mRNAs. It is predicted that more than 30% of protein-coding genes are regulated by miRNAs (Lewis et al. 2005; Xie et al. 2005). MiRNAs act as regulatory switches for reproduction and development, controlling developmental timing, signal transduction, cell fate, and apoptosis. Aberrant expression of miRNAs has been observed in developmental defects (Pearson et al. 2005), cancers, and xenobiotic responses (Berezikov et al. 2006; Johnson et al. 2005; Kosik 2006; Zhang et al. 2007). Current literature shows that miRNA regulation of gene expression might be affected by environmental chemicals such as heavy metals, bisphenol A, nanoparticles, cigarette smoke and air pollution(Avissar-Whiting et al. 2010; Badrnya et al. 2014; Bollati et al. 2010; Fossati et al. 2014; Hou et al. 2016; Huang et al. 2014; Huang et al. 2015; Momi et al. 2014; Vucic et al. 2014). It suggested miRNAs could be novel biomarkers for environmental exposure(Chaudhari et al. 2016; Hou et al. 2011; Jardim 2011; Vrijens et

al. 2015). However, study on miRNA-mediated mechanism after environmental toxicant exposures has still been insufficient. No study has reported microRNA expression modifications related to crude oil/dispersant exposure. In this study, we aims to address this knowledge gap by using the DWH oil spill to understand the effects of environmental crude oil exposure on the miRNA regulatory mechanisms of reproductive toxicity.

Materials and Methods

C. elegans cultivation

The wild-type N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetics Center. Worms were maintained at 20 ºC on NGM (Nematode-Growth-Medium)-agar-plates seeded with *E. coli* strain OP50 as food, according to the standard method previously described by Brenner (Brenner 1974). L4 stage larvae from an age-synchronized culture were used in the experiments. To obtain age-synchronized cultures, eggs from 3-day-old mature adults plates were isolated via bleaching, followed by rinse with M9 buffer (3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1 liter. Sterilized by autoclaving), and the eggs were hatched and allowed to grow to L1-stage larvae in M9 buffer without food. L1-stage larvae were allowed to grow to L4-stage larvae (36 hours) on agar plates with E. coli OP50 as a food source at 20 °C.

Oil and dispersant preparation

Unweathered crude oil from the Macondo well was provided by BP and Corexit 9500A, the primary dispersant used, was provided by NALCO. The stock solution of the dispersant-oil mixture (Dis-Oil) was made as follows: 20 volumes of crude oil and 1 volume of dispersant (according to the approximate proportion in commercial use) were

vortex mixed, shaken overnight in a reciprocating shaker at 120 rpm and then diluted 10 times with K-medium (0.032 M KCl and 0.051 M NaCl). K-medium is a commonly used salt medium suitable for *C.elegans* aquatic toxicity testing (Khanna et al. 1997; Ura et al. 2002). While performing dilution, the parent solution was intensively mixed by inversing the conical tube 25 times and solutions were immediately taken from the middle of the mixed emulsion. To make the dispersant-alone (Dis) stock solution, 20 volumes of K-medium and 1 volume of dispersant were mixed, shaken overnight and then intensively shaken, diluted 10 times with K-medium. Similarly, to make oil-alone (Oil) stock, 20 volumes of crude oil and 1 volume of K-medium were mixed overnight, shaken, and then diluted 10 times with K-medium.

Oil and dispersant exposure treatments for microRNA expression assay

Based on our previous study (Zhang et al. 2013), we found the L4-stage larvae are more sensitive to oil and dispersant compared to that of L1-larvae. We also found that exposure to 50 times dilution of stocking solution significantly inhibit reproduction. Therefore in this study, we made the working solutions of Dis-oil, Dis, and Oil by 50 times dilution of corresponding stock solutions with K-medium for microRNA expression profile assay. K-medium alone was used as control. We treated the wild type N2 L4-stage larvae with oil, dispersant or dis-oil mixture in 15ml centrifuge tubes for 24hr and then rinsed with K-medium twice and collected the worms in 2ml centrifuge tubes. The worms were ready for RNA isolation or were frozen in liquid nitrogen and then stored in -80 °C until use. We did three biological replicates for each treatment.

Genome-wide identification of the aberrant microRNA expression profile using qRT-PCR

Total RNAs were isolated according to the manufacturer's protocol, from each treatment and control using the mirVanaTM miRNA Isolation Kit (Ambion, Austin, TX). The extracted RNAs were evaluated and quantified using the NanoDrop ND-1000 Micro-Volume UV/Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE). Then the aliquoted RNA was stored at -80 ºC immediately until analysis.

Applied Biosystems TaqMan microRNA Assays were employed to detect and quantify miRNAs (Chen et al. 2005). There were two steps in the TaqMan miRNA Assays 1) reverse transcription of the mature miRNA to a longer single-stranded cDNA sequence using a stem-looped primer, and 2) real-time PCR. A single-stranded miRNA cDNA was generated from 500 ng of the total RNA sample by reverse transcription using the Applied Biosystems TaqMan microRNA Reverse Transcription Kit. Every 12 miRNA-specific stem-looped RT primers were mixed to get miRNA cDNAs. Y45F10D.4 and tba-1 were used as reference genes based on our previous studies (Zhang et al. 2013) (Zhang et al. 2012). Relative miRNA expression data were analyzed using the ΔΔCT method (Chen et al. 2005).

Identify miRNAs expression change response to crude oil, dispersant or Dis-Oil mixture exposure

Based on the data obtained from qRT-PCR, sets of miRNAs with up- or down-regulated expression associated with crude oil or dispersant exposure were identified. Differences among treatments were statistically analyzed using ANOVAs. MiRNAs that exhibit sensitive response have been identified.

We used (50-ΔCT) values of each microRNA to generate the heat map by performing R programming. Using heat map clustering, we classified groups with similar expression change trends in a single treatment profile when compared with other profiles.

In silico identification of gene targets of miRNAs with differential expressions

Two computational programs, TargetScan6.2 (Lewis et al. 2003) and Miranda (Betel et al. 2008) were employed to predict selected miRNA targets. This strategy of applying two computational programs for gene target prediction would significantly reduce the potential false positives, since one computational program could over-predict the number of targets of a miRNA. The genes that were predicted by both programs had higher probability of being an authentic target of the miRNA. Two discrete lists of the predicted miRNA targets were first generated by TargetScan and Miranda, respectively. Then, the two lists of miRNA targets were compared with each other and the target genes predicted by both computational programs were selected for further analysis.

Characterization of MiRNA function and regulated gene network by KEGG pathway analyses

In order to better understand miRNA target function and classification as well as the regulatory networks associated with reproduction and other affected biochemical pathways, we made a further target function analysis based on the KEGG pathway database.

Results and Discussions

Genome-wide miRNA expression profiling

The expression levels of 231 miRNA genes were measured after treated with oil, dispersant or oil/dispersant mixture. The primer information of 231 miRNA genes is listed in supplementary materials (Table 3.1). Genes with Ct values higher than 45 in two out of three biological replicates were defined as “undetectable”. Most miRNA genes (173/231, 75%) were detected in all four groups, while four genes (2%) were undetectable in any group (Fig. 1).

Two reference genes, Y45F10D.4 and tba1, were chosen to normalize the Ct values of the 231 miRNA genes according to our previous study (Zhang et al. 2012). (50-ΔCT) values based on those two reference genes were used to construct a heat map using unsupervised hierarchical clustering and performed in R programming (Fig. 2). An overall presentation of the expression of all 231 genes in all four groups with three replicates can be visualized in Fig. 2. Replicates in all treatment groups were clustered together, indicating that our results are consistent. Oil treatment groups were clustered close to control groups, while Oil-Dis treatment groups were most distantly related, implying that a mixture of oil and dispersant had distinct effects from oil or dispersant alone on the expression of miRNAs.

Differentially regulated miRNAs in oil, dispersant and Oil-Dis treatments

Differences in the expression of the 231 tested miRNAs were statistically analyzed using ANOVAs. Comparisons were made among three treatments and also with control. A Venn diagram was used to illustrate the results (Fig. 3). Numbers in black mean miRNAs are regulated in the same direction in different treatments, while those in red

mean miRNAs in opposite directions. Totally 166 (72%) genes were found to be significantly changed in three treatment groups. 59 (59/166, 36%) miRNA genes were up-regulated, while 82 (82/166, 49%) were down-regulated. 25 (25/166, 15%) miRNA genes were differentially regulated, which means that they were up-regulated in one treatment, but were down-regulated in others. For oil treatment, the expression levels of 82 miRNAs were significantly changed (29 up-regulated, 53 down-regulated). For dispersant treatment, the expression levels of 85 miRNAs were significantly changed (72 up-regulated, 13 down-regulated). For Oil-Dis treatment, the expression levels of 130 miRNAs were significantly changed (38 up-regulated, 92 down-regulated). Most of the miRNAs were up-regulated in the dispersant treatment group (72/84, 86%), while most were down-regulated in the Oil-Dis group (92/107, 86%).

Functional analyses of differentially expressed miRNAs through target prediction and KEGG pathway analyses

To better understand the potential regulatory roles of these differentially expressed miRNAs in response to oil, dispersant or Oil-Dis treatments, we performed miRNA target prediction combined with KEGG enrichment analyses.

First, TargetScan6.2 and Miranda were applied to predict the targets of differentially expressed miRNA genes in each treatment individually. Only targets listed in both computational programs were kept for further analyses. 8,701, 9,318 and 10,068 common targets were predicted in oil, dispersant, and Oil-Dis treatments, respectively. Then, a further target function analysis was performed based on the KEGG pathway database. A detailed KEGG enrichment analysis can be found in Table 3.2 and Fig. 4.

Most pathways were involved in all three treatments groups, as shown in Fig. 4. Among those pathways, many are related to environmental information processing,

including ABC transporters, MAPK signaling pathway, Erbb signaling pathway, JAK-STAT signaling pathway, MTOR signaling pathway, Calcium signaling pathway, Wnt signaling pathway, Hedgehog signaling pathway and phosphatidylinositol signaling system (Table 3.2). It indicates that *C. elegans* responds to environmental stimuli, like oil, dispersant or Dis-Oil mixture and activates many pathways. These pathways are related to many important biological processes, such as cell cycle, cell proliferation, differentiation and apoptosis. Some pathways are related to oil uptake and metabolism, such as endocytosis, fatty acid biosynthesis and ABC transporters pathway.

Crude oil, dispersant and Dis-Oil mixture affect fatty acid biosynthesis

In KEGG enrichment analysis, the most affected pathway is fatty acid biosynthesis pathway (Pathway ID: cel00061). Two genes involved in this pathway are *fasn-1* and *pod-2*, both of which catalyze the first two steps in saturated small- and medium-chain fatty acid biosynthesis (Zhang et al. 2011). *fasn-1* is the target of cel-miR-247-5p and *pod-2* is regulated by cel-miR-247-5p and cel-miR-232-3p based on our miRNA target prediction. Both cel-miR-247-5p and cel-miR-232-3p are up-regulated in all three treatment groups with statistical significance. Notably, cel-miR-247-5p (cel-miR-247*) expressed at very low level in the control group, but its expression increased 92, 215 and 47 times in crude oil, dispersant and Dis-Oil mixture, respectively.

Expression of *fasn-1* and *pod-2* is down-regulated due to over-expression of these two miRNAs. Previous study by Li Y. *et. al* indicates that knockdown of *fasn-1* and *pod-2* causes defects in triglyceride production and impairs cuticle formation and molting process in *C. elegans* (Li and Paik 2011). Malfunction in molting process may explain the phenomenon that oil/dispersant-treated worms tend to have small body size. Worms

treated with *fasn-1* RNAi and *pod-2* RNAi have fewer eggs within their bodies, implying that *fasn-1* and *pod-2* also play important roles in reproduction of *C. elegans* (Li and Paik 2011). Lower reproduction ability observed in *fasn-1* RNAi and *pod-2* RNAi treatments is consistent with our previous results observed in oil/dispersant treatment experiments (Zhang et al. 2013). Therefore, oil/dispersant treatments might induce developmental and reproductive defects via miRNA-mediated down-regulation of *fasn-1*and *pod-2*.

Besides the fatty acid biosynthesis pathway, other KEGG pathways, which are related to lipid molecule transportation and metabolism, were also highly involved in exposure of crude oil, dispersant and Dis-Oil mixture. These pathways include ABC transporter pathway and endocytosis pathway.

Crude oil, dispersant and Dis-Oil mixture affected miRNAs mediated regulatory network in the MAPK pathway

In order to further understanding the miRNA-mediated regulatory mechanism in response to crude oil, dispersant and Dis-Oil mixture, we captured a snapshot of the whole miRNA-targets network in MAPK pathway to present regulation complexity (Table 3 & Figure 5). Because the MAPK pathway is interacted with many other environmental information processing pathway, such as Erbb signaling pathway, JAK-STAT signaling pathway, MTOR signaling pathway, Calcium signaling pathway and the Wnt signaling pathway. In addition, the MAPK pathway is involved in proliferation, differentiation and apoptosis and is our interest in Chapter 2. Therefore, instead of showing all the protein-coding genes and their putative miRNAs interactions involved in MAPK pathway, we simplified the problem with focusing on five key players (*let-60, lin-45, mek-2, mpk-1* and *rskn-1*) in MAPK pathway.

The fold change for the significantly changed miRNAs in expression for three different treatments (oil-only, dispersant-only and Dis-Oil mixture) are summarized in Table 3. Those predicted miRNA targets for each individual miRNA are also summarized in Table 3. Based on the miRNA-targets relationship, we built the miRNA regulatory network for the five key genes in the MAPK pathways in different exposure treatments (Figure 5). The results showed that for these five gene targets, nearly all the differentially expressed miRNAs were up regulated in the dispersant exposure and most of the expression-changed miRNAs were down regulated in both oil and Dis-Oil mixture exposure treatments. The Dis-Oil mixture exposure treatment it triggered more miRNAs shown differentially expression, which indicated the mixture of oil and dispersant might cause more complex regulatory mechanisms than the oil-only or dispersant-only exposure.

The miRNAs and their targeted genes were shown complicated regulatory network (Figure 5). One miRNA may regulate many of these five genes as its targets and one miRNA may or may not have the same expression pattern in different exposure treatments. For example, cel-miR-247-5p has multiple putative gene targets (*let-60* and *lin-45*) in the MAPK pathway. Cel-miR-247-5p showed up-regulation in all of the three different exposure treatments while cel-miR-788-3p targeted four genes (*let-60/lin-45/mpk-1/rskn-1*) in MAPK pathway showed down-regulation in both oil and Dis-Oil mixture. Moreover these expression-altered miRNAs also have many other potential targets that are not in the MAPK pathway (data not shown). Some miRNAs may have different expression (fold change) pattern for different exposure treatments. For example, cel-miR-239b-3p targeted mek-2 showed up-regulation in dispersant exposure

treatment and showed down-regulation in Dis-Oil mixture exposure treatment. Meanwhile one gene may be targeted by many miRNAs. In general, the *let-60, lin-45* and *mpk-1* had more targeted miRNAs than the other two genes, *mek-2* and *rskn-1*, in the MAPK pathway. Furthermore, one gene was targeted by more miRNAs in Dis-Oil mixture exposure treatment than that of in oil-only or dispersat-only exposure treatments. For example, *mek-2* was targeted by cel-miR-1021 in oil-only exposure treatment; *mek-2* was targeted by cel-miR-1822-5p and cel-miR-239b-3p in dispersant-only exposure treatment; while in Dis-Oil exposure treatment, mek-2 was targeted by the above three of miRNAs (cel-miR-1021, cel-miR-1822-5p and cel-miR-239b-3p).

In summary, a total of 47-49 out of 53 genes (88.7-92.5%) in the MAPK pathway in *C. elegans* were putatively regulated by expression-changed miRNAs in all three different exposure treatments. Based on the data shown in Chapter 2 (Fig. 11), these five key genes in MAPK pathway didn't show dramatic expression alteration in the Dis-Oil mixture treatment. In Dis-Oil mixture exposure treatment, the expression of those miRNAs that may regulate these five key players showed different regulation directions. Considering many aberrantly expressed miRNAs were involved in the miRNA-target interactions, one possible explanation could be that these miRNAs might serve like a "buffer" to resist the expression changes for their targets. Another publication also suggests that it could be possible that miRNAs buffer fluctuations in gene expression and more faithfully signal outcomes in the context of certain regulatory networks (Ebert and Sharp 2012). It suggests that the mRNA level of protein-coding genes remains in a certain range when face environment information changes might due to multiple

miRNAs play a regulatory role as a whole in a complex miRNAs-targets interactions network.

Conclusion

Our studies showed that the crude oil, dispersant and Dis-Oil mixture induced significantly aberrant expression of miRNAs genome-widely in *C. elegans*. From the miRNA targets prediction and KEGG enrichment analysis of those targets, it suggests that many pathways affected by crude oil, dispersant or Dis-Oil mixture exposures were related to environmental information processing. Our results suggest that microRNAs expression profiles could be used as a reference to evaluate the potential risk of environmental pollution. Further targets prediction and KEGG enrichment analysis of the microRNAs expression profiles could be used in turn to select several microRNAs as a pool of representative biomarkers to indicate environmental pollution. Our work was started with the available miRNAs candidates in miRBase, and the following KEGG analysis was also based on the predicted miRNA targets. Therefore further studies need to take efforts to find the possible miRNA biomarkers for the oil spill environment pollution by both validating the miRNA-target interaction and narrowing down the miRNA biomarker candidates with more powerful bioinformatics analysis.

Reference

(1998) Genome sequence of the nematode C. elegans: a platform for investigating biology. Science 282(5396):2012-8

Allan SE, Smith BW, Anderson KA (2012) Impact of the Deepwater Horizon Oil Spill on Bioavailable Polycyclic Aromatic Hydrocarbons in Gulf of Mexico Coastal Waters. Environmental Science & Technology 46(4):2033-2039 doi:10.1021/es202942q

Anderson SE, Franko J, Lukomska E, Meade BJ (2011) Potential Immunotoxicological Health Effects Following Exposure to Corexit 9500a During Cleanup of The Deepwater Horizon Oil Spill. Journal of Toxicology and Environmental Health-Part a-Current Issues 74(21):1419-1430 doi:10.1080/15287394.2011.606797

Avissar-Whiting M, Veiga KR, Uhl KM, et al. (2010) Bisphenol A exposure leads to specific microRNA alterations in placental cells. Reproductive toxicology 29(4):401-6 doi:10.1016/j.reprotox.2010.04.004

Badrnya S, Baumgartner R, Assinger A (2014) Smoking alters circulating plasma microvesicle pattern and microRNA signatures. Thrombosis and haemostasis 112(1):128-36 doi:10.1160/TH13-11-0977

Barron MG (2012) Ecological Impacts of the Deepwater Horizon Oil Spill: Implications for Immunotoxicity. Toxicologic Pathology(in press) doi:10.1177/0192623311428474

Berezikov E, Thuemmler F, van Laake LW, et al. (2006) Diversity of microRNAs in human and chimpanzee brain. Nature Genetics 38(12):1375-1377

Betel D, Wilson M, Gabow A, Marks DS, Sander C (2008) The microRNA.org resource: targets and expression. Nucleic acids research 36(Database issue):D149-53 doi:10.1093/nar/gkm995

Bispo A, Jourdain MJ, Jauzein M (1999) Toxicity and genotoxicity of industrial soils polluted by polycyclic aromatic hydrocarbons (PAHs). Organic Geochemistry 30(8B):947-952 doi:10.1016/s0146-6380(99)00078-9

Bollati V, Marinelli B, Apostoli P, et al. (2010) Exposure to metal-rich particulate matter modifies the expression of candidate microRNAs in peripheral blood leukocytes. Environmental health perspectives 118(6):763-8 doi:10.1289/ehp.0901300

Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77(1):71-94

Chapman H, Purnell K, Law RJ, Kirby MF (2007) The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Marine pollution bulletin 54(7):827-38 doi:10.1016/j.marpolbul.2007.03.012

Chaudhari U, Nemade H, Gaspar JA, Hescheler J, Hengstler JG, Sachinidis A (2016) MicroRNAs as early toxicity signatures of doxorubicin in human-induced pluripotent stem cell-derived cardiomyocytes. Archives of toxicology doi:10.1007/s00204-016-1668-0

Chen CF, Ridzon DA, Broomer AJ, et al. (2005) Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Research 33(20):e179

Cutter AD, Dey A, Murray RL (2009) Evolution of the Caenorhabditis elegans genome. Molecular biology and evolution 26(6):1199-234 doi:10.1093/molbev/msp048

Ebert MS, Sharp PA (2012) Roles for microRNAs in conferring robustness to biological processes. Cell 149(3):515-24 doi:10.1016/j.cell.2012.04.005

Fossati S, Baccarelli A, Zanobetti A, et al. (2014) Ambient particulate air pollution and microRNAs in elderly men. Epidemiology 25(1):68-78 doi:10.1097/EDE.0000000000000026

Goodbody-Gringley G, Wetzel DL, Gillon D, Pulster E, Miller A, Ritchie KB (2013) Toxicity of Deepwater Horizon source oil and the chemical dispersant, Corexit(R) 9500, to coral larvae. PloS one 8(1):e45574 doi:10.1371/journal.pone.0045574

HHS (1995) Agency for Toxic Substances and Disease Registry. 1995. Toxicological Profile for Polycyclic Aromatic Hydrocarbons. http://www.atsdr.cdc.gov/toxprofiles/tp69.pdf.

Hou L, Barupal J, Zhang W, et al. (2016) Particulate Air Pollution Exposure and Expression of Viral and Human MicroRNAs in Blood: The Beijing Truck Driver Air Pollution Study. Environmental health perspectives 124(3):344-50 doi:10.1289/ehp.1408519

Hou L, Wang D, Baccarelli A (2011) Environmental chemicals and microRNAs. Mutation research 714(1-2):105-12 doi:10.1016/j.mrfmmm.2011.05.004

Huang J, Wu J, Li Y, et al. (2014) Deregulation of serum microRNA expression is associated with cigarette smoking and lung cancer. BioMed research international 2014:364316 doi:10.1155/2014/364316

Huang Y, Lu X, Qu Y, Yang Y, Wu S (2015) MicroRNA sequencing and molecular mechanisms analysis of the effects of gold nanoparticles on human dermal fibroblasts. Biomaterials 37:13-24 doi:10.1016/j.biomaterials.2014.10.042

Jardim MJ (2011) microRNAs: implications for air pollution research. Mutation research 717(1-2):38-45 doi:10.1016/j.mrfmmm.2011.03.014

Johnson SM, Grosshans H, Shingara J, et al. (2005) RAS is regulated by the *let-7* microRNA family. Cell 120(5):635-647

Khanna N, Cressman CP, 3rd, Tatara CP, Williams PL (1997) Tolerance of the nematode Caenorhabditis elegans to pH, salinity, and hardness in aquatic media. Archives of environmental contamination and toxicology 32(1):110-4

Kosik KS (2006) The neuronal microRNA system. Nature Reviews Neuroscience 7(12):911-920

Lee KW, Shim WJ, Yim UH, Kang JH (2013) Acute and chronic toxicity study of the water accommodated fraction (WAF), chemically enhanced WAF (CEWAF) of crude oil and dispersant in the rock pool copepod Tigriopus japonicus. Chemosphere 92(9):1161-8 doi:10.1016/j.chemosphere.2013.01.080

Lewis BP, Burge CB, Bartel DP (2005) Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120(1):15-20

Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP, Burge CB (2003) Prediction of mammalian microRNA targets. Cell 115(7):787-798

Li Y, Paik YK (2011) A potential role for fatty acid biosynthesis genes during molting and cuticle formation in Caenorhabditis elegans. BMB reports 44(4):285-90 doi:10.5483/BMBRep.2011.44.4.285

Momi N, Kaur S, Rachagani S, Ganti AK, Batra SK (2014) Smoking and microRNA dysregulation: a cancerous combination. Trends in molecular medicine 20(1):36-47 doi:10.1016/j.molmed.2013.10.005

Pearson JC, Lemons D, McGinnis W (2005) Modulating Hox gene functions during animal body patterning. Nature Reviews Genetics 6(12):893-904 doi:10.1038/nrg1726

Rico-Martinez R, Snell TW, Shearer TL (2013) Synergistic toxicity of Macondo crude oil and dispersant Corexit 9500A((R)) to the Brachionus plicatilis species complex (Rotifera). Environmental pollution 173:5-10 doi:10.1016/j.envpol.2012.09.024

Sriram K, Lin GX, Jefferson AM, et al. (2011) Neurotoxicity Following Acute Inhalation Exposure to The Oil Dispersant Corexit EC9500A. Journal of Toxicology and Environmental Health-Part a-Current Issues 74(21):1405-1418 doi:10.1080/15287394.2011.606796

Torres ES, Andrade CV, Fonseca EC, Mello MA, Duarte MEL (2003) Insulin impairs the maturation of chondrocytes in vitro. Brazilian Journal of Medical and Biological Research 36(9):1185-1192

Ura K, Kai T, Sakata S, Iguchi T, Arizono K (2002) Aquatic acute toxicity testing using the nematode Caenorhabditis elegans. Journal of Health Science 48(6):583-586 doi:10.1248/jhs.48.583

Vella MC, Slack FJ (2005) C. elegans microRNAs (September 21, 2005), WormBook, ed. The C. elegans Research Community, WormBook, doi/10.1895/wormbook.1.26.1, http://www.wormbook.org. .

Vella MCaS, F.J. (2005) C. elegans microRNAs

Vrijens K, Bollati V, Nawrot TS (2015) MicroRNAs as potential signatures of environmental exposure or effect: a systematic review. Environmental health perspectives 123(5):399-411 doi:10.1289/ehp.1408459

Vucic EA, Thu KL, Pikor LA, et al. (2014) Smoking status impacts microRNA mediated prognosis and lung adenocarcinoma biology. BMC cancer 14:778 doi:10.1186/1471-2407-14-778

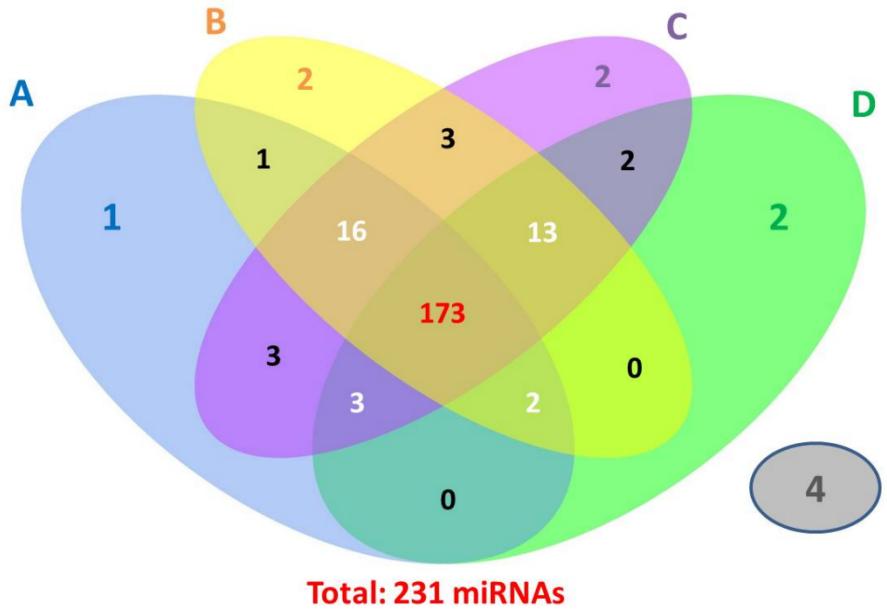
Xie XH, Lu J, Kulbokas EJ, et al. (2005) Systematic discovery of regulatory motifs in human promoters and 3 ' UTRs by comparison of several mammals. Nature 434(7031):338-345

Zhang BH, Pan XP, Cobb GP, Anderson TA (2007) microRNAs as oncogenes and tumor suppressors. Developmental Biology 302(1):1-12

Zhang H, Abraham N, Khan LA, Hall DH, Fleming JT, Gobel V (2011) Apicobasal domain identities of expanding tubular membranes depend on glycosphingolipid biosynthesis. Nature cell biology 13(10):1189-201 doi:10.1038/ncb2328

Zhang Y, Chen D, Ennis AC, et al. (2013) Chemical dispersant potentiates crude oil impacts on growth, reproduction, and gene expression in Caenorhabditis elegans. Archives of toxicology 87(2):371-82 doi:10.1007/s00204-012-0936-x

Zhang Y, Chen D, Smith MA, Zhang B, Pan X (2012) Selection of Reliable Reference Genes in Caenorhabditis elegans for Analysis of Nanotoxicity. PLoS One 7(3):e31849



A: Control ;
B: Oil treatment;
C: Dispersant treatment;
D: Dispersant-Oil mixture treatment.

Fig. 3-1.

Venn diagram showing the numbers of miRNAs expressing in each group. 173 miRNA genes were detected in all four groups, while four genes could not be found in any group.

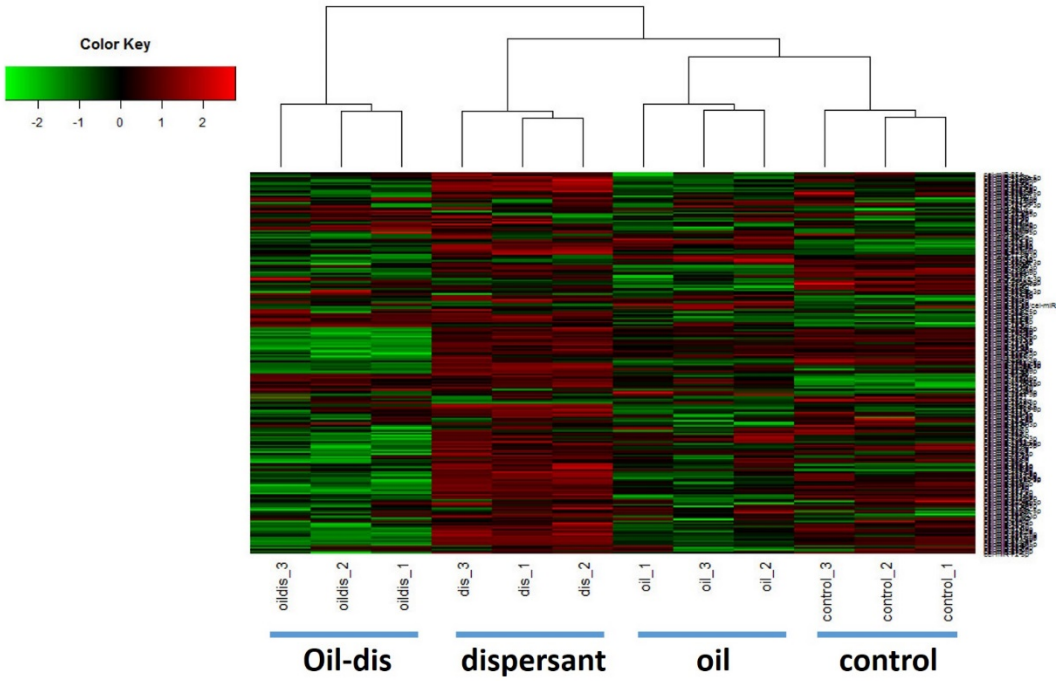


Fig. 3-2

Oil/dispersant induces the aberrant expression of miRNAs in *C. elegans*. Heat map showing expression profiles of 231 miRNAs. From left to right, three biological replicates of: Oil-Dis, dispersant and oil treatment groups. (50-ΔCT) values based on two reference genes (Y45F10D.4 and tba-1) were used to construct a heat map using unsupervised hierarchical clustering and performed in R programming. In the figures, color red, green, and black represent up-regulation, down-regulation and no change, respectively. Expression levels for miRNAs at different biological replicates and in different treatment groups were compared by its Z scores and are presented according to inlaid color keys.

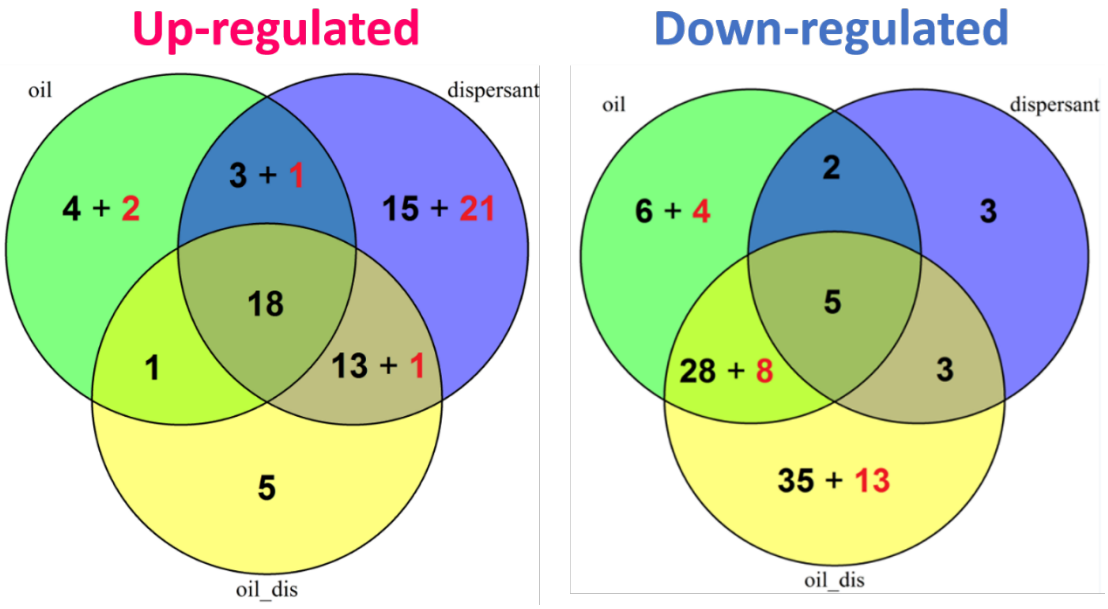


Fig. 3-3

Venn diagram showing the number of miRNAs which are significantly up-regulated or down-regulated using ANOVA analysis in oil, dispersant and Oil-Dis treatments. Numbers in black mean miRNAs are regulated in the same direction in different treatments, while those in red mean miRNAs in opposite directions.

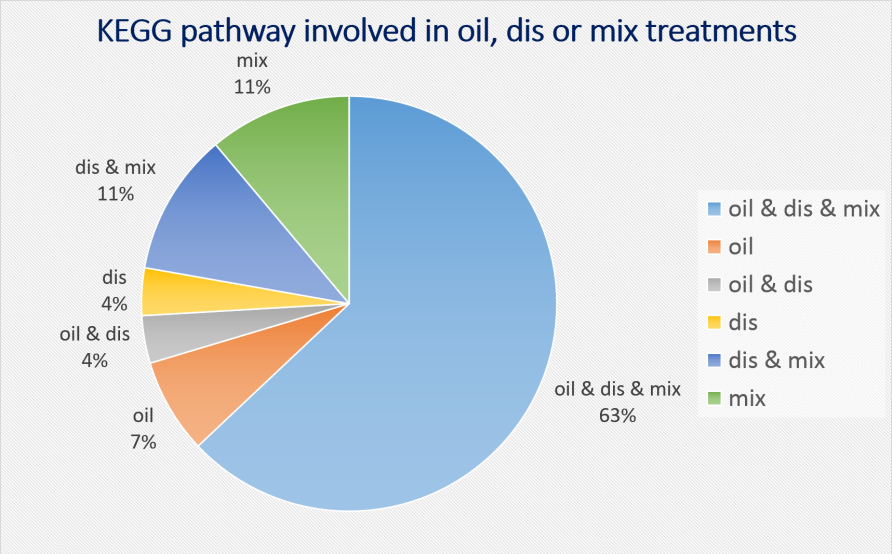


Fig. 3-4

Pie chart showing pathways that are involved significantly in combinations of each treatment group. Most pathways are involved in all three treatment groups.

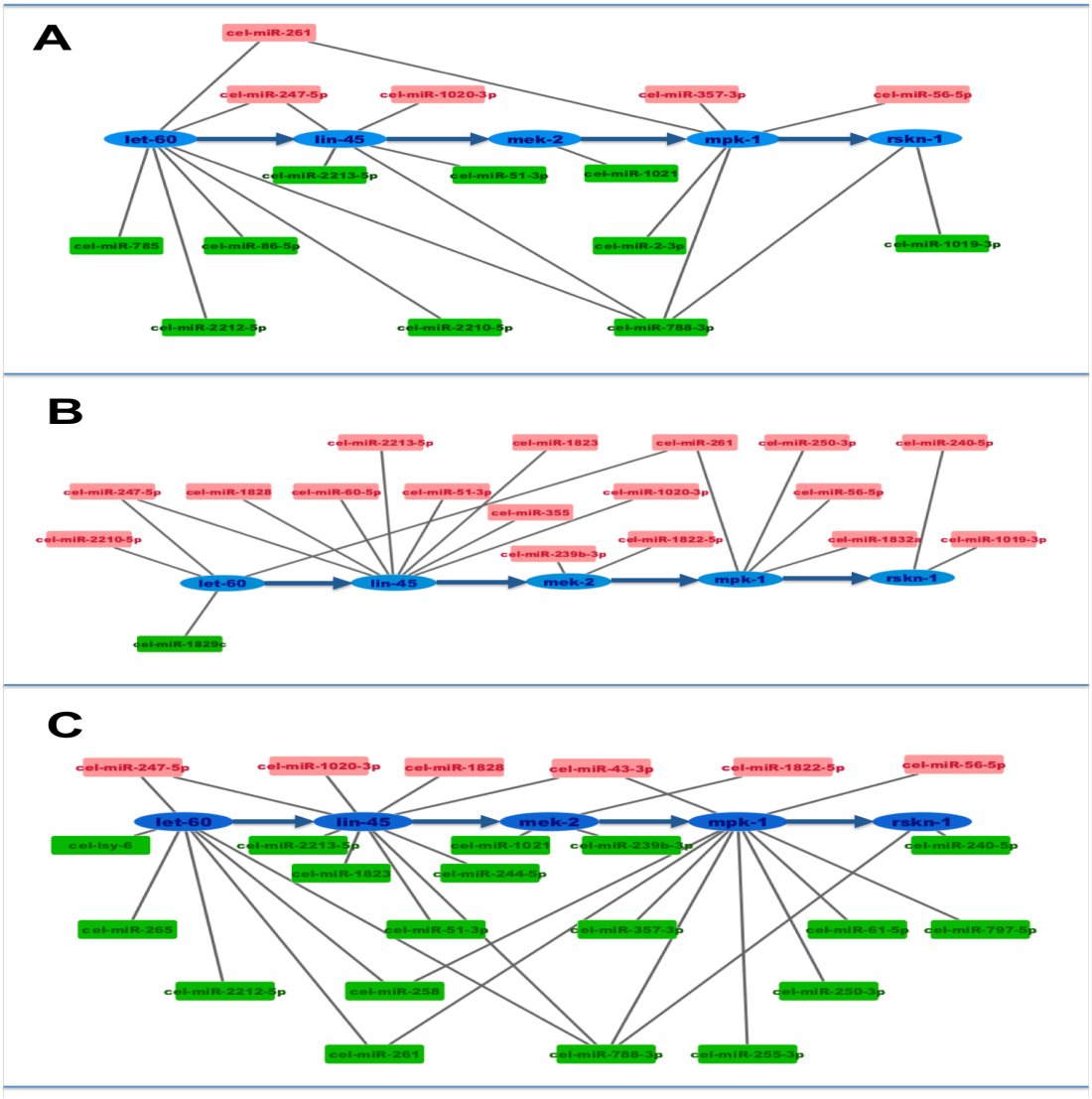


Fig. 3-5

MicroRNA mediated gene regulatory network in MAPK pathway. A: oil only treatment; B: dispersant only treatment; C: oil and dispersant mixture treatment. Blue color showed the key regulatory protein-coding genes in MAPK pathway in *C. elegans*, which *are let-60, lin-45, mek-2, mpk-1* and *rskn-1*. Red color indicated those microRNAs were up regulated in the corresponding treatment while the green color indicated those microRNAs were down regulated in the corresponding treatment.

Table 3-1 Primers used in qRT-PCR for 231 microRNAs in *C. elegans*.

miRNA name	RT primer	Forward primer
cel-let-7-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTAAGG	GCGGCGGTGAACTATGCAATTTTC
cel-let-7-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAACTAT	GCGGCGGTGAGGTAGTAGGTTG
cel-lin-4-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTACCC	GCGGCGGACACCTGGGCTCTCC
cel-lin-4-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCACAC	GCGGCGGTCCCTGAGACCTCAAG
cel-lsy-6	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGAAAT	GCGGCGGTTTTGTATGAGACGC
cel-miR-1-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACATA	GCGGCGGTGGAATGTAAAGAAG
cel-miR-1018	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGTAA	GCGGCGGAGAGAGATCATTGGAC
cel-miR-1019-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGGAA	GCGGCGGCTGTAATTCCACATTG
cel-miR-1019-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAAAATG	GCGGCGGGTGAGCATTGTTCGAG
cel-miR-1020-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTGAAA	GCGGCGGATTATTCTGTGACAC
cel-miR-1021	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGAGG	GCGGCGGAAGTGAGATCATGTG
cel-miR-1022-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATGGC	GCGGCGGAAGATCATTGTTAGG
cel-miR-124-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTGGCAT	GCGGCGGTAAGGCACGCGGTG
cel-miR-124-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTAAA	GCGGCGGGCATGCACCCTAGTG
cel-miR-1817	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATGAT	GCGGCGGTAGCCAATGTCTTCTC

cel-miR-1818	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAAAATC	GCGGCGGTGTGGTCTTCATG CC
cel-miR-1819-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTCCATC	GCGGCGGTGGAATGATTGA GCTTG
cel-miR-1820-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGAACAT	GCGGCGGTTTTGATTGTTTTT C
cel-miR-1821	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCTACCT	GCGGCGGTGAGGTCTTATAG TTAG
cel-miR-1822-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAGAGTT	GCGGCGGGAGCTGCCCTCA G
cel-miR-1822-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGCCGAT	GCGGCGGAGTTTCTCTGGGA AAG
cel-miR-1823	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTTACTC	GCGGCGGTACTGGAAGTGTT TAG
cel-miR-1824-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAAGTTG	GCGGCGGTGGCAGTGTTTCT CC
cel-miR-1828	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACACTATC	GCGGCGGACTGGAAGCATTT AAG
cel-miR-1829a-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAATAGA	GCGGCGGCAACCATTGGAAT TTC
cel-miR-1829a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTACAAA	GCGGCGGAAGGGGACTTCT AATTG
cel-miR-1829b	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTACAAC	GCGGCGGAAGCGATCTTCTA GATG
cel-miR-1829c	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTACAAC	GCGGCGGAAGCGAAATTCAA GATG
cel-miR-1830-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGCCTAG	GCGGCGGCGAGGTTTCACG TTTTC
cel-miR-1832a	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACATCATC	GCGGCGGTGGGCGGAGCGA ATC
cel-miR-1832b-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACATCAGC	GCGGCGGAGTGGGCAGAGC GATTC
cel-miR-1832b-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCAGCGAATCGCT

	CGACAAAGTG	CGG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCGAGGCTTGCGA
cel-miR-1833	CGACGCACAC	AAT
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGAGATCAACCAT
cel-miR-1834-3p	CGACTTGGAT	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATCACAGCCAG
cel-miR-2-3p	CGACGCACAT	CTTTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGATGCACAGGCTC
cel-miR-2207-3p	CGACTGTGTG	AATG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGTGAATTGAGAC
cel-miR-2207-5p	CGACCTTATA	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGATGCAGTTTCTGG
cel-miR-2208a-3p	CGACTGAAGT	
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAGTGTACCCGA
cel-miR-2208a-5p	CGACGGATAT	ATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGATGCAGATTTTGG
cel-miR-2208b-3p	CGACTGAAGT	TAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGAGATCAGCGG
cel-miR-2209a-3p	CGACTGTAGT	TTAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGAGTGTAACCAC
cel-miR-2209a-5p	CGACGAAGGA	TCTTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGAGATGAGCGG
cel-miR-2209b-3p	CGACTGAAGC	TTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGTGTAACAACTC
cel-miR-2209b-5p	CGACGAAGGA	TTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAAAGACCACCG
cel-miR-2209c-3p	CGACTGTAGT	GTTAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGAGTGTAACCGC
cel-miR-2209c-5p	CGACAAACAA	ACG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAAGTCGATTGC
cel-miR-2210-3p	CGACGTGGGT	TC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGGCAGATCAAT
cel-miR-2210-5p	CGACCCTAAA	C

cel-miR-2211-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTTTCTC	GCGGCGGTCAGGTAGAATTT AG
cel-miR-2211-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCGTCAG	GCGGCGGCCTCCATCTATTC TC
cel-miR-2212-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGATGGC	GCGGCGGAAGTGGCATTTGA TAAG
cel-miR-2212-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCAAAGT	GCGGCGGTGGCAGATCATA GGC
cel-miR-2213-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTTAGGC	GCGGCGGAAGCTGTAAGAG GAC
cel-miR-2213-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTCAAAC	GCGGCGGTGGCGGACTCTT CAC
cel-miR-2214-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAACGCC	GCGGCGGCATTGACAACAAC TTG
cel-miR-2214-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACCCCATT	GCGGCGGATTCGGTCCGGA GTC
cel-miR-2215-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAAACAA	GCGGCGGAGAATCGTAGCG CGTG
cel-miR-2215-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGGAGCA	GCGGCGGACAGCACGTGTT ACG
cel-miR-2216-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACAGGCAC	GCGGCGGCTATCTACTTAAA ATG
cel-miR-2216-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGCCTAC	GCGGCGGGCACATTTTAAGT CG
cel-miR-2217-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACACCGAA	GCGGCGGTCGACCCTTGTG CCTG
cel-miR-2217-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACGATCGA	GCGGCGGCAGAGTGGGCAG TCG
cel-miR-2218a-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTACAAA	GCGGCGGAGGCCAGAATAG TGTAG
cel-miR-2218a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA CGACTGAGGC	GCGGCGGCAAACTACAAGTT TT
cel-miR-2218b-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAATTTGTAGTTT

	CGACTCTCAC	GTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGACTACAAACTA
cel-miR-2218b-5p	CGACGAAAAT	CATC
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cel-miR-2219-3p	CGACCTTCAG	GAAAG
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cel-miR-2219-5p	CGACGACGAT	GC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCAATTGTTTGTG
cel-miR-2220-3p	CGACCTGTAA	GAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGTAAGACCATAAA
cel-miR-2220-5p	CGACGATAAA	C
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCAAGTGATACCA
cel-miR-2221	CGACACTAGC	GAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAATGGCACTGCA
cel-miR-228-5p	CGACCCGTGA	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGAAAGGTATCG
cel-miR-229-3p	CGACCTATGA	GGTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAATGACACTGGTT
cel-miR-229-5p	CGACCGATGG	ATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGTATTAGTTGTGC
cel-miR-230-3p	CGACTCTCCT	GAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGACTTGGTCGGCG
cel-miR-230-5p	CGACTAATAT	ATT
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAGCTCGTGATC
cel-miR-231-3p	CGACTTCTGC	AAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAATGCATCTTA
cel-miR-232-3p	CGACTCACCG	AC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTGAGCAATGCG
cel-miR-233-3p	CGACCCGCAC	CATG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTATTGCTCGAGA
cel-miR-234-3p	CGACAAGGGT	ATAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATTGCACTCTCC
cel-miR-235-3p	CGACTCAGGC	CC

cel-miR-236-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCGTC	GCGGCGGTAATACTGTCAGG
cel-miR-237-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTGT	GCGGCGGTCCCTGAGAATTCTC
cel-miR-238-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTGAA	GCGGCGGTTTGTACTCCGATGC
cel-miR-238-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCTTTG	GCGGCGGTGGATGTTCTCGGAC
cel-miR-239a-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCAGTA	GCGGCGGTTTGTACTACACATAG
cel-miR-239b-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTTTTG	GCGGCGGGCACTTTTGTGGTGTG
cel-miR-239b-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCAGTAC	GCGGCGGTTTGTACTACACAAAAG
cel-miR-240-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCGAA	GCGGCGGTACTGGCCCCCAAATC
cel-miR-240-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATTCT	GCGGCGGCGAGGATTTTGAGAC
cel-miR-241-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATTT	GCGGCGGTGAGGTAGGTGCGAG
cel-miR-242	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGAAG	GCGGCGGTTGCGTAGGCCTTTG
cel-miR-243-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATATC	GCGGCGGCGGTACGATCGCGGC
cel-miR-243-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTACGA	GCGGCGGTATCTCGGTGCGATC
cel-miR-244-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCCTTT	GCGGCGGTACTGCTTTTCAGC
cel-miR-244-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCATACC	GCGGCGGTCTTTGGTTGTACAAAG
cel-miR-245-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAGCTA	GCGGCGGATTGGTCCCCTCCAAG
cel-miR-246-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTACATGTTTCGG

	CGACGCTCCT	GTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGACTAGAGCCT
cel-miR-247-3p	CGACAGAAGA	ATTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAGAGAAAAGTTT
cel-miR-247-5p	CGACGGTAAT	C
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cel-miR-248	CGACTGAGCG	GG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACAGGACTTTT
cel-miR-249-3p	CGACGGCAAC	GAG
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cel-miR-250-3p	CGACTGCCAA	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTAAGTAGTGGTG
cel-miR-251	CGACAATAAG	CC
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cel-miR-252-5p	CGACTTACCT	CC
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cel-miR-253-3p	CGACCCTTCC	GTG
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cel-miR-253-5p	CGACGGTCAG	CAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGCAAATCTTTCG
cel-miR-254	CGACCCTACA	CG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAACTGAAGAGAT
cel-miR-255-3p	CGACCTGTAA	TT
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cel-miR-256	CGACTACAGT	AAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGAGTATCAGGAG
cel-miR-257	CGACTCACTG	TAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGGTTTTGAGAGG
cel-miR-258	CGACAAAAGG	AATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAATCTCATCCTA
cel-miR-259-5p	CGACTGCTAC	ATC
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cel-miR-260	CGACCTACAA	TCTTG

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cel-miR-261	CGACCGTGAA	TTC
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cel-miR-262	CGACATCAGA	TTC
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cel-miR-264	CGACCATAAC	TTG
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cel-miR-265	CGACATACCA	GGTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGGCAAGACTTT
cel-miR-266	CGACGCTTTG	GGC
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cel-miR-267	CGACTGCAGC	CTG
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cel-miR-268	CGACACCAAA	AAG
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cel-miR-269	CGACAGTTTT	GC
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cel-miR-270	CGACCTCCAC	CAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCGCCGGGTGGG
cel-miR-271	CGACAATGCT	AAAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGTAGGCATGGG
cel-miR-272	CGACCAAACA	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGCCCGTACTGT
cel-miR-273	CGACCAGCCG	GTC
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cel-miR-2953-3p	CGACGCTACA	TTC
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cel-miR-2953-5p	CGACATTTTC	GTG
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cel-miR-34-3p	CGACGGGTGG	ACTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGGCAGTGTGGT
cel-miR-34-5p	CGACCAACCA	TAG
cel-miR-35-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTGGA

	CGACACTGCT	AAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCAATTGCCATGTG
cel-miR-353	CGACAATACC	TTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGACCTTGTTTGTTG
cel-miR-354	CGACAGGAGC	CTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTTGTTTTAGCCT
cel-miR-355	CGACCATAGC	GAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTGAGCAACGCG
cel-miR-356a	CGACTGATTT	AAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAATGCCAGTC
cel-miR-357-3p	CGACACTCCT	GTTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGATTGGTATCCCTG
cel-miR-358-3p	CGACGACCTT	TC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGACCTGGCCAGGC
cel-miR-358-5p	CGACACAGTT	ATTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACTGGTCTTTC
cel-miR-359	CGACTTCGTC	TC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTGAA
cel-miR-36-3p	CGACCATGCG	AATTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGACCGTAATCC
cel-miR-360-3p	CGACTTGTGA	CGTTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTGTGACCGTTGT
cel-miR-360-5p	CGACTGACCG	TAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTGAA
cel-miR-37-3p	CGACACTGCA	CAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGTGGGTGTCCG
cel-miR-37-5p	CGACGCACCG	TTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGAGAA
cel-miR-38-3p	CGACACTCCA	AAAC
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cel-miR-39-3p	CGACCAAGCT	AATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATCATCGATCAC
cel-miR-392-3p	CGACTCATCA	GTG

	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTGTA
cel-miR-40-3p	CGACTTAGCT	CATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTGAA
cel-miR-41-3p	CGACTAGGTG	AAATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACCGGGTTAA
cel-miR-42-3p	CGACTCTGTA	CATC
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cel-miR-42-5p	CGACCTTCAC	TTT
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATCACAGTTTAC
cel-miR-43-3p	CGACGCGACA	TTG
cel-miR-44-3p/cel-	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGACTAGAGACA
miR-45-3p	CGACAGCTGA	CATTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGTCATGGAGTC
cel-miR-46-3p	CGACTGAAGA	GCTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAGAGAGCCGTC
cel-miR-46-5p	CGACCTGTCA	TATTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGTCATGGAGGC
cel-miR-47-3p	CGACTGAAGA	GCTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAAGAGAGCAGTC
cel-miR-47-5p	CGACACTGTC	TATTG
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cel-miR-48-3p	CGACTGCGAG	CTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAGGTAGGCTC
cel-miR-48-5p	CGACTCGCAT	AGTAG
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cel-miR-49-3p	CGACTCTGCA	AAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGATATGTCTGGT
cel-miR-50-5p	CGACCCCAAG	ATTC
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cel-miR-51-3p	CGACTGCACC	TAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTACCCGTAGCTC
cel-miR-51-5p	CGACAACATG	CTATC
cel-miR-52-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCACCCGTACATAT

	CGACAGCACG	G
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCACCCGTACATTT
cel-miR-53-5p	CGACAGCACG	G
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTACCCGTAATCTT
cel-miR-54-3p	CGACCTCGGA	C
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTACCCGTATAAGT
cel-miR-55-3p	CGACCTCAGC	TTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTACCCGTAATGTT
cel-miR-56-3p	CGACCTCAGC	TC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGCGGATCCAT
cel-miR-56-5p	CGACTACAAC	TTTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTACCCTGTAGATC
cel-miR-57-5p	CGACACACAC	GAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAGATCGTTCA
cel-miR-58-3p	CGACATTGCC	GTAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGCCCTACTCTTC
cel-miR-58-5p	CGACTGAGAT	GC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCGAATCGTTTAT
cel-miR-59-3p	CGACCATCAT	CAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCGTCCTGAAAAC
cel-miR-59-5p	CGACTTCCGT	G
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATTATGCACATT
cel-miR-60-3p	CGACTGAACT	TTC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAACTGGAAGAGT
cel-miR-60-5p	CGACATTTTA	GCC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGACTAGAACCG
cel-miR-61-3p	CGACGATGAG	TTAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGGTTACGGGG
cel-miR-61-5p	CGACGGACTA	CTTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGATATGTAATCT
cel-miR-62	CGACCTGTAA	AG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATGACACTGAAG
cel-miR-63-3p	CGACTTTCCA	CG

	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCTAACTCGTCG
cel-miR-63-5p	CGACTGACTA	GTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGTGCAACGATCA
cel-miR-64-3p	CGACGCATGC	GTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATGACACTGAAG
cel-miR-64-5p	CGACTTCGGT	CG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCTGCTACGCGCA
cel-miR-65-3p	CGACGCATGG	GTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATGACACTGAAG
cel-miR-65-5p	CGACTTCGGT	CG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCATGACACTGATT
cel-miR-66-5p	CGACTCACAT	AG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCACAACCTCCTA
cel-miR-67-3p	CGACTCTACT	G
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAATACGTCGTTG
cel-miR-70-3p	CGACATGGAA	GTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTATCACTATTCTG
cel-miR-71-3p	CGACGCGAAA	
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAAAGACATGG
cel-miR-71-5p	CGACTCACTA	GTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGAGGCAAGATGTT
cel-miR-72-5p	CGACTCAGCT	GGC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGCAAGATGTA
cel-miR-73-3p	CGACACTGAA	GGC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGCAAGAAATG
cel-miR-74-3p	CGACTGTAGA	GCAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTAAAGCTACCAA
cel-miR-75-3p	CGACTGAAGC	CC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCAGTCGGTTGCA
cel-miR-75-5p	CGACTTTAAG	AGC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTCGTTGTTGATG
cel-miR-76-3p	CGACTCAAGG	AAG
cel-miR-77-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTCATCAGGCCAT

	CGACTGGACA	AG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGAGGCCTGGT
cel-miR-78	CGACGCACAA	TG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGGCACAATCTG
cel-miR-784-5p	CGACTCTACG	CGTAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAGTGAATTGTT
cel-miR-785	CGACTCTACA	TTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAATGCCCTGAAT
cel-miR-786-3p	CGACATTGAA	GATG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCGAATATCAGTTG
cel-miR-786-5p	CGACGTAAAT	GG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTAAGCTCGTTTTA
cel-miR-787-3p	CGACCGAAAG	G
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGGAAATGGATTA
cel-miR-788-3p	CGACCGATTC	GAATC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCCGCTTCTAACT
cel-miR-788-5p	CGACCTGCAA	TC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTCCCTGCCTGGG
cel-miR-789	CGACACAATT	TCAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGATAAAGCTAGGTT
cel-miR-79-3p	CGACAGCTTT	AC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGCTTGGCACTCGC
cel-miR-790-5p	CGACCGCGGT	GAAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTTGGCACTCCG
cel-miR-791-3p	CGACTGCCTT	CAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTTGAAATCTCTTC
cel-miR-792-3p	CGACTCTGAA	AAC
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAGGTATCTTAG
cel-miR-793	CGACTCTGTC	TTAG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAGGTAATCATC
cel-miR-794-5p	CGACAGTGAC	GTTG
	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGTGAGGTAGATTG
cel-miR-795-5p	CGACAAGCTC	ATC

cel-miR-796	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTACTA	GCGGCGGTGGAATGTAGTTGAG
cel-miR-797-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATTTCA	GCGGCGGTTTTCATTGGTTTCTG
cel-miR-797-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTTCT	GCGGCGGTATCACAGCAATCAC
cel-miR-798	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGTC	GCGGCGGTAAGCCTTACATATTG
cel-miR-799	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCACTA	GCGGCGGTGAACCCTGATAAAG
cel-miR-80-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGCT	GCGGCGGTGAGATCATTAGTTG
cel-miR-80-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTTCAG	GCGGCGGAGCTTTCGACATGATTC
cel-miR-800-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGGCAG	GCGGCGGCAAACTCGGAAATTG
cel-miR-81-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTAGC	GCGGCGGTGAGATCATCGTGAAAG
cel-miR-81-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCTCAG	GCGGCGGCGGTTTTCACCGTGATC
cel-miR-82-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACTGGC	GCGGCGGTGAGATCATCGTGAAAG
cel-miR-83-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTACTG	GCGGCGGTAGCACCATATAAATTC
cel-miR-84-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCCGAG	GCGGCGGCACAATGTTTCAAC
cel-miR-84-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTACAAT	GCGGCGGTGAGGTAGTATGTAA
cel-miR-85-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCACGA	GCGGCGGTACAAAGTATTTG
cel-miR-86-5p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGACTGT	GCGGCGGTAAGTGAATGCTTTG
cel-miR-87-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATA	GCGGCGGGTGAGCAAAGTTT

	CGACGCACAC	CAG
cel-miR-90-3p	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGGGGC	GCGGCGGTGATATGTTGTTTG

Table 3-2 KEGG pathway enrichment analysis. Detailed significant involved pathway information in three treatment groups. Column one, two and six gave the IDs, names and classes of each individual pathway. One specific treatment group was checked if that pathway was involved significantly.

Pathway ID	pathway name	oil	dis	mix	class
cel00061	FATTY ACID BIOSYNTHESIS	√	√	√	Metabolism; Lipid metabolism
cel00533	GLYCOSAMINOGLYCAN BIOSYNTHESI- KERATAN SULFATE	√	√	√	Metabolism; Glycan biosynthesis and metabolism
cel00532	GLYCOSAMINOGLYCAN BIOSYNTHESIS - CHONDROITIN SULFATE / DERMATAN SULFATE	√	√	√	Metabolism; Glycan biosynthesis and metabolism
cel02010	ABC TRANSPORTERS	√	√	√	Environmental Information Processing; Membrane transport
cel04010	MAPK SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel04012	ERBB SIGNALING PATHWAY	√	√	√	Environmental Information

					Processing; Signal transduction
cel04144	ENDOCYTOSIS	√	√	√	Cellular Processes; Transport and catabolism
cel04630	JAK-STAT SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel04150	MTOR SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel04020	CALCIUM SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel03022	BASAL TRANSCRIPTION FACTORS	√	√	√	Genetic Information Processing; Transcription
cel04310	WNT SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel00534	GLYCOSAMINOGLYCAN BIOSYNTHESIS - HEPARAN SULFATE / HEPARIN	√	√	√	Metabolism; Glycan biosynthesis and metabolism

cel04120	UBIQUITIN MEDIATED PROTEOLYSIS	√	√	√	Genetic Information Processing; Folding, sorting and degradation
cel00512	O-GLYCAN BIOSYNTHESIS	√	√	√	Metabolism; Glycan biosynthesis and metabolism
cel04340	HEDGEHOG SIGNALING PATHWAY	√	√	√	Environmental Information Processing; Signal transduction
cel04070	PHOSPHATIDYLINOSITOL SIGNALING SYSTEM	√	√	√	Environmental Information Processing; Signal transduction
cel03450	NON-HOMOLOGOUS END-JOINING	√			Genetic Information Processing; Replication and repair
cel04512	ECM-RECEPTOR INTERACTION	√			Environmental Information Processing; Signaling molecules and interaction
cel00630	GLYOXYLATE AND DICARBOXYLATE METABOLISM	√	√		Metabolism; Carbohydrate metabolism
cel00072	SYNTHESIS AND DEGRADATION		√		Metabolism; Lipid metabolism

	OF KETONE BODIES			
cel00020	CITRATE CYCLE (TCA CYCLE)	√	√	Metabolism; Carbohydrate metabolism
cel04350	TGF-BETA SIGNALING PATHWAY	√	√	Environmental Information Processing; Signal transduction
cel00562	INOSITOL PHOSPHATE METABOLISM	√	√	Metabolism; Carbohydrate metabolism
cel00523	POLYKETIDE SUGAR UNIT BIOSYNTHESIS		√	Metabolism; Metabolism of terpenoids and polyketides
cel03020	RNA POLYMERASE		√	Genetic Information Processing; Transcription
cel00640	PROPANOATE METABOLISM		√	Metabolism; Carbohydrate metabolism

Table 3-3 Significantly changed miRNAs and their targets in MAPK pathway*. Targets shown here are *let-60, lin-45, mek-2, mpk-1* and *rskn-1.*

	Fold change			MiRNA targets		
miRs	mixture	dispersant	oil	mixture	dispersant	oil
cel-miR-247-5p	46.502	214.65	91.82	let-60/lin-45	let-60/lin-45	let-60/lin-45
cel-miR-1020-3p	20.325	36.93	36.78	lin-45	lin-45	lin-45
cel-miR-43-3p	12.831			lin-45/mpk-1		
cel-miR-1828	9.549	10.51		lin-45	lin-45	
cel-miR-1822-5p	8.804	16.74		mek-2	mek-2	
cel-miR-56-5p	3.936	49.9	9	mpk-1	mpk-1	mpk-1
cel-miR-261	0.615	1.76	6.95	let-60/mpk-1	let-60/mpk-1	let-60/mpk-1
cel-miR-265	0.450			let-60		
cel-miR-357-3p	0.332		4.91	mpk-1		mpk-1
cel-lsy-6	0.304			let-60		
cel-miR-240-5p	0.268	15.12		rskn-1	rskn-1	
cel-miR-250-3p	0.193	7.71		mpk-1	mpk-1	
cel-miR-258	0.178			let-60/mpk-1		
cel-miR-61-5p	0.174			mpk-1		
cel-miR-239b-3p	0.171	5.05		mek-2	mek-2	
cel-miR-1021	0.141		0.17	mek-2		mek-2
cel-miR-788-3p	0.124		0.14	let-60/lin-45/mpk-1/rskn-1		let-60/lin-45/mpk-1/rskn-1
cel-miR-1823	0.107	7.04		lin-45	lin-45	
cel-miR-51-3p	0.047	5.82	0.01	lin-45	lin-45	lin-45
cel-miR-255-3p	0.046			mpk-1		

cel-miR-244-5p	0.016			lin-45		
cel-miR-2213-5p	0.011	3.04	0.08	lin-45	lin-45	lin-45
cel-miR-797-5p	0.004			mpk-1		
cel-miR-2212-5p	0.003		0.19	let-60		let-60
cel-miR-60-5p		19.05			lin-45	
cel-miR-2210-5p		10.68	0.41		let-60	let-60
cel-miR-1832a		6.54			mpk-1	
cel-miR-1019-3p		5.58	0.05		rskn-1	rskn-1
cel-miR-355		4.15			lin-45	
cel-miR-1829c		0.09			let-60	
cel-miR-86-5p			0.21			let-60
cel-miR-785			0.1			let-60
cel-miR-2-3p			0.003			mpk-1