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Comparative developmental toxicity of conventional oils and diluted bitumen on early life stages of the rainbow trout (*Oncorhynchus mykiss)*

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ABSTRACT

Petroleum hydrocarbons are widely used and transported, increasing the risks of spills to the environment. Although conventional oils are the most commonly produced, the production of unconventional oils (i.e. diluted bitumen or dilbit) is increasing. In this study, we compared the effects of conventional oils (Arabian Light and Lloydminster) and dilbits (Bluesky and Clearwater) on early life stages of a salmonid. To this end, aqueous fractions (WAF: water accommodated fraction) of these oils were extracted using mountain spring water. Rainbow trout *(Oncorhynchus mykiss)* larvae were exposed to 10 and 50% dilutions of these WAFs from hatching (340 DD; degree days) until yolk sac resorption (541 DD). Exposure to WAFs increased skeletal malformations (both dilbits) and hemorrhage (both conventional oils and Bluesky) and decreased head growth (Arabian Light). In addition, increases in EROD activity and DNA damage were measured for all oils and an increase in *cyp1a* gene expression was measured for Arabian Light, Bluesky and Clearwater. The PAH and C_{10}—C_{50} concentrations were positively correlated to total larval EROD activity, whereas concentrations of total hydrocarbons, VOCs, PAHs, and C_{10}—C_{50} were positively correlated to *cyp1a* expression. Total hydrocarbon, VOC, and C_{10}—C_{50} concentrations were also negatively correlated to larval growth. This study supports that petroleum hydrocarbons are toxic to early developmental stages of rainbow trout and show that their degree and spectrum of toxicity depends on their chemical composition.

1. Introduction

Bitumen is considered an unconventional oil because of its high viscosity, which implies that it needs heating or dilution in order to be transported through pipelines. The dilution of bitumen leads to different types of bitumen oils, for example, diluted bitumen (dilbit), which results from the addition of natural gas condensates (20–30% v/v), and synthetic bitumen (synbit), containing up to 50% of synthetic chemicals (Dew et al., 2015). Bitumen, like some other heavy oils, is extracted from source rocks as light or medium oils. The processes that follow, including water washing, sand removal, bacterial degradation, or evaporation sometimes lead to the loss of light organic compounds, resulting in its transformation into a heavy oil. Heavy oils contain the asphaltic fraction, which is made of resins, asphaltenes, and pre-asphaltenes (Meyer et al., 2007). The composition of bitumens and conventional crude oils also differs. Bitumens have fewer saturates, more resins, and more asphaltenes than conventional crude oils. The only fraction that is constant between the two types of oils is the aromatic fraction (Woods et al., 2008). As the production and global consumption of petroleum keeps increasing, the risk of oil spills is also rising (Ball and Truskewycz, 2013). Assessing the environmental impact of oil spills is a big challenge, because crude oil composition is highly variable, involving complex mixtures of chemicals with different environmental behavior and toxicity.

Several studies have documented the toxicity of a wide variety of bitumen and conventional crude oils on fish embryos. Exposure to crude oil and dilbits can increase mortality of early life stages in rainbow trout *(Oncorhynchus mykiss),* Japanese medaka *(Ozyzias latipes),* fathead minnow *(Pimephales promelas),* zebrafish *(Danio rerio)*, pink salmon (*Oncorhynchus gorbuscha)* and pacific herring (*Clupea pallasii)* (Carls et al., 1999; Perrichon et al., 2016; Philibert et al., 2016). It can also lead to a delay of hatching in zebrafish embryos (Perrichon et al., 2016;

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Philibert et al., 2016). Several studies have reported an induction of developmental anomalies including skeletal malformations, pericardial and yolk sac edemas in different fish species including zebrafish, fathead minnow, Japanese medaka and Australian rainbow fish (*Melanotaenia fluviatilis*) (Madison et al., 2020; B.N. 2015; McDonnell et al., 2019; Perrichon et al., 2016; Philibert et al., 2016; Pollino and Holdway, 2002). Moreover, exposure of fish embryos to conventional and unconventional oils causes a disruption in swimming behavior, as reported for zebrafish in two different studies. Zebrafish larvae exposed to mixed sweet blend and heavy oil WAFs showed a decrease in bottom-dwelling and distance moved compared to control unexposed larvae (Perrichon et al., 2016; Philibert et al., 2016). It was also showed that exposure to crude oils and oil compounds can lead to an increase in EROD activity, oxidative stress and DNA damage in rainbow trout (Brinkmann et al., 2013; Gagné et al., 2011; Le Bihanic et al., 2014b; McNeill et al., 2012). Lastly, studies on fathead minnow, zebrafish, and Japanese medaka pointed out that exposure to constituents extracted from dilbits like Access Western Blend (AWB) and/or Cold Lake Blend (CLB), affected the level of transcription of genes involved in xenobiotic metabolization (*cyp1a, gst*), oxidative stress response (gsr, sod) and cell homeostasis (*p53, hsp70*) (B.N. Madison et al., 2015; McDonnell et al., 2019). Although there are several studies about the effects of conventional crude oils and dilbits on the early life stages of various fish species, very few actually discuss the differences of toxicity spectrum between those two types of oils.

Rainbow trout is an ideal fish model for toxicity assays on early life stages (ELS). It is commercially available at all life stages throughout the year in Europe and North America, it is easy to maintain in the laboratory, its development is well-known and its ELS are sensitive to numerous pollutants, including PAHs (Le Bihanic et al., 2014b; Valotaire and Borel, 2017).

This study aimed to compare the toxicity of two conventional crude oils (Arabian Light and Lloydminster) with that of two dilbits (Bluesky and Clearwater) to early life stages of rainbow trout. Arabian Light was chosen as it is one of the most produced oils worldwide and Lloydminster was chosen because its density is similar to that of the dilbits. Bluesky and Clearwater are two oil sands mined in the oil sands region in Alberta (Canada), with a high sulfur concentration. They were chosen because their composition is similar to that of the most common dilbits circulating in Canada, the CLB and the Western Canadian Select (WCS), which were also implicated in the Kalamazoo River Spill (Crude Quality Inc, 2010; Deshpande et al., 2018; US Energy Information Administration, 2021). The toxicity of the water accommodated fractions (WAFs) of the four oils was tested on larval development, swimming behavior, EROD (ethoxyresorufin-O-deethylase) activity, DNA damage and on the expression of genes related to the AhR (aryl hydrocarbon receptor), EROD activity, cell integrity and oxidative stress. The oils were prepared following the WAF protocol at a concentration of 156 mg/L, an environmentally-realistic concentration after an oil spill (up to 530 mg/L for the DeepWater Horizon oil spill, Sammarco et al., 2013). Moreover, WAF are prepared only by mixing oil in water (in contrast to CEWAF in which a chemical dispersant is added), which corresponds to what happens in the environment after an oil spill (Adams et al., 2020; Perrichon et al., 2016). To compare the toxicity of the conventional and unconventional oils, we compared the number of endpoints significantly affected by the exposure to each oil type. The chemical composition of the aqueous fractions was also analyzed to investigate relationships between the concentrations of the oil components and their toxicity.

2. Materials and methods

2.1. Chemicals

Cedre (Brest, France) supplied the Arabian Light crude oil, while the Lloydminster crude oil and Bluesky and Clearwater dilbits were provided by Crude Quality Inc. (Edmonton, Alberta, Canada). For WAF preparation, 156 mg of each oil was added to 1 L of spring water (Laqueuille) in a 1 L glass bottle and the oils' aqueous components were extracted following the standardized protocol by Singer (Singer et al., 2000). This concentration was chosen because it represents realistic environmental concentrations after an oil spill (Perrichon et al., 2016). The solutions were left under magnetic stirring (350 rpm) for 24 h in the dark at room temperature before being poured into a separation funnel and left there for one hour in the dark. The solutions were then diluted at 10 and 50% and these final solutions were used for the rainbow trout exposure experiment.

2.2. Embryo exposure

Two experiments using the same conditions were performed. The first experiment involved the exposure of rainbow trout sac fry to the crude oil WAFs, while the second experiment involved exposure to the dilbit WAFs. INRA-PEIMA (INRA Experimental Fish Farm of the Monts d'Arrée, Sizun, France) provided 1000 rainbow trout embryos at the eyed stage for each exposure (280° days, DD, number of days x daily temperature). Each treatment was replicated three times, except for the control group, which was replicated 4 times. For each replicate, 25 embryos were laid in a 700 mL glass jar containing 500 mL of diluted WAF. The exposure started when the hatching period began (340 DD) and ended after 17 days when the yolk sac was just resorbed (541 DD). Throughout the exposures, embryos were kept in the dark in a climate chamber (Thirode, Poligny, France) at 12 °C. Air bubbling in each jar ensured proper oxygenation. Dissolved oxygen was measured daily with a fiber optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany). The hardness and pH of the WAFs were not monitored during the experiment, but the Laqueuille spring water had a hardness of 19.1 ppm of $CaCO_3$ and a pH of 7.7. For each replicate, 80% of the exposure solution was replaced by a fresh solution daily. At the end of the exposures, the larvae that were not used for biomarker analyses were euthanized with a lethal dose of ethyl 4-aminobenzoate (benzocaine, 120 mg/L, Sigma-Aldrich, St Quentin Fallavier, France).

2.3. Chemical analysis

Fresh WAFs were prepared for chemical analysis following the protocol described in Section 2.1, then split into aliquots for the following analyses. A glass bottle was filled with 800 mL of the solution (acidified to pH 2 with hydrogen sulfate) and was stored in the dark to quantify the PAH concentrations. In addition, two glass vials were filled with 40 mL of the solution and stored in the dark, to measure the concentrations of VOCs (volatile organic compounds), and C_{10}—C_{50} fractions. The CEAEQ (center d'expertise en analyze environnementale du Québec, Ministère de l'Environnement et de la Lutte contre les changements climatiques, Canada) performed the VOC measurements following the MA. 400-COV 2.0 protocol, the PAH measurements following the MA. 400-HAP 1.1 protocol and the C10-C50 hydrocarbons following the MA. 400-HYD 1.1 Rev. 3 protocol (CEAEQ, 2016b, 2016a; B.N. 2015). Details on the chemical analysis methods and quality controls are supplied in the Supplementary Information (Table S1).

2.4. Phenotypic effects

Larval survival was monitored and dead individuals were removed daily. Mortality was calculated as the number of dead individuals over the total number of embryos at the beginning of the experiment. At the end of the exposures, nine individuals (541 DD) for each replicate were sedated with carbonated water and biometrics as well as malformations were recorded using a Leica MZ75 microscope and the software ToupView 3.7. From the pictures taken, the total body and head lengths were measured as described by Weeks-Santos et al. (2019). Larvae were also observed to detect developmental defects such as spinal and cranio-facial deformities, edema, cardiac anomalies, and hemorrhages

according to Le Bihanic et al. (Le Bihanic et al., 2014b).

2.5. Swimming behaviour

At the end of the exposure, the swimming behavior of larvae (541 DD) was analyzed using a DanioVision Image Analysis system (version 12.0, Noldus). Nine larvae per replicate were individually placed in 6-well microplates containing 5 mL of the exposure water. Microplates were placed in the recording chambers, which were previously set at 12 °C. The larvae were acclimated for one hour in the dark in the climate chamber and then for 10 min in the DanioVision chamber before starting the video tracking. The video recording lasted 30 min with a dark/light/dark cycle of 10 min each and at 12 °C. The swimming performance of each larva was assessed from their mobility status (highly mobile, mobile and non-mobile) and the distance moved over each 10 min period following the protocol published by Weeks-Santos et al. (2019).

2.6. EROD activity

The in vivo EROD activity was measured on four isolated larvae per replicate using the protocol developed by Le Bihanic et al. (2013) and adapted by Gaaied et al. (2019). For each series of samples, a freshly prepared resorufin standard range (0; 0.625; 1.25; 2.5; 5; 10 nM) was added for EROD activity calculation and a positive control was run together to ensure that the test performed well. The positive control consisted of four larvae per replicate exposed for 2 h at 12 °C in the dark to a 100 nM BaP solution. For EROD activity measurements, larvae were exposed individually in a 24-well plate containing 1.2 mL of 7-ethoxyreforufin and the plate was incubated at 12 °C in the dark. One hour later, the solution was replaced again by 1.2 mL of freshly prepared 7-ethoxyresorufin. At T0 and T0+4 h, 100 µL of the medium was sampled in duplicate and transferred to two wells of a 96-well plate. Fluorescence was quantified using the FLUOstar OPTIMA reader at 560 nm and 580 nm for excitation and emission wavelengths, respectively. The activity was calculated using the fluorescence data at $T = 4$ h and was expressed in% of the EROD activity relative to the EROD activity measured in the control group.

2.7. DNA damage

The comet assay was performed on blood cells according to the protocol adapted by Le Bihanic (2014a). At the end of the exposures, 2 to 3 µL of blood from six randomly chosen larvae (541 DD) per replicate were sampled using a heparinized pipette. The blood samples were diluted in 200 µL of cryopreservation solution (250 mM sucrose, 40 mM trisodium citrate, 5% DMSO, pH 7.6) and frozen in liquid nitrogen. The protocol for the preparation of the slides and the migration of the blood cells was described by Weeks-Santos et al. (2019). The slides were observed using an epifluorescence microscope (Olympus BX51). One hundred nuclei were randomly chosen and the level of DNA damage was measured using the Comet Assay IV software. Results (Tail Intensity) were expressed as the amount of DNA in the tail of the comets. Nuclei with no apparent head and a diffuse tail were considered as being heavily degraded and were counted as “hedgehog cells”.

2.8. S9 preparation

At the end of the exposure period, total proteins were extracted from three pools of two larvae at 541 DD from each replicate. The yolk sac was firstly removed and the larvae were homogenized on ice in 250 µL of a chilled phosphate buffer (0.1 M; pH 7.5) using the MoBiTec G50 Tissue Grinder set at 3000 rpm. Nine hundred µL of the phosphate buffer were added to the tissue extract and then centrifuged at 9000 g for 25 min. The supernatant was isolated (S9 fraction) and 20 µL were diluted in 1 mL of ultra-pure water for protein analysis. Two other tubes containing 500 µL of the S9 fraction were stored at −80 °C for further analyzing lipid peroxidation and protein carbonyl content.

Total protein concentration was measured on the diluted S9 fraction following the method of Lowry et al. (1951). Bovine Serum Albumin (BSA) was used as a standard. Measurements were done using a BIO-TEK Synergy HT microplate reader and the KC4 3.3 Rev 10 software.

2.9. Protein carbonyl content and lipid peroxidation

The protein carbonyl is a measurement of protein oxidation. It was measured on freshly thawed S9 fractions following the spectrophotometric method of Augustyniak et al. (2015) and adapted to trout larvae by Weeks-Santos et al. (2019). In this case, the carbonyl content was measured using the BIO-TEK Synergy HT microplate reader and the KC4 3.3 Rev 10 software at 370 nm. The results were expressed as the percentage of protein carbonyl content relative to the control.

Lipid peroxidation was measured on freshly thawed S9 fraction using the TBARS assay developed by Buege and Aust (1978). The method was adapted to a microplate reader and trout larvae by Weeks-Santos et al. (2019). The TBARS assay is a spectrophotometric method that quantifies the level of MDA (malondialdehyde), the major lipid oxidation product. MDA was measured using the BIO-TEK Synergy HT microplate reader and the KC4 3.3 Rev 10 software at 530 nm. The results were expressed as the percentage of thiobarbituric acid reactive species (TBARS) relative to the control.

2.10. Gene expression analysis

At the end of the exposures, total RNA was extracted from a pool of 5 larvae per replicate (541 DD) using the TRIzol/chloroform extraction protocol (Life Technologies). The RNA integrity was then measured using multiple RNA Nano Chips (Bioanalyser 2011, Agilent), and only the samples having an RNA Integrity Number (RIN) over 6 were kept for gene expression analysis. The retro-transcription into cDNA was done using the iScript™ Reverse Transcription Supermix (BioRad) following the manufacturer’s instructions. Total RNA content was measured with the Thermo Fisher Scientific Nanodrop 2000. Each sample was then diluted to get 1 µg of RNA in 16 µL of solution. Four µL of the iScript Reverse Transcription Supermix (BioRad) were added to the mix. The samples were then centrifuged for 10 s before being retro-transcribed in an Eppendorf MasterCycle for 5 min at 25 °C, 20 min at 46 °C, and 1 min at 95 °C. cDNA samples were diluted 40-fold before gene expression analysis. Expression of seven target genes was investigated and specific pairs of primers were designed (Supplementary Information, Table S3). Real time PCR were performed using the iTaq Universal SYBR Green One-Step Kit (BioRad) with 10 µL of SYBR Green, 4 µL of cDNA and 6 µL of primers and RNase-free water. The resulting solutions were centrifuged for 10 s before performing qPCR in the BioRad C1000 Touch Thermal Cycler CFX95 Real-Time system and the protocol followed is indicated in the Supplementary Information (Table S4). On each plate, a standard curve was prepared using the mix of all the samples’ cDNA. Results and standard curves were analyzed using the Bio-Rad CFX Manager 3.1 software. For each plate and gene, the standard curve always had a $R^2 > 0.970$. Two different housekeeping genes were used (*ef1α* and *rpl8*) for qPCR calibration and were found to be stable over all exposure conditions. The relative expression of each gene of interest was measured following the relative quantification using the standard curve method. Each gene of interest’s relative expression was normalized according to the mean value of the expression of both housekeeping genes. The relative expression of each target gene in the different treatments was expressed as a fold-change to the level of expression of the same gene in the non-contaminated larvae.

2.11. Statistical analysis

For statistical analysis, each replicate was considered as an independent sample. All data were expressed as means ± SE (Standard

Error). Statistical analyses were performed using RStudio. The normality of data distribution and the homogeneity of variances were verified using the Shapiro-Wilks test ($p < 0.05$) and the Levene test ($p < 0.05$), respectively. In both experiments and for all treatments and endpoints, ANOVA's prerequisite conditions were met and two-way ANOVA analyzes were carried out ($p < 0.05$), followed by a Tukey post-hoc test ($p < 0.05$). Spearman's correlation was used to study the correlations between concentrations of PAHs, VOCs, C_{10}—C_{50}, or total hydrocarbons (the sum of the hydrocarbons measured) and biological effects on the larvae (Rs: Spearman correlation number, $p < 0.05$). Power and sample size analyses were performed using the pwr2 and effectsize packages in RStudio, respectively.

3. Results

3.1. Chemical composition of the WAFs

Arabian Light presents the highest hydrocarbon concentration, followed by Bluesky, Clearwater, and Lloydminster (Fig. 1 and Table 1). The four WAFs were similar regarding their global chemical composition. Indeed, the VOCs were the most represented fraction (55.6 – 68.3%), followed by C_{10}—C_{50} aliphatic hydrocarbons (26.7 – 38.7%), and PAHs (3.1 – 12.1%). However, the four WAFs were different regarding the exact composition and concentration of their VOC and PAH fractions (Supplementary Information, Figures S1 and S2 and Tables S5 and S6). For example, the Arabian Light and Bluesky WAFs were richer in naphthalene (and its derivatives) than the other two WAFs and both dilbit WAFs presented higher levels of benzene than the crude oil WAFs. The major compounds in the VOC fraction were the monocyclic aromatic hydrocarbons benzene, toluene, ethylbenzene and xylene (BTEX), while PAH fractions were dominated by naphthalene and alkylated PAHs.

3.2. Developmental effects

None of the dilutions from conventional oils or dilbits had significant effects on larval survival compared to their control groups (Tables 2, 3 and S1). However, there was a small but significant decrease (≤10%) of larval survival in the 50% WAF compared to the 10% WAF for both dilbits (Table 3). Exposure to the Arabian Light WAFs decreased biometrics of the larva (i.e., head length, head/body length ratio), with effects of the oil composition and concentration. In addition, the larvae of the 50% Arabian Light WAF group had a significantly smaller head size compared to the other treatment groups, and a lower head/body length ratio than larvae of the control group and of the 10% Arabian Light WAF group. Larvae exposed to the Arabian Light WAF did not exhibit any significant increase in global malformation rate. Exposure to 50% Lloydminster WAF led to a noticeable increase of hemorrhages compared to the control group. More severe effects were observed after exposure to the dilbit WAFs compared to those from conventional oils (Tables 2 and 3). Indeed, both WAFs, regardless of the concentration, increased developmental anomalies (29–36% vs 14% for the control), and notably, increased skeletal malformations frequency (30–41% vs 11% for the control). In addition, the Bluesky WAF induced a significant increase of hemorrhages and craniofacial malformations compared to the control group, reaching 40% at 10% of Bluesky WAF (vs 11–14% for the control group, pictures are available in the supplementary material, Figure S3). For the craniofacial malformations, a dose-dependent effect of the Bluesky WAF was also observed ($p = 0.04$, 40% versus 18.5% for 10 and 50% WAF, respectively). A rise in craniofacial deformities (33%) and hemorrhages (30%) was also observed after exposure to Clearwater WAFs but the individual variability was higher than for Bluesky, preventing identification of statistically significant differences. No effects were observed on larval survival and biometrics for either dilbit.

Table 1
VOC, C_{10}—C_{50}, PAHs and total hydrocarbon concentrations (µg/L) measured in the water accommodated fractions of the different oils by GC–MS ($n = 1$). The complete list of PAHs and hydrocarbons analyzed and their concentrations can be found in Tables S4 and S5.

Analytes	Concentration (µg/L)			
	Arabian Light	Lloydminster	Bluesky	Clearwater
VOCs	954.4	479.2	942.8	794.8
C_{10}—C_{50}	500	333	400	333
PAHs	199.8	49.1	156.3	35.7
Alkylated PAHs	82.6	18.7	62.0	13.0
Sulfur-containing PAHs	9.2	2.1	7.6	2.3
Total hydrocarbons	1654.2	861.3	1499.1	1163.5

3.3. Photomotor response

None of the oil treatments affected swimming behavior of the larvae

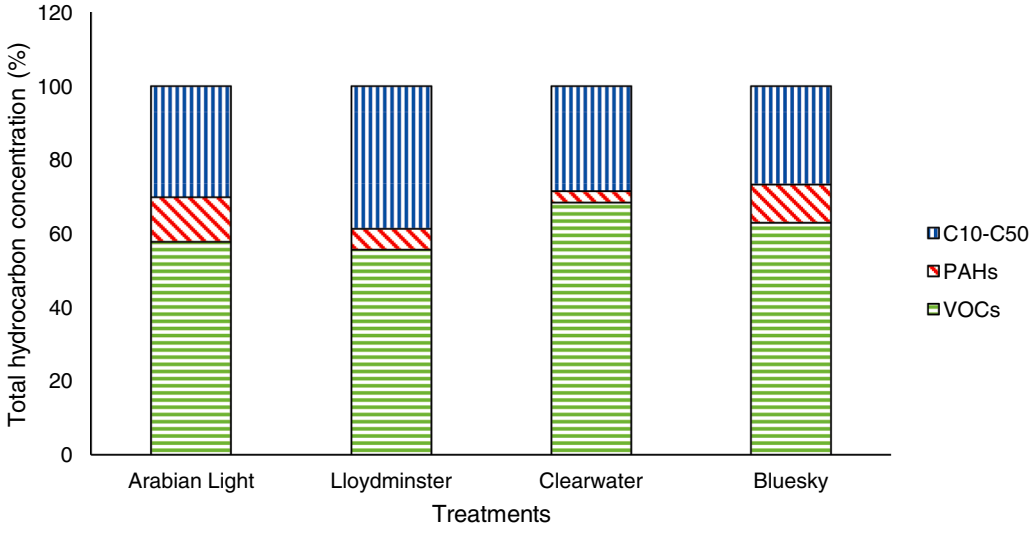

Fig. 1. Chemical composition (% of the total hydrocarbon content) of the undiluted water accommodated fraction (WAF 100%) prepared for four different oils ($n = 1$). Composition was analyzed in terms of the VOCs (volatile organic compounds), PAHs, and C_{10}—C_{50} (hydrocarbons with between 10 and 50 carbon atoms). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Developmental endpoints in rainbow trout larvae following exposure to the water accommodated fractions of Arabian Light and Lloydminster oils. Values are means ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; A, B: concentration effect; a, b: combined effect of petroleum and concentration) (Control $n = 4$, aqueous fractions $n = 3$; Two-way ANOVA, $p < 0.05$).

	Treatments Control	10% WAF Arabian Light	50% WAF Arabian Light	10% WAF Lloydminster	50% WAF Lloydminster
Larval survival (%)	94.0 ± 5.2^{a}	93.3 ± 2.3^{a}	94.7 ± 2.3^{a}	92.0 ± 4.0^{a}	94.7 ± 9.2^{a}
Body length (mm)	21.4 ± 0.2^{AB}	21.4 ± 0.3^{B}	20.7 ± 0.1^{A}	21.5 ± 0.4^{B}	21.1 ± 0.2^{A}
Head length (mm)	5.2 ± 0.1^{β,AB,b}	5.2 ± 0.1 ^{β,AB,b}	**4.8 ± 0.0^{α,A,a}**	5.11 ± 0.0^{αβ,B,b}	5.1 ± 0.1^{αβ,A,b}
Ratio head/body length (%)	24.3 ± 0.3 ^{β,AB,b}	24.2 ± 0.1 ^{β,AB,b}	**23.2 ± 0.1** ^{α,A,a}	23.8 ± 0.3 ^{αβ,B,ab}	23.9 ± 0.2 ^{αβ,A,ab}
Developmental anomalies (%)					
Total	18.6 ± 13.5^{a}	18.7 ± 5.3^{a}	22.6 ± 3.0^{a}	20.3 ± 5.1^{a}	27.4 ± 9.5^{a}
Skeletal	25.0 ± 21.0^{a}	29.6 ± 6.4^{a}	40.7 ± 12.8^{a}	37.0 ± 12.8^{a}	37.0 ± 12.8^{a}
Craniofacial	25.0 ± 21.0^{a}	29.6 ± 6.4^{a}	40.7 ± 12.8^{a}	37.0 ± 12.8^{a}	33.3 ± 11.1^{a}
Hemorrhages	22.2 ± 9.1^{α,a}	33.3 ± 0.0 ^{αβ,ab}	40.7 ± 12.8 ^{αβ,ab}	33.3 ± 11.1 ^{β,ab}	**51.9 ± 6.4^{β,b}**

Table 3
Developmental endpoints in rainbow trout larvae following the exposure to the water accommodated fractions of Bluesky and Clearwater oils. Values are means ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; A, B: concentration effect; a, b, c: combined effect of petroleum and dose) (Control $n = 4$, aqueous fractions $n = 3$; Two-way ANOVA, $p < 0.05$).

	Treatments Control	10% WAF Bluesky	50% WAF Bluesky	10% WAF Clearwater	50% WAF Clearwater
Larval survival (%)	97.0 ± 2.0^{AB,ab}	100.0 ± 0.0^{A,a}	90.7 ± 4.7^{B,ab}	96.0 ± 4.0^{A,ab}	88.0 ± 4.0^{B,b}
Body length (mm)	24.7 ± 0.4^{a}	24.8 ± 0.7^{a}	24.0 ± 0.5^{a}	24.8 ± 0.8^{a}	24.3 ± 0.7^{a}
Head length (mm)	5.4 ± 0.2^{a}	5.4 ± 0.2^{a}	5.3 ± 0.1^{a}	5.5 ± 0.2^{a}	5.3 ± 0.1^{a}
Ratio head/body length (%)	22.1 ± 0.7^{a}	21.9 ± 0.4^{a}	22.1 ± 0.2^{a}	21.9 ± 0.5^{a}	21.8 ± 0.2^{a}
Developmental anomalies (%)					
Total	14.3 ± 9.8^{α}	**29.3 ± 2.3^{β}**	**35.4 ± 4.9^{β}**	**33.8 ± 16.6^{β}**	**36.5 ± 5.4^{β}**
Skeletal	11.1 ± 9.1^{α}	**40.7 ± 6.4^{β}**	**37.0 ± 12.8^{β}**	**40.7 ± 6.4^{β}**	**29.6 ± 17.0^{β}**
Craniofacial	11.1 ± 9.1^{α,AB,a}	**40.7 ± 6.4^{β,A,b}**	**18.5 ± 12.8^{β,B,ab}**	33.3 ± 11.1^{αβ,A,ab}	22.2 ± 11.1^{αβ,B,ab}
Hemorrhages	13.9 ± 5.6^{α}	**40.7 ± 12.8^{β}**	**25.9 ± 6.4^{β}**	29.6 ± 17.0^{αβ}	14.8 ± 6.4^{αβ}

(i.e., distance swum, speed, or mobility) compared to their control groups (data not shown). Although statistical analyses indicated, the larvae exposed to the Arabian Light WAFs had a significantly lower mobility than the larvae exposed to the Lloydminster WAFs, neither of them were statistically different from their control group.

3.4. EROD activity

The EROD activity was significantly induced in rainbow trout larvae exposed to WAFs from both conventional crude oils (Arabian Light and Lloydminster) and dilbits (Bluesky and Clearwater) (Fig. 2). Moreover, the level of EROD activity was much higher for the conventional oils than for the dilbits (around 100 times the control value versus 5 times, respectively). In addition, there were concentration-dependent effects for both conventional crude oils, but not for the dilbits. This induction was significant from the lowest tested concentration of WAFs (10%).

3.5. DNA damage and oxidative stress

Comet assays were performed to measure DNA damage (Fig. 3). All the treatments significantly increased DNA damage at the two tested concentrations. An effect of the conventional oils (Arabian Light and Lloydminster) was observed compared to the control group alongside with a combined effect of the Arabian Light at 50% of WAF compared to the control group. In addition, both dilbits WAFs (Bluesky and Clearwater) induced an increase in DNA damage. No concentration-dependent effects were observed for those oils. While the number of hedgehog cells was not affected by exposure to the WAFs of conventional oils or Clearwater dilbit, the Bluesky WAF significantly induced the formation of hedgehog cells compared to the control group. In addition, composition-dependent and concentration-dependent effects were observed, with a higher number of hedgehog cells after the larvae were exposed to Bluesky at 50% of WAF compared to the control group (Fig. 4).

Exposure to the different oil treatments tested did not induce any oxidative stress (data not shown).

3.6. Gene expression analysis

The expression level of five target genes was analyzed in the whole body of 15 larvae per condition. When larvae were exposed to the dilbits, the expression of *ahr2* increased by 2-fold following Bluesky exposure compared to Clearwater exposure, but no difference with the control group was observed for any of the conditions (data not shown). The expression of *cyp1a* levels was significantly higher (61-fold) in larvae exposed to Arabian Light WAFs compared to their control group and a concentration effect was observed (Fig. 5). After exposure to 50% of Arabian Light WAF, the *cyp1a* level increased by approximately 60-fold compared to all the other groups. Similarly, Bluesky and Clearwater WAFs increased *cyp1a* levels by 4-fold and 2-fold, respectively, compared to the control group. A concentration-dependent induction was also observed. Larvae exposed to 50% of Bluesky WAF showed a higher *cyp1a* expression level compared to the other groups (2- to 4-fold). No statistically significant changes were noted for *ahr2, nfe2.1*, and *arnt*.

3.7. Relationship between chemical composition and effects of WAFs

Spearman correlations were used to investigate relationships between the chemical composition of the WAFs and the biomarkers of effects measured on the rainbow trout larvae at the end of the exposures (541 DD) (Table 4). Only larval growth, EROD activity, and *cyp1a* expression were significantly correlated to the chemical composition of the WAFs. Positive correlations were observed between the gene expression level of *cyp1a* and all compound families examined. Positive correlations were also observed between EROD activity and the PAH and C_{10}—C_{50} concentrations. In contrast, weak but significant negative correlations were observed between larval growth (based on the total length of the larvae) and the total hydrocarbon, VOC, and C_{10}—C_{50} concentrations.

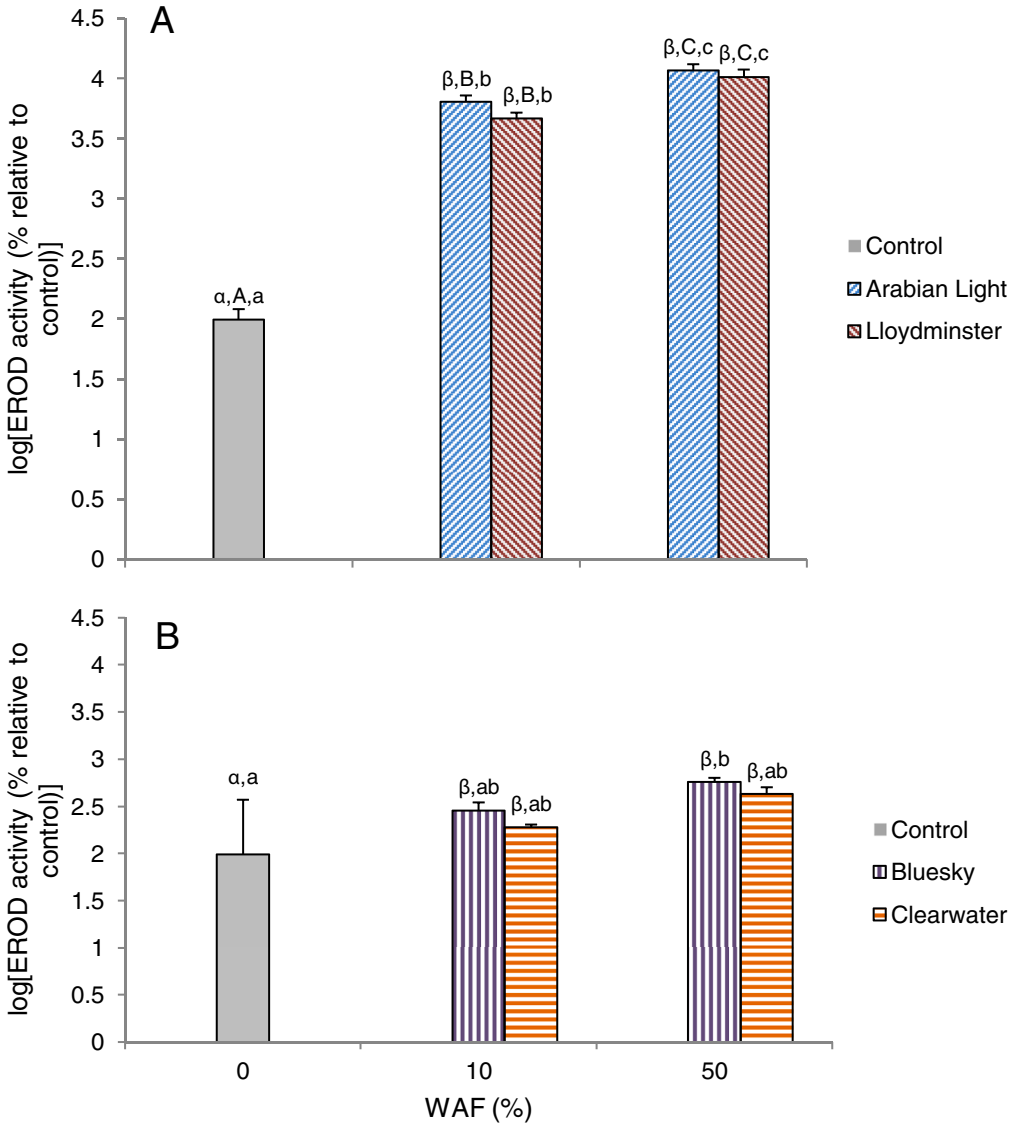

Fig. 2. In vivo EROD activity (% relative to control) measured in rainbow trout larvae following exposure to the water accommodated fractions of conventional crude oils (A) and dilbits (B). Values are means ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; A, B, C: concentration effect; a, b, c: combined effect of petroleum and concentration) (Control $n = 4$ and aqueous fractions $n = 3$; Two-Way ANOVA, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

4.1. Chemical composition of the WAFs

Analysis by GC–MS and GC-FID highlighted important differences in composition among the four WAFs studied. Previous studies have shown that hydrocarbons are the principal constituents in conventional crude oils and dilbits (Brooks et al., 1988; Wang et al., 2003). Typically, PAHs and hydrocarbon levels are higher in the WAFs extracted from conventional crude oils compared to those extracted from dilbits, in which other chemicals were added to facilitate extraction or transport (Philibert et al., 2016). Conventional crude oils are known for containing high levels of saturated hydrocarbons, which was the case with Arabian Light, whose total hydrocarbon concentration was the highest. In contrast, Lloydminster, the other conventional oil studied, contained the lowest concentration of hydrocarbons among the four oils examined here. The modification of the composition can result from the evaporation of the volatile compounds during the transport of the crude oil, in agreement with a study that reported lower levels of saturated hydrocarbons after transport of crude oils (Brooks et al., 1988). Variations in VOC concentrations were also observed among our WAFs. Indeed, dilbits contained more VOCs compared to the conventional oils. These differences in VOC content between dilbits and conventional oils were less pronounced among the different WAFs, which can be caused by a loss of the light weight molecular (LWM) compounds during the aqueous fraction extraction process (Philibert et al., 2016). In our study, the Arabian Light WAF was the one with the highest VOC content, closely followed by the two dilbit WAFs. This is consistent with Arabian Light being considered a light crude oil with greater VOC content. For dilbits, VOCs are added to the crude oil to make it more fluid and facilitate its transport (Brooks et al., 1988; Dew et al., 2015; Wang et al., 2003).

Available literature suggests that the PAH fraction is similar among various crude oils in terms of quantity (Dew et al., 2015; Woods et al., 2008). However, GC–MS and GC-FID analyses of our WAFs revealed differences in PAH composition among the oils studied. The dominant PAHs in our WAFs were naphthalene and its derivatives, which agrees with other studies (Perrichon et al., 2016; Philibert et al., 2016).

4.2. Toxicity of the WAFs and relationships with their chemical composition

Our data show that the dilbit WAFs induced more sublethal effects than those from the conventional crude oils on early life stages of rainbow trout (Table 5). The Bluesky WAF was globally the most toxic, followed by Clearwater, Arabian, and Lloydminster WAFs. Power analyses conducted on larval biometrics and malformations reported values

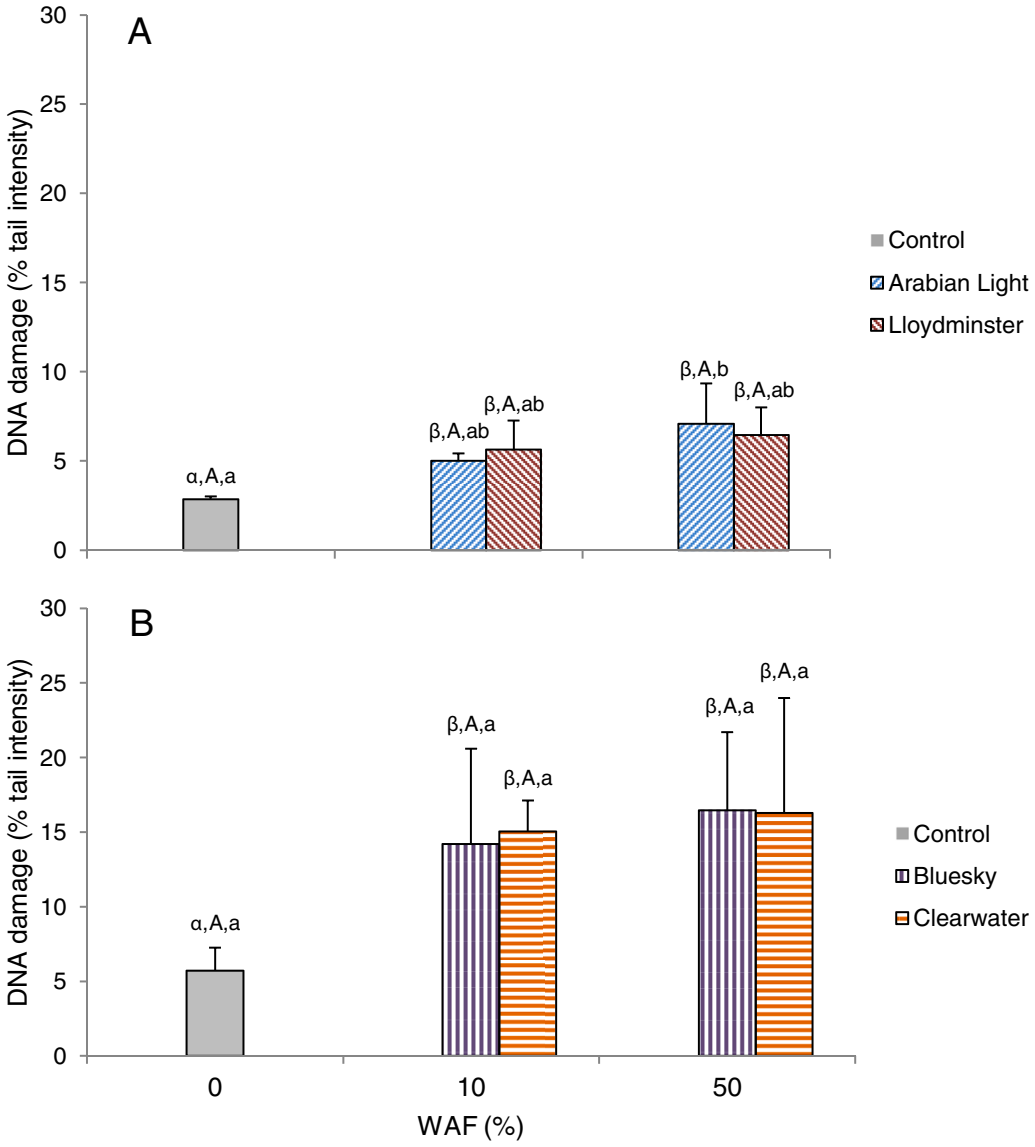

Fig. 3. DNA damage (tail intensity,%) measured in rainbow trout larvae following exposure to the water accommodated fractions of conventional crude oils (A) and dilbits (B). Values are means ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; A, B, C: concentration effect; a, b, c: combined effect of petroleum and concentration) (Control $n = 4$ and aqueous fractions $n = 3$; Two-Way ANOVA, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that were consistently lower than 50%. Overall, our analyses indicated that although the risk of false positives remained low, the low sample size and data variability were more likely to generate false negatives (i.e. concluding an absence of difference between means when indeed there was one). It is likely, therefore, that our study underestimated the effects of oil exposure on larval biometrics and malformations.

None of the WAFs studied induced a significant mortality, likely due to the low concentrations used. Several studies have reported mortality in fish embryos exposed to oil WAFs, but the concentrations used were much higher (Alderman et al., 2018; Philibert et al., 2016). Arabian Light was the only oil which led to a decrease in larval growth, as previously reported in another study where that oil was used to spike sediments (Le Bihanic et al., 2014b). In another study, dilbit WAFs (Cold Lake Summer Blend) did induce a decrease in larval growth on sockeye salmon, but the PAH concentration used by Alderman et al. (2018) was at least two to three times higher than the concentration measured in the Bluesky and Clearwater WAFs in our study. Spearman correlations revealed negative relationships between larval size and VOC and total hydrocarbon concentrations, in agreement with a study on zebrafish in which a decrease in growth has been reported after exposure to various hydrocarbons and VOCs (Perrichon et al., 2016). The lower size of larvae after WAF exposure may be linked to a reallocation of energy from growth towards detoxification processes. This hypothesis is consistent with the induction of EROD activity and in *cyp1a* transcription level, indicating an activation of these processes. Reallocation of energy could cause long-term health effects including post-exposure mortality (Perrichon et al., 2016).

The results of our study agree with others highlighting that Bluesky and Clearwater WAFs induced malformations with a rise of skeletal and craniofacial deformities alongside with a rise in hemorrhages (Alderman et al., 2018; B.N. Madison et al., 2015; McDonnell et al., 2019; Philibert et al., 2016). Furthermore, in most studies on fish embryos, crude oil WAF exposure induced edemas and skeletal malformations (Adams et al., 2014; Perrichon et al., 2016; Philibert et al., 2016). However, following Arabian Light and Lloydminster WAF exposures we did not observe inductions of edemas or skeletal deformations but a trend of increase in hemorrhages, which was only significant for the Lloydminster WAF at 50%. The relatively low developmental anomalies observed in our study could probably stem for our exposure design that did not include developing embryos. However, even subtle deformities can have long-term consequences. For example, skeletal deformities can affect blood flow and spinal cord function. Craniofacial deformities may also affect jaw development and interfere with feeding, causing growth retardation and sometimes death

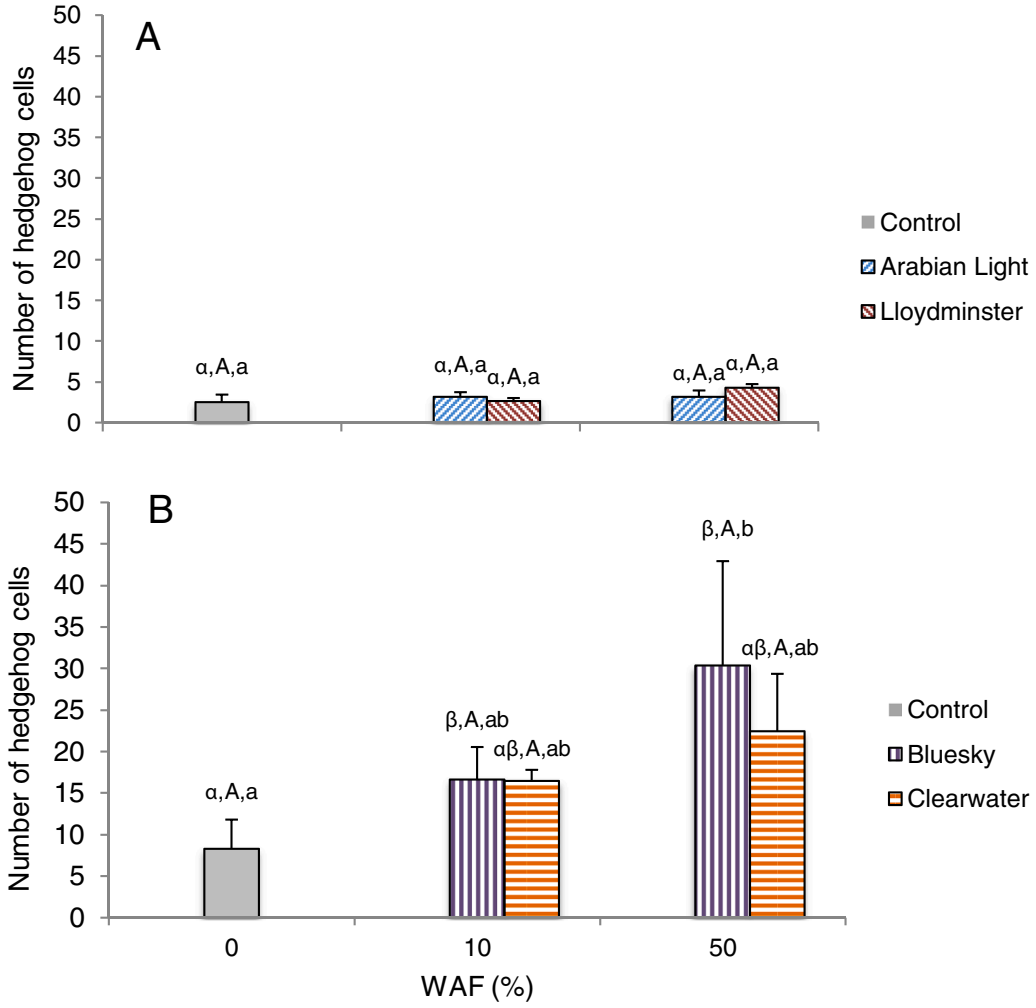

Fig. 4. Number of hedgehogs in rainbow trout larvae's blood cells following exposure to the water accommodated fraction of conventional crude oils (A) and dilbits (B). Values are means ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; A, B, C: concentration effect; a, b, c: combined effect of petroleum and concentration) (Control $n = 4$ and aqueous fractions $n = 3$; Two-Way ANOVA, $p < 0.05$).

(Boglione et al., 2013). Our statistical analysis did not reveal any correlation between malformations and the global chemical composition of the WAFs (Table 4). This suggests that other oil components including other organic chemical families (polar compounds such as resins and asphaltenes) and metals (As, Cd, Cr, Hg, Pb, Sb, Se, V, etc.) might be involved in the induction of malformations that we observed in larvae exposed to dilbit WAFs. Another hypothesis relies on the differences of composition among the different chemical groups. Exposure to 3-rings PAHs can lead to an increase of malformations in fish embryos (Adams et al., 2014; Le Bihanic et al., 2014b). Alkyl PAHs are also frequently pointed out as possible chemicals involved in developmental defects (Barjhoux et al., 2014; Mu et al., 2014; Sørensen et al., 2019). In their recent study, Sørensen et al. (2019) reported accumulation and toxicity of monaromatic petroleum hydrocarbons in Atlantic haddock and cod embryos. Interestingly, in this study, benzene was two to three times more concentrated in the WAFs from both dilbits than in the WAFs from both crude oils (Table S5).

Abnormal swimming behavior have already been documented after exposure of fish embryos to PAHs (Knecht et al., 2017; Le Bihanic et al., 2015) and crude oils (Stieglitz et al., 2016). This behavioral effect in early developmental stages can stem from neuromuscular, skeletal or cardiac malformations (Le Bihanic et al., 2015; Stieglitz et al., 2016) and from swim bladder inflation defect (Price and Mager, 2020). In the present study, swimming behavior of WAF-exposed larvae were not significantly different from control ones.

These differences with the results of other studies for malformations and swimming behavior suggest that the starting point and the duration of exposures are key factors for oil toxicity. In the studies performed by Adams et al. (2014) and Le Bihanic (2014b) on rainbow trout, the exposure started before hatching, while the exposures in our study started at the beginning of the hatching period. Since organogenesis is already complete at hatching (Valotaire and Borel, 2017), this could explain that the formation of edemas and swimming behavior were not affected in our study.

The expression of *cyp1a* was the most responsive gene following WAF exposures, except for Clearwater. Variations in the expression levels of this gene have been reported in other studies after an exposure to pollutants, including petroleum products (B.N. Madison et al., 2015; McDonnell et al., 2019). High *cyp1a* mRNA levels measured were consistent with high CYP1A activity measured via the in vivo EROD activity assay. Several studies have demonstrated that oils are potent EROD activity inducers, even at low doses (Brinkmann et al., 2013; McNeill et al., 2012). These two biomarkers showed that Arabian Light was the strongest inducer of both EROD activity and *cyp1a* expression. The pattern was the same for both markers with Arabian Light and Lloydminster (conventional oils) being stronger inducers than Bluesky and Clearwater (dilbits). Some previous works suggest that activation of the AhR signaling pathway could elicit long-term adverse effects. Indeed, a study performed on pink salmon *(Oncorhynchus gorbuscha)* embryos pointed out that a CYP1A induction in early developmental

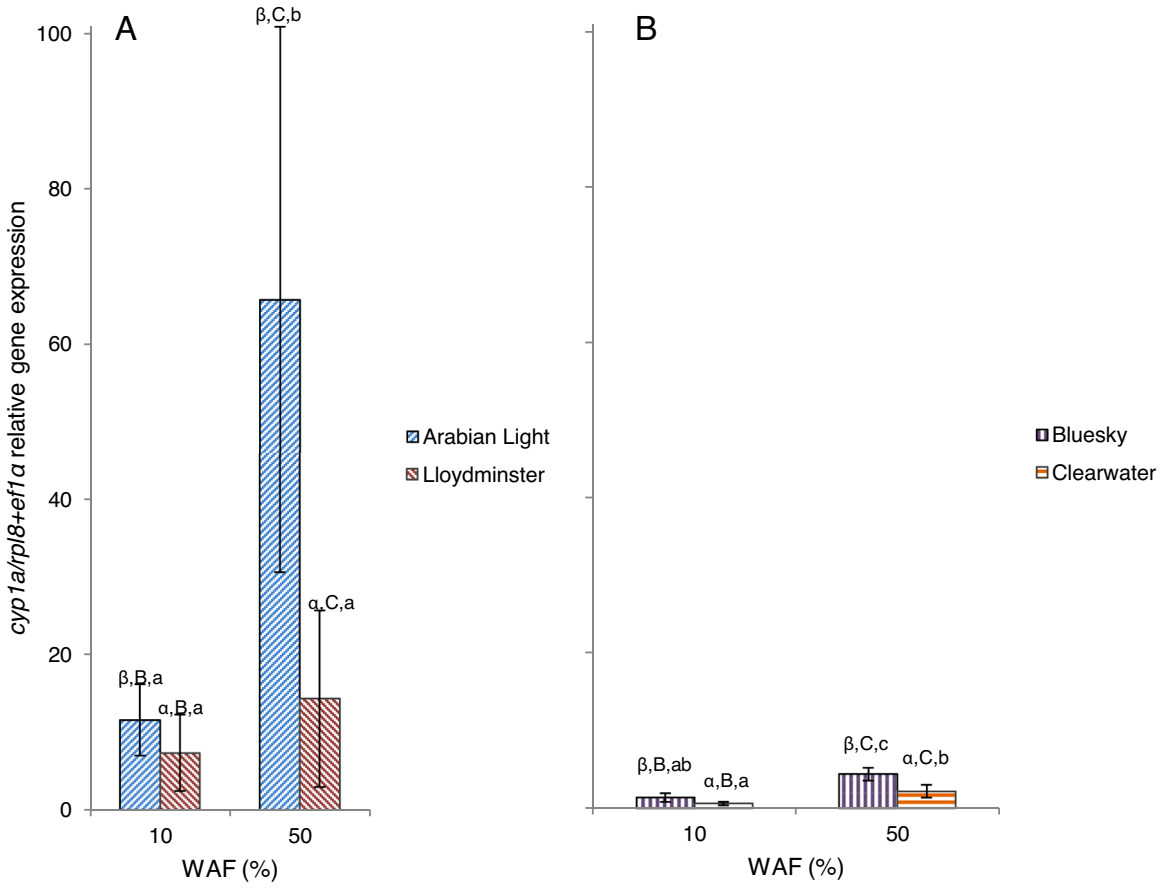

Fig. 5. Relative expression of the gene *cyp1a* following exposure of rainbow trout larvae to the water accommodated fractions of conventional oils (A) and dilbits (B). Gene expression was normalized by the mean value of the expression of two housekeeping genes (*ef1α* and *rpl8*) and by the mean value of the relative expression of the control group. Values are Mean ± SE. Different letters indicate differences between conditions (α, β: petroleum effect; B, C: concentration effect; a, b, c: combined effect of petroleum and concentration). The control is considered α,A, a ($n = 4$) and the aqueous fractions have a $n = 3$ (Two-way ANOVA, $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 4
Matrix of Spearman's correlation coefficients between the concentrations of the main classes of chemicals in the water accommodated fractions of the four oils studied (combined) and the biomarkers measured in rainbow trout larvae after exposures (541 DD). Values indicated are the Rs (Spearman's correlation coefficient) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

	Total hydrocarbon	VOCs	PAHs	C_{10}—C_{50}
Larval size	- 0.19*	**- 0.19***	- 0.18	**- 0.20***
Hemorrhages	- 0.53	- 0.53	- 0.33	- 0.45
EROD activity	0.16	0.16	**0.57*****	**0.39****
cyp1a expression	0.45**	**0.45****	**0.8*****	**0.67*****
DNA damage	0.07	0.07	0.05	0.06
Hedgehog cells	- 0.004	- 0.004	- 0.14	- 0.08

stages is correlated with long-term health effects and reduced survival (Carls et al., 2005). Spearman correlations revealed positive relationships between the total PAH concentration and EROD activity and *cyp1a* expression. It is now well-established that PAHs and other petroleum hydrocarbons can bind to the Ah receptor (AhR), causing an increase in *cyp1a* expression and thus inducing the synthesis of the CYP1A protein that is in charge of EROD activity (Denison and Nagy, 2003; McNeill et al., 2012; Nebert et al., 2004). The positive correlations that we observed between the C10-C50 hydrocarbon fraction and *cyp1a* expression and EROD activity may therefore also be related to the activation of the AhR pathway.

Our study did not reveal an induction of lipid peroxidation or protein carbonylation in WAF-exposed trout larvae, in contrast to DNA damage that was clearly detected. Petroleum hydrocarbons are known to induce DNA damage. For instance, when PAHs enter cells, they are metabolized and their metabolites are responsible for some of the DNA damage (Fallahtafti et al., 2012; Regoli et al., 2002). In our study, comet assays performed on larval blood cells indicated that the four different oil WAFs induced DNA damage, in agreement with the literature (Gagné et al., 2011; Le Bihanic et al., 2014b). The induction of DNA damage has major implications for the long-term health of individuals. Indeed, DNA damage repair is energetically costly and if the damage is not repaired, it can elicit mutations and chromosomic aberrations, leading to cell death or physiological organ dysfunction (Devaux et al., 2011). However, we could not identify any significant correlation between DNA damage and the chemical composition of the WAFs measured by GC–MS and GC-FID. Petroleum is also composed of resins and asphaltenes and these were not measured in this study (Brooks et al., 1988; Wang et al., 2003). Although a study has suggested that these components do not make an important contribution to oil toxicity (Adams et al., 2014), we cannot exclude an effect of these chemicals on DNA damage in our study. The absence of clear relationships between DNA damage and WAF chemical composition could also be due to undetected synergistic interactions among the chemicals present in the complex mixtures making up the WAFs (Bliss, 1939).

5. Conclusions

This study supports a growing body of literature indicating that when petroleum hydrocarbons end up in the aquatic environment, some of

Table 5
Summary of significant biomarker responses to each oil's WAF (10 and 50%) exposure on rainbow trout at early development stages in comparison to controls (0: no effect; -: negative effect (decrease of the biomarker); +: positive effect (increase of the biomarker)).

	Treatments							
	Arabian Light		Lloydminster		Bluesky		Clearwater	
	10%	50%	10%	50%	10%	50%	10%	50%
Survival	0	0	0	0	0	0	0	0
Larval size	0	–	0	0	0	0	0	0
Malformations	0	0	0	0	+	+	+	+
Hemorrhages	+	+	+	+	+	+	0	0
Skeletal malformations	0	0	0	0	+	+	+	+
Swimming behavior	0	0	0	0	0	0	0	0
EROD activity	+	+	+	+	0	+	+	+
DNA damage	0	+	+	+	+	+	+	+
Oxidative stress	0	0	0	0	0	0	0	0
Cyp1a gene expression	0	+	0	0	0	+	0	+

their constituents can dissolve and cause deleterious effects in fish species such as rainbow trout. The increase in *cyp1a* expression and EROD activity in WAF-exposed trout larvae clearly suggests that PAHs and probably other toxic components of the oils studied penetrated inside the cells of our fish. Various endpoints were affected by the WAF components, including a decrease in the larval growth and an increase in skeletal malformations and DNA damage. Our study also compared the toxicity of dilbits with that of conventional crude oils and highlighted the highest toxicity of dilbits. Finally, these experiments pointed out a correlation between oil components such as PAHs and EROD activity and *cyp1a* expression, alongside a negative correlation between the VOCs content and larval growth. Future studies are required with other types of oils and more detailed chemical analyses to had better understanding of the relationships between toxicity and chemical composition. Fractioning the oils and testing their toxicity with an effect-directed analysis (EDA) could also give more information on the toxicity of the different chemical fractions found in oils.

CRediT authorship contribution statement

Magali Schiano Di Lombo: Investigation, Formal analysis, Data curation, Writing – original draft. **Shannon Weeks-Santos:** Methodology, Writing – review & editing. **Christelle Clérandeau:** Methodology, Formal analysis. **Gaëlle Triffault-Bouchet:** Formal analysis, Data curation, Writing – review & editing. **S. Langlois Valérie:** Methodology, Data curation, Writing – review & editing. **Patrice Couture:** Funding acquisition, Conceptualization, Project administration, Supervision, Data curation, Writing – review & editing. **Jérôme Cachot:** Funding acquisition, Conceptualization, Project administration, Supervision, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105937.

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