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Assessment of the effectiveness of Can-Gel™ in reducing crude oil hydrocarbon release into sea water and subsequent toxicity in juvenile steelhead trout (*Oncorhynchus mykiss*).

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Executive Summary

Can-Gel™ is a new product that complexes with crude oil to form a gel-like substance that can prevent oil leaks from tankers and remove spills from water surfaces. This experiment was designed to compare the effects of light crude oil (LCO) directly with that of LCO gelled with the Can-Gel™ product (CG). LCO and CG were mixed with sea water to create water-accommodated fractions (WAFs) of hydrocarbons that partition to the water. Total petroleum hydrocarbon and selected polycyclic aromatic hydrocarbons (PAH) were quantified in both the LCO- and CG-WAF and demonstrated that CG released fewer hydrocarbons and PAHs to the water than LCO. Juvenile steelhead trout (*Oncorhynchus mykiss*) were immersed for 48 h in exposure sea water containing 0.1-10% (v/v) of either LCO- or CG-WAF. A significant increase in toxicity was observed in both 10% WAF treatments, with LCO-WAF being more toxic than CG-WAF. mRNA abundance of genes indicating estrogenicity, PAH toxicity, stress, or metal responses in the liver or caudal fin was not altered upon exposure to either WAF, in part due to high inter-individual variation in responsiveness to hydrocarbon challenge. These results indicate that the Can-Gel™ product is not more toxic than crude oil on its own, and therefore an effective means to reduce the amount of hydrocarbon introduced into marine environments.

Introduction

Oil and related hydrocarbon products are transported over water at an estimated 2-2.4 billion tonnes per year (Global Endeavours, 2014). Oil spills are a constant threat to marine life and have occurred both in the production (e.g. BP Oil in the Gulf of Mexico) and transport (e.g. ExxonValdez in Prince William Sound, Alaska) of crude oil. Can-Gel™ is a product designed by Global Endeavours Ltd. to help in the prevention and remediation of oil spills, as it causes the immobilization of oil into a more manageable gel-like substance. Can-Gel™ can be applied to oil in tankers to prevent or lessen leakage or directly to oil spills after which the gelled oil is removed from the water and recovered by reversing the gelling process. This contrasts with current remediation methods that disperse the oil quickly into the water, allowing marine animals to come in contact with potentially high concentrations of dispersed oil as well as the dispersant itself.

The purpose of the present research is to investigate the potential toxicity of Can-Gel™ to biota in the marine environments it is designed to help protect. Rainbow trout are a species commonly found in the wild and are widely used in aquatic toxicology studies. While rainbow trout (*Oncorhynchus mykiss*) are usually found and studied in a freshwater context, some populations undergo a smolting process to enable them to migrate into the ocean. Experimentally, rainbow trout can be smolted as juveniles to acclimate them to sea water. These acclimated fish are then referred to as steelhead trout. As such, the availability of these animals and their associated molecular tools can still be taken advantage of in the context of a marine environment.

In the present study, water accommodated fractions (WAFs) of light crude oil (LCO) and LCO complexed with Can-Gel™ product (CG) were prepared using equal amounts of LCO and juvenile steelhead trout were immersed in a WAF dilution series over a 48 h time period to determine toxic effects. Crude oils consist of a highly complex mixture of hydrocarbons, including polycyclic aromatic hydrocarbons (PAH) which are toxic and can even have estrogenic properties (Sievers et al, 2013). In addition to investigating toxicity, Can-Gel™ was evaluated for its effectiveness in reducing the release of hydrocarbons including PAHs to the water.

The toxicity due to LCO- or CG-WAF was evaluated in two ways. The first was with physiological endpoints including mortality, morbidity, length and weight, while the second was with molecular endpoints including known indicator targets. Quantitative real-time polymerase chain reaction (qPCR) was used to investigate the abundance of select gene transcripts. Cytochrome P450 1A (*cyp1a*) is regulated by aryl hydrocarbon receptor (*ahr*), and both are considered responsive to PAHs (Vrabie et al, 2009). We used vitellogenin envelope protein gamma (*vepg*) as a known indicator for estrogenicity (Veldhoen et al, 2013) since PAHs can elicit estrogenic effects (Mortensen & Arukwe, 2008). Oxidative stress can also be a consequence of exposure to PAHs or heavy metals in crude oil (Deer et al, 2010; US EPA, 2011), therefore catalase (*cat*) (Deer et al, 2010) and metallothionein A and B (*mta, mtb*) (Veldhoen et al, 2013) transcript levels were also investigated.

Materials and Methods

Experimental Animals

Juvenile rainbow trout were supplied by Miracle Springs Inc. Rainbow Trout Farm (certified disease free; Mission, BC, Canada) and kept in freshwater at the University of Victoria Outdoor Aquatics Facility until they were of a size to be smolted (~35 g). The smolting process consisted of adding 1 L of synthetic sea salt (Instant Ocean sea salt, Aquarium Systems Inc, Mentor, OH, USA) to the 360 L holding tanks each day for 7 d, followed by 2 L salt a day for 3 d. After 10 days of salt spiking, the fresh water inflow is gradually switched to sea water over an 8 h period. No mortalities were observed as a result of the smolting process. Animals were then kept outdoors at 10°C in recirculating sea water, and fed Skretting Biotrout (Vancouver, BC, Canada) daily until 24 h prior to exposures. Sea water parameters were 425 ppm hardness, 240 ppm alkalinity, and 29.5 ppt salinity. All animals were cared for according to the guidelines of the Canadian Council on Animal Care using an approved protocol by the Animal Care Committee at the University of Victoria.

WAF Preparation

Light Cardium Crude was provided by Global Endeavours Ltd. (Creston, BC, Canada), along with the proprietary Can-Gel™ solutions 327 and 448. The LCO was weathered to reduce loss of volatiles during the exposures by exposing it to air for 24 h, causing a 1.5 % loss in weight. The CG was created immediately before addition to the WAF. Can-Gel™ 327 (a cross-linker) was added at 1.5 % (v/v) to weathered LCO and thoroughly mixed. Following this, 1.5 % (v/v) of Can-Gel™ 448 (a gelling agent) was added and mixed until the Teflon-coated magnetic stir bar was stilled.

WAF preparation was adapted from the standardized protocol for creating WAF without chemical dispersants (Singer et al, 2000). Equivalent volumes of weathered LCO were used to prepare both WAFs. Either LCO or CG was added to exposure sea water at room temperature at a ratio of 1:9 in a 20 L PP carboy, mixed with a magnetic stir bar without a vortex for 23 h, and allowed to settle for 1 h before siphoning the WAF from the bottom of the carboy. Treatments were prepared at 10x final concentration by mixing WAF with sea water. Sea water alone was used as the negative control. All treatment stocks were added directly to the exposure tanks in a 1/10 dilution and allowed to adjust to 10°C with aerators for ~2 h prior to fish distribution. Final treatment concentrations were 0 (negative control), 0.1, 1 and 10 % (v/v) either LCO-WAF or CG-WAF in exposure sea water.

Exposure Setup

Two exposures were conducted at the Outdoor Aquatics Facility at the University of Victoria (Victoria, BC, Canada). Juvenile steelhead trout were immersed in either LCO-WAF or CG-WAF for 48 h without water change. Five animals were placed into glass tanks containing 16 L exposure sea water with 0 – 10 % (v/v) WAF in exposure sea water. Each treatment included 3 replicate tanks to give n=15 per treatment, and fish were distributed evenly among all treatments. The tanks were placed in a circulating water bath to maintain the temperature at 10.1°C throughout the exposure. Dissolved oxygen and pH were maintained at >80% and 8.5 respectively, and nitrogenous wastes (ammonium, nitrate, nitrate) were monitored daily to ensure they did not rise to toxic levels.

Daily observations were taken where mortality and morbidity were assessed according to the Aquatics Facility's standard operating procedure (SOP #OA1009: Endpoints Setting, Monitoring and Humane Termination for Experiments Using Fish). Mortalities were removed immediately from the tank. On day 2, morbid animals were scored a 2, and non-morbid animals that were demonstrating other evidence of poor health (e.g. scales sloughing off) were scored 1 for correlation analysis.

After 48 h exposure, animals were euthanized with a 0.03% tricaine methane sulfonate according to Aquatics SOP# OA2003 Euthanasia – Fish. Length and weight were measured, and a tissue section from liver and 2 mm caudal fin biopsies were collected and preserved in RNAlater (Life Technologies Corp., Carlsbad, CA, USA) for subsequent analysis of transcripts.

H_2O Chemistry

Total petroleum hydrocarbon (TPH) measurement was carried out by ALS laboratories (Vancouver, BC, Canda) using the British Columbia Ministry of Water, Land and Air Protection (BC WLAP) methods for "Extractable Petroleum Hydrocarbons in Water by GC-FID" (version 2.1, July 1999) and "Silica Gel Cleanup of Extractable Petroleum Hydrocarbons" (Draft, October 23, 2003). Before fish distribution on day 0 of the exposures, 1 L of exposure water was collected from a representative tank in each treatment (excluding 0.1 % WAF) as well as the 100% WAF and preserved with sodium bisulfate tablets. Silica gel cleanup was applied to the samples to selectively remove most naturally occurring organics, and samples were then extracted with dichloromethane prior to capillary column gas chromatography with flame ionization detection (GC-FID).

Selected PAHs were measured by ALS Laboratories according to EPA analytical methods EPA 3510 and 8270 (Separatory Funnel Liquid-Liquid Extraction; Semivolatile Organic Compounds by GC-MS). Before treatment addition, 1 L of water was collected from both 100% LCO- and CG-WAF and preserved with sodium bisulfate. The water samples were extracted with dichloromethane and then analyzed by gas chromatography with mass spectrometry detection (GC-MS). Concentrations were validated through the use of PAH surrogates.

Spectrofluorometric analysis was conducted to give a general indication of hydrocarbon content in all tanks, based on fluorescence of compounds with conjugated double bonds in UV light, which includes PAHs. On day 0, before fish distribution, 6 mL aliquots were taken from each tank, including the 100% WAF, and preserved with 6 mL of 100% ethanol. Total fluorescence was determined using a Cary Eclipse Fluorescence Spectrophotometer (Varian, Missisauga, ON, Canada), according to a protocol adapted from Greer et al (2012) "Supplemental SOP- S1: Analysis of Saline Water Samples Containing Oil by Fluorescence". Excitation wavelength and emission range were 280 nm and 295-470 nm respectively. The concentration was determined by comparing samples to a standard curve created using LCO dissolved in hexanes and serially diluted with a 1:1 sea water to 100% ethanol mixture, with the following equation: $y=0.8705x + 0.4941$.

RNA Processing and qPCR

Total RNA was isolated from RNAlater-preserved liver and 2 mm caudal fin biopsies using the TRIzol reagent as described by the manufacturer (Invitrogen Canada Inc., Burlington, ON, Canada). Liver tissue was subsectioned into approximately 3 mm cubes prior to transfer into TRIzol. Liver and caudal fin were mechanically disrupted with a Retsch MM301 Mixer Mill (Fisher Scientific Ltd, Ottawa, ON, Canada) at 20 Hz for 6 and 12 min respectively, with 180° rotation of chambers every 3 min. Liver was disrupted in 500 µL TRIzol with a 3 mm tungsten-carbide bead, in safe-lock Eppendorf 1.5 mL microcentrifuge tubes, while caudal fin biopsies were disrupted in 300 µL TRIzol, with a 1 mm bead in 0.5 ml tubes. Glycogen (20 µg) was added to each caudal fin homogenate immediately prior to precipitation of RNA. Isolated total RNA was resuspended in 80 µL (liver) and 15 µL (caudal fin) diethyl-pyrocarbonate-treated water (Ambion, cat# AM9922) and stored at -80°C. Total cDNA was synthesized with 1 µg of total RNA with the High Capacity cDNA Synthesis Kit as described by the manufacturer (Life Technologies), and diluted 20-fold prior to qPCR analysis. Three pooled "no reverse transcription" (NoRT) controls were created for each tissue to determine if any contaminating genomic DNA (gDNA) was present to contribute to the target signal.

Abundance of mRNA transcripts was determined with qPCR analysis as previously described (Veldhoen et al, 2013) using the comparative cycle threshold analysis (ΔΔCt)(Livak & Schmittgen, 2001). Each gene target was run on either CFX Connect Real-Time PCR Detection Systems (Bio-Rad Laboratories Ltd., Mississauga, ON) or Stratagene MX3005P machines (Agilent Technologies, La 45 Jolla, CA, USA). The primers used for *cyp1a* amplification were previously described (Jonsson et al, 2010) and run using the same conditions as all other primers in the present study with the exception of a 64°C annealing temperature over 32 cycles to reduce non-specific amplification. No cDNA template controls (NTC) and inter-plate standards were run on every plate to ensure reagent purity and consistency. The NoRT controls demonstrated that there was insufficient gDNA contamination to interfere with cDNA amplification signal. Caudal fin transcript abundance was normalized to the geometric mean of invariant transcripts ribosomal proteins L8 (*rpl8*) and S10 (*rps10*), and eukaryotic elongation factor 1a (*eef1a*), with the addition of beta actin (*actb*) for liver. Samples with poor mRNA quality as determined through high normalizer geometric means were removed from analysis (n=3 in caudal fin only), as well as mRNA targets with melt curves indicating the presence of more than one primary amplicon (in *mta* only).

Statistical Analysis

All data was determined to be non-parametric through evaluation with the Shapiro-Wilk test for normality and Levene's test for heteroscedasticity (SYSTAT ver. 13, Systat Software Inc., Chicago, IL, USA). Length, width, and all qPCR data were analyzed with Kruskal-Wallis followed by pairwise Mann-Whitney U tests. Mortality and mortality/morbidity were evaluated with Fisher's Exact test (www.quantitativeskills.com/sisa/index.htm), using one-sided p-values. Spearman's Rho was used to determine correlation between morbidity and other molecular and physiological endpoints. Statistical significance was designated at $p \leq 0.05$ in all cases.

Results and Discussion

H_2O Chemistry

TPH was measured to determine the overall release of hydrocarbons into the WAF from LCO or CG, and to ensure that fish were being exposed to hydrocarbon (Figure 1). The 100% LCO-WAF contained 1.88 mg/L hydrocarbon in the C10-19 fraction, and 1.08 mg/L in the C19-32 fraction. The TPH in the 100% CG-WAF was slightly increased 119.7% in the smaller C10-19 fraction and significantly decreased 35% of the LCO-WAF in the larger C19-32 fraction, with an overall decrease of 88.9% when both fractions were combined. The spectrofluorometric values corresponded well to those obtained from GC-FID analysis, at 3.29 and 2.60 mg/L in LCO- and CG-WAF respectively, showing a corresponding decrease of 79.1% hydrocarbon in the CG-WAF. The similar trend observed with both analytical methods validates the use of spectrofluorometry as a quick and cost-effective method to detect hydrocarbons in all exposure tanks. Furthermore, the measurable hydrocarbon abundance in the 10% WAF treatments follows the fraction patterns in their respective 100% WAF for each exposure (Figure 1). This indicates that the hydrocarbons in the exposure treatments were representative of each unique WAF, and demonstrates that the fish were directly exposed to measurable concentrations of hydrocarbon.

The abundance of selected PAHs were also measured in both 100% LCO- and CG-WAF (Table 1). Only three were above their respective detection limits: fluorene, naphthalene, and phenanthrene (see structures in Figure 2). These three hydrocarbons also all show a decrease in abundance in the CG-WAF compared with that from the LCO (65.4% for Σ PAHs). Along with dibenzothiophene (not measured), these are the highest measured PAHs in multiple types of crude oils and in their related WAFs (Greer et al, 2012; Kerambrun et al, 2012; Wu et al, 2012). The similarity in PAH composition between these different crude oils suggests that the observed reduction due to complexing with the Can-Gel™ product would not be limited to the light cardium crude used in the present study.

The three analytical methods used in the present study all demonstrate that the Can-Gel™ product reduces the abundance of hydrocarbon released into sea water, particularly in the larger carbon fraction. The slight increase in the smaller carbon fraction (C10-19) in the CG-WAF was not reflected in the individual PAHs which all fall within that size fraction. This could be potentially explained by release of small carbon-

based molecules present in the proprietary Can-Gel™ formulation, which are C10-12 in size. However, an overall decrease in hydrocarbon release was seen when the LCO was gelled with Can-Gel™.

Physiology

The Can-Gel™ product was successful in decreasing the toxicity due to crude oil exposure. Mortality and morbidity combined were significantly increased in the 10% WAF treatments in both exposures (Table 2). However when all concentrations were combined compared with the exposure controls, a significantly higher number of animals were affected due to exposure to LCO-WAF compared to the CG-WAF exposure ($p = 0.039$ and 0.350 respectively). Furthermore, significant mortalities and morbidities were observed when comparing the two WAF exposures to each other ($p = 0.042$).

This decreased toxicity could be due to multiple factors. First, there was an overall decrease in the TPH in the CG-WAF, indicating the juvenile steelheads were exposed to fewer toxic hydrocarbons. Furthermore, there was a proportionally larger decrease in the larger carbon fraction (C19-32), and based on the lethal concentration of 50% of animals (LC_{50}) in aquatic animals, compounds in the larger hydrocarbon fractions are more toxic than smaller ones (Battelle Business Solutions, 2007, <www.mass.gov/eea/docs/dep/cleanup/laws/tphbat.pdf>). Furthermore, in an *in vitro* screening assay with fish hepatoma cells, Cyp1a enzymatic activity was only induced with larger ≥ 3-ring PAHs (Fent & Batscher, 2000). The CG-WAF also had decreased PAHs, specifically fluorene, phenanthrene and naphthalene. These have all been shown to bioaccumulate in European sea bass muscle tissue, and were associated with inflammatory reactions and immune depletion (Bado-Nilles et al, 2011). These factors could all contribute to the decreased toxicity observed with Can-Gel™ complexed LCO.

LC_{50} values could not be calculated as no concentrations produced lethality at or above 50%. The LCO-WAF concentrations used in the present study were chosen to be sublethal to maximize the utility of the molecular endpoints, which cannot be reliably determined in necrotic tissues. At similar concentrations (10% v/v WAF prepared with equivalent techniques), Greer et al (2012) and Wu et al (2012) saw 0% normality at

swim up and significant mortality in fish embryos after 22 and 14 d, respectively. Therefore, with a much shorter time period (48 h) and developmentally older animals (juveniles), the comparative reduction in mortality rates at the highest concentration was expected.

Length and weight were not altered during the exposures (Table 2), likely due to the relatively short period of exposure. In a chronic exposure with juvenile sea bass, Kerambrun et al (2012) found that while no change in length was observed after 48 h exposure to Arabian LCO, there was a significant decrease in growth rate during the 28 d depuration phase. This indicates that an exposure period of 48 h may be too short to discern significant changes in growth. Furthermore, the relatively low n of 15 may have resulted in statistical power too low to detect differences. Further studies with higher animal numbers would address this issue.

It should be noted that preliminary results indicate that both Can-Gel™ 327 and 448 are highly toxic when not complexed, even when pH-adjusted to neutral from their highly acidic starting material (data not shown). Care should be taken for proper use and storage on tankers.

Transcript Abundance

The abundance of the selected mRNA transcripts was not significantly altered by exposure to either LCO- or CG-WAF (Figure 3). The major detoxification system in response to hydrocarbon insult is mediated by Cyp1a, via its transcriptional regulator Ahr (Vrabie et al, 2009). As a transcription factor, *ahr* mRNA abundance may be more tightly regulated and therefore less dynamic than *cyp1a* in response to hydrocarbon insult, as seen presently (<2-fold change in either direction), and with previous work involving exposure of *O. mykiss* fry to diesel (Mos et al, 2008). Exposure of rat hepatoma cells to petroleum oils also indicated that *ahr* mRNA responses to complex mixtures were orders of magnitude lower than pure PAH compounds (Vrabie et al, 2009). While not significantly different across treatments, a large range in *cyp1a* transcript abundance was observed in the liver (>300-fold) and caudal fin (>100-fold). When the *cyp1a* abundance of all animals was combined, it was significantly negatively correlated with morbidity in both liver and caudal fin (Figure 4; Spearman's rho = -0.363

and -0.400, respectively; $p < 0.01$). This large range could indicate significant inter-individual variability in ability to cope with the WAF exposure, as morbid animals tended to have very low amounts of *cyp1a* transcript. This decrease in *cyp1a* mRNA abundance corresponds with the effect of dibenzothiophene, a major component of crude oils, on juvenile *O. mykiss* (Wozny et al, 2010). It should also be noted that mortalities (presumably the most sensitive animals) were not included in the present analysis, and therefore were not able to contribute to statistical analysis. Increasing the number of animals could help counter the large range in responsiveness. In the future, because of the close relationship between liver and caudal fin *cyp1a* abundance with respect to morbidity, multiple caudal fin biopsies taken from live animals over time could be a non-lethal method to identify animals which are in poor health leading to mortality due to exposure.

The remaining mRNA targets were all indicators of specific biological responses. There was no change in *vepg* abundance, indicating no estrogenic activity in response to either WAF. The lack of abundance change in *cat* mRNA indicates that oxidative stress was not significantly present in the liver and caudal fin. Also, given that petroleum products often have heavy metals as additives, the non-responsiveness of *mta* and *mtb* indicate that they were not present at levels capable of induction in the WAF generated from LCO or CG.

Conclusions

In the present study we compared the effects of LCO alone with that of LCO complexed with Can-Gel™ product. We were able to determine that Can-Gel™ directly reduces the amount of hydrocarbon released into sea water, particularly the C19-C32 fraction and PAHs. Can-Gel™ also indirectly can reduce hydrocarbon pollution as the gelling of the LCO allows for its removal from the sea water completely, as opposed to more traditional oil spill remediation methods that increase the dispersion of the oil into the sea water.

We were able to determine that Can-Gel™ solidified LCO decreases the associated toxicity of LCO alone. While there was a high degree of inter-individual variation in the *cyp1a* response in liver and caudal fin, it was associated with morbidity,

and therefore non-lethal sampling of the caudal fin could be used as an indicator of hydrocarbon-induced toxicity over time. The present study supports the use of Can-Gel™ to prevent and help clean up crude oil spills in a salt water context. The reduction in the amount of hydrocarbon in sea water will be greatly beneficial to the preservation of marine life, especially in the context of increasing exploitation and transportation of crude oils.

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Figures

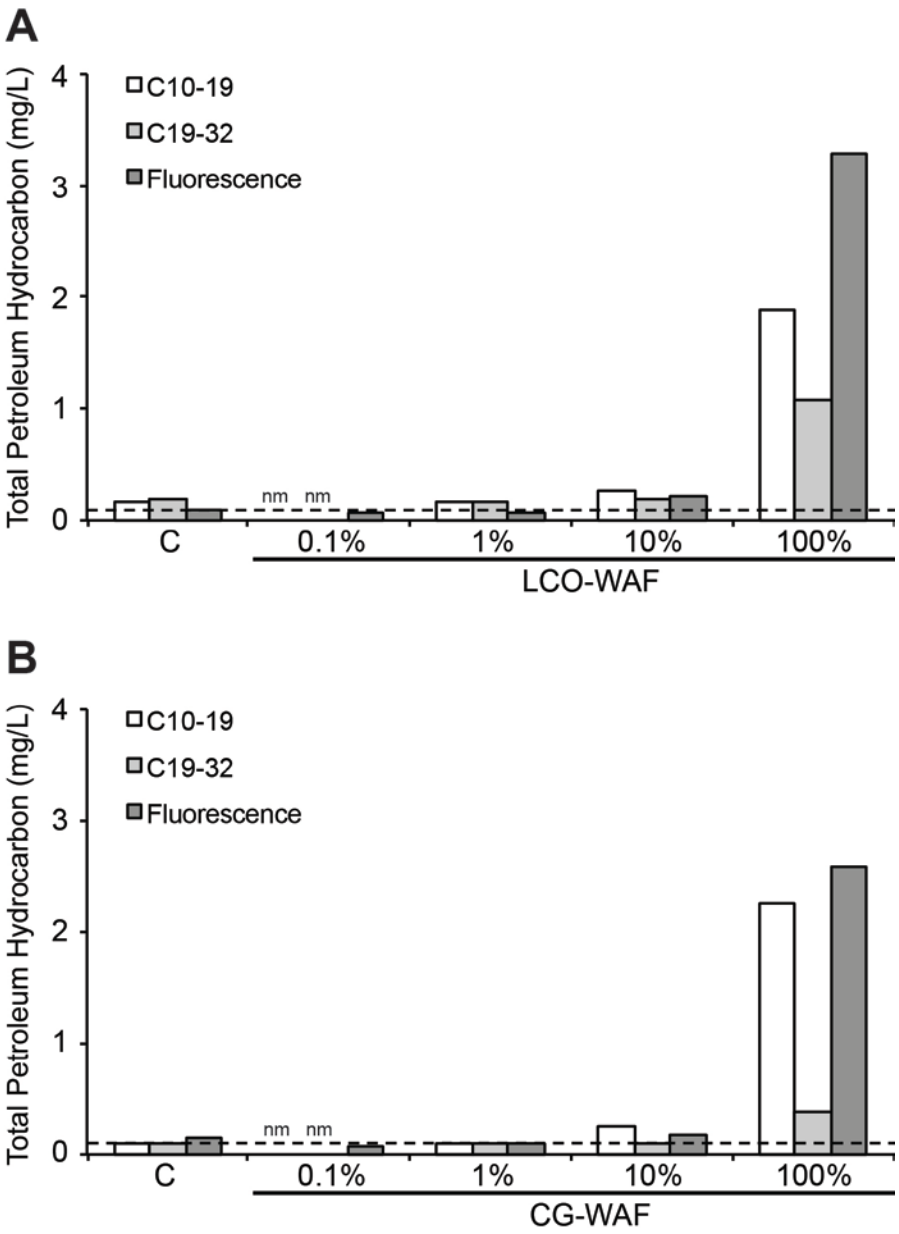

Figure 1 – Total petroleum hydrocarbons measured in exposure water and 100% water accommodated fraction (WAF) prepared with light crude oil neat (LCO; **A**) or complexed with Can-Gel™ (CG; **B**). Carbon fractions C10-19 and C19-32 were determined by GC-FID, with a minimum detection limit of 0.1 mg/L (dashed line), unless not measured (nm). Hydrocarbon content in each exposure tank and the 100% WAF was determined by spectrofluorometry using a prepared standard curve.

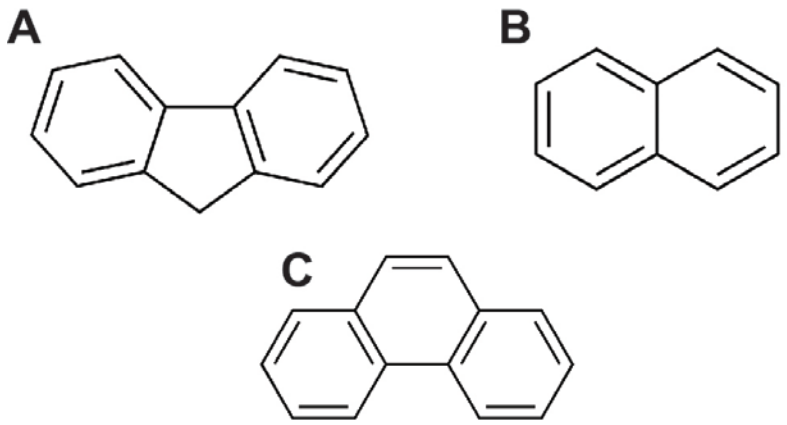

Figure 2 – Molecular structure of fluorene (**A**), naphthalene (**B**) and phenanthrene (**C**). Images from Sigma Aldrich (www.sigmaaldrich.com).

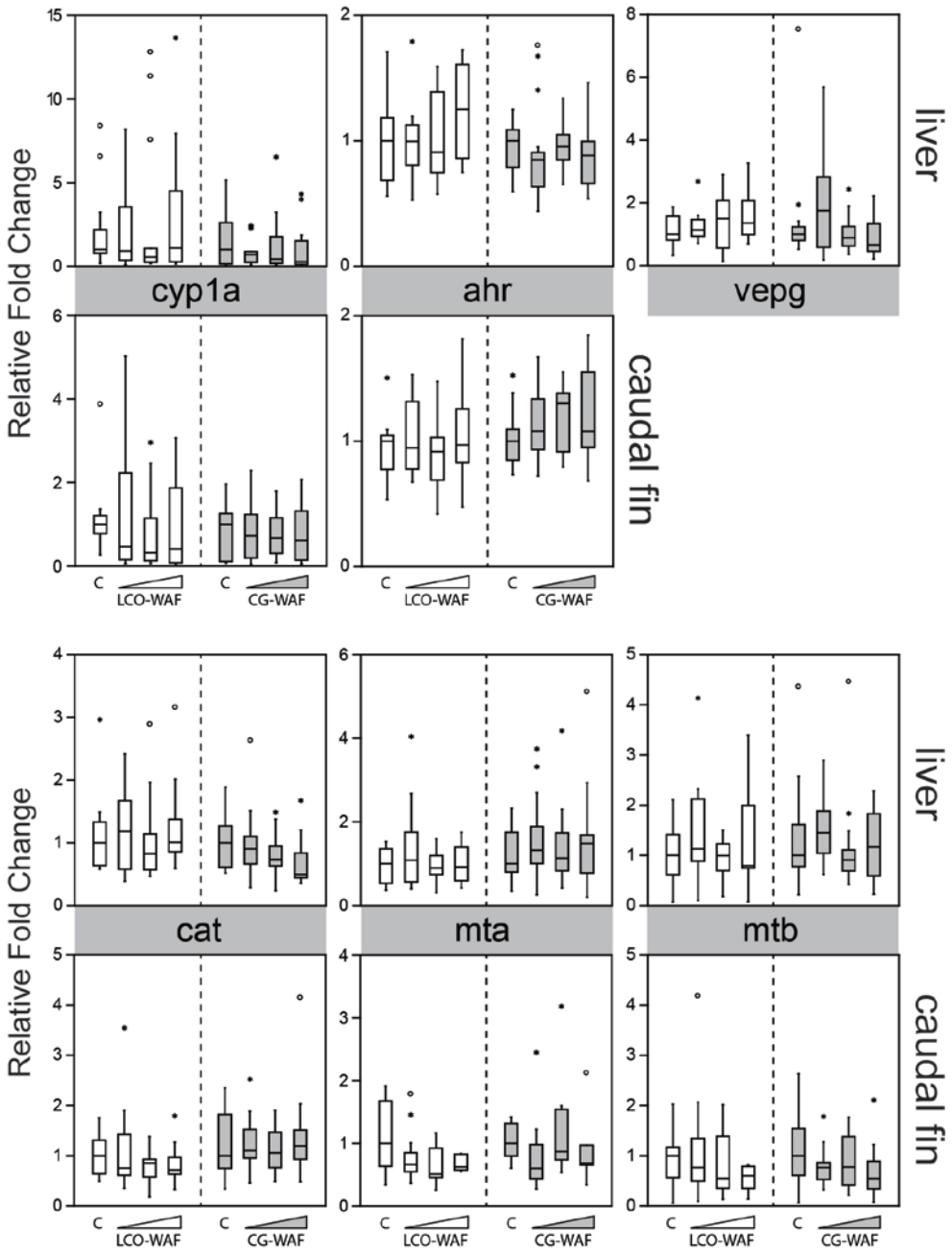

Figure 3 – Relative mRNA abundance of target genes in liver and caudal fin of juvenile *O. mykiss* exposed to LCO- (white) or CG-WAF (grey) for 48 h. Increasing treatment concentrations (0.1, 1, 10% v/v WAF in exposure sea water) represented by a bevel. The central bar represents the median, the box encloses 50% of data points, and the remaining 50% are enclosed by the whiskers, with the exclusion of outliers (°) and extreme values (*). C=control, cyp1a=cytochrome P450 1A, ahr=aryl hydrocarbon receptor, vepg=vitellogenin envelope protein gamma, cat=catalase, mta=metallothionein A, mtb=metallothionein B.

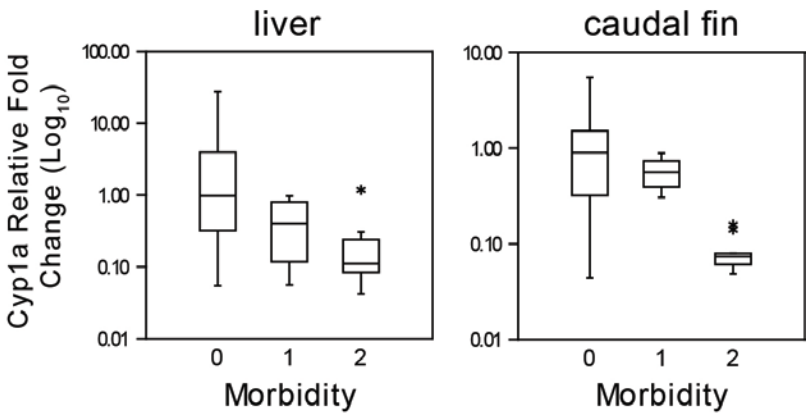

Figure 4 – Morbidity significantly correlates with decreased cyp1a mRNA abundance in the liver and caudal fin. See Figure 3 for boxplot details.

Tables

Table 1 – Polycyclic aromatic hydrocarbon (PAH) content in 100% LCO- or CG-WAF. Analyzed by GC-MS, with limit of detection indicated for each compound.

Compound	100% LCO-WAF (µg/L)	100% CG-WAF (µg/L)
Acenaphthene	<0.20	<0.20
Acenaphthylene	<0.050	<0.050
Acridine	<0.050	<0.050
Anthracene	<0.060	<0.060
Benz(a)anthracene	<0.050	<0.050
Benzo(a)pyrene	<0.010	<0.010
Benzo(b)fluoranthene	<0.050	<0.050
Benzo(g,h,i)perylene	<0.050	<0.050
Benzo(k)fluoranthene	<0.050	<0.050
Chrysene	<0.050	<0.050
Dibenz(a,h)anthracene	<0.050	<0.050
Fluoranthene	<0.050	<0.050
Fluorene	1.220	0.793
Indeno(1,2,3-c,d)pyrene	<0.050	<0.050
Naphthalene	48.7	31.9
Phenanthrene	0.727	0.451
Pyrene	<0.050	<0.050
Quinoline	<0.20	<0.20
Σ PAHs	50.647	33.144

Table 2 - Mortality, morbidity, length and weight of juvenile *O. mykiss* exposed to 0.1-10% (v/v) LCO- or CG-WAF for 48 h. Significance from exposure controls at $p \leq 0.05$ (*), and n=15 per treatment.

Exposure	Treatment	Mortality[a]	Mortality/ Morbidity[a,b]	Length (cm)[c]	Weight (g)[c]
LCO-WAF	Ctrl	1 (6.7)	1 (6.7)	16.4 ± 0.2	55.4 ± 1.9
	0.1% WAF	3 (20)	5 (33.3)	16.0 ± 0.2	53.8 ± 2.3
	1% WAF	1 (6.7)	2 (13.3)	16.2 ± 0.2	52.7 ± 2.1
	10% WAF	5 (33.3)	8 (53.3)*	16.1 ± 0.2	51.0 ± 2.7
CG-WAF	Ctrl	1 (6.7)	1 (6.7)	16.0 ± 0.2	49.4 ± 2.1
	0.1% WAF	0	1 (6.7)	16.3 ± 0.1	52.4 ± 1.2
	1% WAF	0	0	15.7 ± 0.2	46.1 ± 1.6
	10% WAF	3 (20)	6 (40)*	16.3 ± 0.2	52.3 ± 2.3

a - Frequency (percentage)

b - Includes dead and morbid animals

c - Mean ± SEM