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Metabolic responses produced by crude versus dispersed oil in Chinook salmon pre-smolts via NMR-based metabolomics

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ABSTRACT

Crude oil spills from tankers remain a serious threat along coastal California. Resource managers require information on the acute toxicity of treated and untreated oil, and their sublethal effects on wildlife. This investigation compared the toxic actions of the water-accommodated fraction (WAF) and the chemically-enhanced WAF (CEWAF; Corexit 9500) of Prudhoe Bay crude oil in pre-smolt Chinook salmon (*Oncorhynchus tshawytscha*) via nuclear magnetic resonance (NMR)-based metabolomics. Metabolite profiles from muscle samples, after 96 h exposures, were measured using 1D ^{1}H NMR and compared via principal component analysis. It was determined that both WAF and CEWAF produced similar profiles in which amino acids, lactate and ATP comprised the highest intensity signals. Overall, metabolic substrates and growth measurements did not show residual effects of short-term exposure on long-term development. In conclusion, the 96 h LC_{50}s indicate dispersant application significantly decreased hydrocarbon potency and identified metabolites may be bio-indicators of hydrocarbon stress from hydrocarbon exposure.

1. Introduction

Chinook (or king) salmon (*Oncorhynchus tshawytscha*) is an anadromous species found within the Sacramento-San Joaquin River Delta system (Eschmeyer and Herald, 1983). Within the Delta's freshwater streams, offspring undergo morphological, physiological and behavioral transformations during their seaward migration, preparing them for a dramatic change in habitat (Cech and Moyle, 1988). During key periods, particularly when pre-smolts (the parr-smolt stage) are migrating from the San Pablo and Suisun Bay estuaries (located within the Delta) towards San Francisco Bay, their populations are susceptible to accidental oil spills. Resource agencies responsible for spill response require information on the potential risk of treating such a spill with chemical dispersants. Therefore, this investigation compares the potential impacts of physically – versus chemically – dispersed petroleum on migrating salmon to model a spill within the Bay area.

Chemical dispersants are perceived to be efficient tools to treat and disperse many oil spills. Their complex mixture of surfactants and solvents aid their ability to lower the water and oil interfacial tension, facilitating phase mixing, micelle formation and surface dispersion (Singer et al., 1995). It is generally thought that the dissolved fraction, consisting of relatively low molecular weight hydrocarbons, particularly monocyclic aromatic hydrocarbons (MAHs) and small polycyclic aromatic hydrocarbons (PAHs), represents the main toxic fraction, as they are the most bioavailable to aquatic organisms. It is also assumed that highly lipophilic compounds will be suspended as droplets or sorb to particulate and/or organic matter, making them less bioavailable (Mielbrecht et al., 2005). In addition, chemical dispersion has been shown to introduce five times more total petroleum hydrocarbons (TPH) into the water column when compared to natural dispersion (Cohen et al., 2001). Mielbrecht et al. (2005) suggest that hydrocarbons entrapped within micelles are potentially less bioavailable than those sorbed to particulates, as toxicity is generally reduced when a larger hydrophilic complex (i.e. micelle) is formed by surfactants, potentially reducing hydrocarbon diffusion across membrane surfaces, such as gills.

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The direct effects of a toxic stressor such as petroleum usually involve alterations in metabolic functions, cellular components, or impaired vital functions (Adams, 1990). Sublethal stress primarily occurs at the molecular and biochemical levels, altering enzymes and cell membranes to induce structural and functional responses, which can impair processes such as metabolism and osmoregulation, ultimately affecting an organism's survival, growth and reproduction (Adams, 1990). Petroleum hydrocarbons have been found to disrupt metabolism in fish, for example increasing respiration due to gill damage (Pollino and Holdway, 2002).

The Federal Endangered Species Act currently classifies all salmon species as "threatened"; therefore it is important to characterize the toxic actions of oiled freshwater and seawater on the early life stages of salmon if a spill was to occur during their seaward migration.

Lin et al. (2009) studied the impacts of crude versus dispersed oil on salmon post-smoltification and found that crude oil was 20 times more potent than dispersed oil based on total hydrocarbon concentrations. Currently, there is little information on the impacts on salmon prior to smoltification. Therefore, the objective of this study was to compare the metabolic effects of the water-accommodated fraction (WAF; via physical dispersion) versus the chemically-enhanced water-accommodated fraction (CEWAF; via Corexit 9500) of Prudhoe Bay crude oil (PBCO) on the metabolic health and survival of Chinook salmon pre-smolts. Based on previous results (Lin et al., 2009), the hypothesis tested was whether the application of Corexit 9500 would reduce the toxic impacts of crude oil in pre-smolt salmon. Information from this study may provide useful information to response personnel for decision-making should a spill occur in northern San Francisco Bay.

2. Materials and methods

2.1. Organisms

Chinook salmon pre-smolts (*O. tshawytscha*, average FL=12 cm) were obtained *gratis* from the California Department of Fish & Game (CDFG), Nimbus Hatchery (Rancho Cordova, CA, USA) and housed at the Marine Pollution Studies Laboratory (Granite Canyon, CA, USA) in 1.2 m (dia.) tanks (ca. 1000 L of freshwater) under a partially-closed freshwater re-circulating system and quarantined until use. They were fed commercial salmon pellets *ad libitum* twice daily, antibiotic-treated salmon pellets dosed with formalin (up to ca. 2 weeks prior to exposure experiments) to limit bacterial infection and treated once with a 5% formalin solution (ca. 4 weeks prior to exposure experiments) to eradicate a ciliated protozoan infection.

2.2. WAF and CEWAF preparation

Full details of WAF and CEWAF preparations are presented in Lin et al. (2009). Testing was conducted using PBCO obtained from Resource Technology Corporation (Laramie, WY, USA), and the dispersant Corexit 9500 was obtained *gratis* from Nalco/Exxon Energy Chemicals, L.P. (Sugar Land, TX, USA). Briefly, WAFs of unweathered PBCO were prepared by layering a known mass of PBCO onto a standard volume (22 L) of laboratory well water in a 23 L polycarbonate carboy (Singer et al., 2000). Using a standardized low-energy mixing method, with a mixing rate of 110 ± 10 rpm provided by magnetic stirrers, was sufficient to provide circulation throughout the bottle, for 24 h, without creating any discernable vortex (Singer et al., 1998). Mixing energies used to prepare CEWAFs were increased to create a vortex 20–25% of water depth, providing sufficient mixing energy for dispersion. Dispersion was carried out at a nominal oil-dispersant ratio of 10:1 where known volumes of oil and dispersant were delivered in sequence into the center of the vortex using beakers and micropipettes (Singer et al., 1998). Exact masses were calculated by difference. The total 24 h preparation time, as used for WAFs, consisted of 18 h of mixing followed by 6 h of settling, which allowed the largest oil droplets to resurface (Singer et al., 2000).

2.3. Hydrocarbon analysis

Full details of the hydrocarbon analysis are presented in Lin et al. (2009). Briefly, exposure concentrations of WAF and CEWAF were characterized for total petroleum hydrocarbon (TPH: C_{10}–C_{36}) using a Hewlett-Packard 6890 gas chromatograph fitted with a flame ionization detector (GC/FID) and a Hewlett-Packard 6890-5973 gas chromatograph with a mass spectrometer (GC/MS) containing a 7695 purge and trap concentrator and a Teledyne Tekman autosampler, following modified methods described in EPA methods 8015 and 8260 (US EPA SW-846). WAF and CEWAF samples were liquid–liquid extracted ($3\times$) with dichloromethane (DCM), combined and brought to a standard volume. Extracts were not concentrated in order to retain any volatile compounds. Test samples were quantitated against PBCO standards (Payne, 1994), to better represent the number and relative proportions of DCM-soluble compounds within the oil. A stock solution was prepared by adding a measured mass of oil directly into a sealed, septum-capped vial containing DCM (4.5 mL); it was then serially diluted volumetrically with DCM directly into septum-capped vials via gas-tight syringes. Concentrations, based on the total response of the samples (with background response correction and minus the dispersant components), were designated as the total hydrocarbon content (THC=BTEX C_6–C_9 compounds+TPH C_{10}–C_{36}); where BTEX are the volatiles benzene, toluene, ethylbenzene and xylenes and TPH is the total petroleum hydrocarbon content—specifically C_{10}–C_{36} compounds. This technique did not directly quantify individual hydrocarbons and thus was not biased by quantifying specific target analytes (Girling et al., 1994).

Individual PAHs were extracted and quantified following modified methods described in EPA methods 3500 and 3510 (US EPA SW-846). Briefly, 1 L samples (and sample bottles) were subjected to liquid–liquid extraction with methylene chloride ($3\times$) after addition of deuterated surrogates (naphthalene-d_8, biphenyl-d_{10}, acenaphthene-d_{10}, phenanthrene-d_{10}, pyrene-d_{10}, benz[a]anthracene-d_{12}, perylene-d_{12} and benzo[ghi]perylene-d_{12}; for target correction), dried with anhydrous sodium sulfate and brought to a final volume of 1.0 mL in isooctane. Internal standards (1-methylnaphthalene-d_{10}, acenaphthylene-d_8, fluorene-d_{10}, dibenzothiophene-d_8, chrysene-d_{12} and benzo[a]pyrene-d_{12}; at100 ng/mL) were added by syringe, to the extract before analysis by GC/MS, using selected ion monitoring. The trace level substituted PAH analysis method employed was developed and validated by the Water Pollution Control Laboratory (WPCL) and is based on modifications of EPA method 8270 (US EPA SW-846).

2.4. Oil exposures

Exposure protocols followed methods developed for marine early life stages by Singer et al. (1998) and adapted for salmon by Lin et al. (2009). Following WAF or CEWAF preparation, ca. 6 L of test solution was directly transferred from three replicate 20 L carboys into three replicate 18 L exposure chambers. Test solutions comprised of composite solutions from the replicate carboys to minimize between replicate variability in hydrocarbon concentrations. Range-finding experiments were conducted to determine the range of concentrations of WAF or CEWAF, which bracketed the LC_{50}s.

Tests involved six treatments: five WAF (oil loadings of 0.5, 1.0, 2.0, 4.0 and 8.0 g/L) or CEWAF (oil loadings of 0.0625, 0.125, 0.25, 0.5 and 1.0 g/L) and a freshwater control, three replicates per treatment and eight salmon per replicate. Exposures were conducted in sealed 18 L polycarbonate flow-through exposure chambers. After each 96 h test, skeletal muscle tissue was dissected from two surviving fish, from each replicate tank, and was flash frozen in liquid N2 for metabolomic analysis. Remaining survivors (survival shown in supporting information Table S1) were transferred to culture tanks for long-term growth studies. At grow-out phase initiation, fish were acclimated to full seawater salinity ($\sim$33‰) by daily 5‰ incremental increases and fed commercial pellets *ad libitum* twice daily. After 3 month, growth of surviving fish was quantified and selected fish were sacrificed for metabolomic analysis. All experiments were conducted following institutional guidelines and approved by the UC Davis Animal Care and Use Committee.

2.5. Metabolite extraction

Metabolite extraction methods followed Lin et al. (2007), with modifications. Frozen tissue samples were ground in a liquid N2-cooled mortar and lyophilized overnight. The homogenized dry powder was weighed and extracted using 20 mL/g (dry mass) of ice cold methanol–water (2:1) and vortexed for 15 s ($3\times$ each), being placed on ice in between. Following centrifugation (12,000g, 10 min., 4 °C), the supernatant (0.60 mL) was lyophilized overnight. Each extraction was re-suspended in a phosphate buffer (pH 7.4) prepared in D_2O containing 1 mM sodium 3-trimethylsilyl proprionate–2, 2, 3, 3 – d_4 (TMSP) as an internal chemical shift standard, centrifuged and analyzed by NMR.

2.6. 6 ^{1}H NMR metabolomics and spectral pre-processing

Metabolomic analyzes were conducted as described previously (Viant, 2003), with some modifications. One-dimensional (1D) ^{1}H NMR spectra of muscle tissue extracts were measured at 500.11 MHz, with pre-saturation of the residual water resonance, using an Avance DRX-500 spectrometer (Bruker, Fremont, CA). Spectra were phased, baseline corrected, and calibrated (TMSP, 0.0 ppm) using XWIN-NMR (version 3.1; Bruker) and Topspin (version 1.3; Bruker) software; upgrade between the two Bruker software packages occurred mid-way through analysis and NMR parameters remained consistent for each sample. Peaks within the ^{1}H NMR spectra were assigned by comparison to tabulated chemical shifts and peak multiplicities (Fan, 1996) and by use of Chenomx NMR software (Professional Edition, version 4.6, Chenomx, Inc.).

Spectra were converted to a format for multivariate analysis using custom-written *ProMetab* software (Viant, 2003) in MATLAB (version 7, The MathWorks, Natick, MA, USA). Each spectrum was segmented into chemical shift bins between 0.2 and 10.0 ppm, corresponding to a bin width of 0.005 ppm (2.5 Hz). The spectral area within each bin was integrated and bins representing the residual water peak (from 4.60 to 5.20 ppm) were removed. The total spectral area (TSA) of the remaining bins was normalized to 1 in order to facilitate comparison between the spectra. Binned data were log transformed, forming a matrix (i.e. one sample per row and one bin per column) and the columns were mean-centered before multivariate analysis.

2.7. Statistical analysis of the NMR data

Principal component analysis (PCA) of pre-processed NMR data was conducted using the PLS_Toolbox (version 3.5; Eigenvector Research, Manson, WA, USA) within MATLAB. Potential outliers and similarities between metabolic fingerprints were examined for WAF and CEWAF datasets in an unsupervised manner. This pattern recognition technique employs an algorithm to calculate the highest amount of correlated variation along the first principal component (PC1), with subsequent principal components (PC2, PC3 etc.) containing smaller amounts of variance. One-way ANOVA was conducted on the identified metabolites contributing to the variance along the PC axes, at which the metabolite peak areas were tested for differences between doses (SAS Institute Inc., version 9.2; Cary, NC, USA). The analysis was accomplished using the "Proc Mixed" procedure and a student residual >2 determined outliers (additional information shown in supporting information Table S3).

3. Results

3.1. Acute toxicity and mortality Measurements (96 h)

Exposures to the WAF or CEWAF of PBCO affected the percent survival of pre-smolts, as shown in supporting information Table S1. THC (C_6–C_{36}) was quantified for Time 0 (T_0) samples and used to calculate LC_{50}s for each of the three WAF and CEWAF tests. The 96 h LC_{50}s for the WAF tests were 6.5, 9.9 and 6.2 mg/L THC (mean $LC_{50}=7.6$ mg/L $\pm$ 1.19 THC), while the 96 h LC_{50}s for the CEWAF tests were 60.5, 48.2 and 37.0 mg/L THC (mean $LC_{50}=48.6$ mg/L $\pm$ 6.79 THC). Thus, pre-smolts were more sensitive to WAF than its corresponding CEWAF (from Corexit 9500).

Individual PAHs were measured in the first and third WAF and CEWAF experiments. T_0 results (Tables 1 and 2) showed greater PAH concentrations in CEWAF relative to WAF. Overall, naphthalenes dominated in both WAF and CEWAF; however dibenzothiophenes, phenanthrene/anthracenes (C_1-C_4) and fluorenes dominated in CEWAF. This suggests that although WAF tests contained more oil (i.e. higher initial oil loading), higher concentrations of PAHs were present in solution following application of Corexit 9500. The BTEX data (Tables 5 and 6) for each test show that benzene concentrations were highest in WAF for all oil loadings, but toluene, ethylbenzene and xylenes concentrations were highest for the lowest oil loadings when compared to CEWAF. Overall, resultant THC and TPH concentrations were greatest for each CEWAF oil loading.

To assess impacts of short-term exposures on long-term, growth surviving pre-smolts from each acute test were weighed upon test termination, placed in culture tanks (0.91 m dia.) for 3 month and fed *ad libitum* twice daily. Continual growth (as wet weight) was observed in all control fish, except for those from the third WAF test. The reason for the lack of growth is unclear as all were fed the same; none of the control fish showed any signs of distress. Growth was observed in all WAF- and CEWAF-treated fishes at rates similar to the control fish. However, hatchery raised fish may vary genetically resulting in varied growth, as observed by the control fish from the third WAF test. Growth data do not indicate residual effects of short-term WAF or CEWAF exposure on long-term growth in either the WAF or CEWAF exposed fish.

Table 1
Relative concentrations of selected PAHs in WAF as measured in all five test PBCO loading concentrations at time 0 (T_0). Each concentration is an average of the measured PAHs in the first and third WAF experiments.

PAHs	T_0 (ug/L)				
	0.5	1.0	2.0	4.0	8.0
Naphthalene	41.7	36.2	33.7	47.7	30.3
2-Methylnaphthalene	21.2	16.3	16.0	22.3	19.1
1-Methylnaphthalene	17.4	13.8	13.1	18.4	16.0
2,6-Dimethylnaphthalene	3.4	2.8	3.2	3.4	19.3
2,3,5-Trimethylnaphthalene	0.5	0.4	0.5	0.6	4.1
C1-naphthalenes	40.2	31.3	30.3	42.4	36.5
C2-naphthalenes	16.3	14.0	12.8	17.9	17.6
C3-naphthalenes	4.0	3.6	4.2	4.4	5.2
C4-naphthalenes	0.6	0.6	0.7	0.8	6.0
Biphenyl	2.7	2.1	2.5	2.7	14.2
Acenaphthylene	0.0	0.1	0.1	0.1	0.5
Acenaphthene	0.1	0.2	0.2	0.2	1.1
Fluorene	0.7	0.6	0.7	0.7	4.7
1-Methylfluorene	0.5	0.5	0.5	0.5	3.7
C1-fluorenes	0.9	0.8	0.9	1.0	6.6
C2-fluorenes	0.2	0.4	0.4	0.5	3.3
C3-fluorenes	0.0	0.2	0.2	0.2	1.8
Dibenzothiophene	0.7	0.6	0.7	0.7	4.9
4-Methyldibenzothiophene	0.1	0.2	0.3	0.3	1.9
C1-dibenzothiophenes	0.7	0.6	0.7	0.7	5.3
C2-dibenzothiophenes	0.2	0.5	0.6	0.6	4.3
C3-dibenzothiophenes	0.1	0.3	0.4	0.4	3.0
Phenanthrene	0.7	0.5	0.6	0.7	4.7
1-Methylphenanthrene	0.1	0.2	0.2	0.2	1.2
C1-Phenanthrene/anthracene	0.6	0.6	0.7	0.8	5.1
C2-phenanthrene/anthracene	0.5	0.6	0.6	0.6	4.1
C3-phenanthrene/anthracene	0.1	0.2	0.2	0.2	1.5
C4-phenanthrene/anthracene	0.0	0.0	0.0	0.1	0.5

3.2. ^{1}H NMR spectra showing petroleum actions on muscle extracts

1D ^{1}H NMR spectra of muscle extracts are shown in supporting information Fig. S1. NMR peak assignments (supporting information Table S2) were identified by chemical shifts, peak multiplicity and use of Chenomx NMR Software. Metabolite classes observed from both acute tests and 3 month growth studies included amino acids (e.g. alanine), nucleotides (e.g. ATP) and TCA cycle intermediates (e.g. succinate), of which amino acids, lactate and ATP comprised the highest intensity signals. Although spectra contained well-resolved peaks, the congestion between chemical shifts 2 and 4 ppm could potentially limit identification of additional metabolites.

3.3. Acute and long-term dose–response of muscle metabolites

Metabolic profiles are capable of providing a snapshot of the current physiological state of cells, yielding useful information of the metabolic actions of pollutants. Often, profiles are analyzed by the unsupervised method of PCA, yielding a graphical output that depicts the metabolic similarities and differences between the sample spectra. Here, the clustering of samples within a PCA

Table 2
Relative concentrations of selected PAHs in CEWAF as measured in all five test PBCO loading concentrations at time 0 (T_0). Each concentration is an average of the measured PAHs in the first and third CEWAF experiments.

PAHs	T_0 (ug/L)				
	0.0625	0.125	0.3	0.5	1.0
Naphthalene	32.4	51.3	90.0	177	275
2-Methylnaphthalene	45.2	60.1	115	241	392
1-Methylnaphthalene	35.6	48.3	89.6	184	293
2,6-Dimethylnaphthalene	16.4	21.8	50.7	115	193
2,3,5-Trimethylnaphthalene	4.5	6.8	19.0	38.9	69.9
C1-naphthalenes	80.3	112	212	441	929
C2-naphthalenes	77.9	109	225	488	1150
C3-naphthalenes	44.0	71.2	171	401	916
C4-naphthalenes	14.1	20.9	56.6	135	228
Biphenyl	6.2	7.9	15.8	33.2	53.9
Acenaphthene	0.7	0.5	0.0	4.1	5.0
Fluorene	2.7	1.9	7.5	16.1	26.8
1-Methylfluorene	4.7	6.8	15.6	36.7	61.5
C1-fluorenes,	8.3	11.9	27.6	63.6	107
C2-fluorenes	10.0	15.1	37.8	86.1	152
C3-fluorenes	8.7	13.8	32.7	75.7	156
Dibenzothiophene	4.3	6.0	13.4	29.4	48.5
4-Methyldibenzothiophene	4.8	7.1	17.6	40.8	68.4
C1-dibenzothiophenes	9.6	14.0	34.7	79.6	134
C2-dibenzothiophenes	10.9	16.0	41.7	97.4	166
C3-dibenzothiophenes	7.3	11.2	28.6	67.5	115
Phenanthrene	5.2	7.0	16.7	36.1	60.9
1-Methylphenanthrene	3.2	2.3	11.3	26.1	44.9
3,6-Dimethylphenanthrene	1.0	0.9	0.0	9.8	17.5
C1-phenanthrene/anthracene,	12.8	18.4	46.7	107	183
C2-phenanthrene/anthracene	18.1	26.6	68.4	160	274
C3-phenanthrene/anthracene	10.3	14.9	39.2	90.6	154
C4-phenanthrene/anthracene	4.7	7.8	18.9	44.3	75.3
C1-fluoranthene/pyrenes	1.9	1.5	7.9	18.0	32.4
Benz[a]anthracene	0.0	0.3	0.0	0.0	6.4
Chrysene	0.5	0.7	0.0	4.1	7.7
C1-chrysenes,	1.0	0.8	0.0	9.4	15.7
C2-chrysenes	0.9	0.6	0.0	5.6	14.6
C3-chrysenes,	0.7	0.0	0.0	7.5	12.8

scores plot (Figs. 1 and 2), indicate that different concentrations of physically or chemically dispersed oil appear to induce similar metabolic responses as the controls.

The 96 h WAF PCA scores plot shows separation between the three WAF tests along the PC1 axis (with 30.53% of the variability in the dataset explained by this axis; Fig. 1a) with slight overlap between the WAF 2 and WAF 3 tests. The 96 h CEWAF PCA scores plot shows separation among the three CEWAF tests along PC1 and PC2 (which account for 29.71% and 15.63% of variance, respectively; Fig. 1b). These scores plots represented an unanticipated result. Replicate tests were expected to result in similar metabolomes with minimal variation; however those of the control fish, between replicate tests, appear to change, resulting in a decrease in their PC1 scores. This may indicate growth or development of the fish during the experiments. Furthermore, the 96 h toxicity tests do not display a metabolic dose–response relationship between concentrations, indicating that the variation in metabolomes may be induced by similar dissolved concentrations of hydrocarbons within the water and not the total hydrocarbon concentrations themselves.

Scores plots for the 3 month grow-out study displayed similar trends with regards to separation and metabolome overlap. The 3 month WAF PCA scores plot shows overlap of tests 1 and 2 along PC1 and PC2 (explaining 32.09% and 15.01% of variance; Fig. 2a) with minimal separation. The 3 month CEWAF PCA scores plot indicates clear separation of CEWAF 1 from tests 2 and 3 along PC1 (explaining 63.74% variance; Fig. 2b). As for the 96 h tests, growth and development of individual fish and hydrocarbon concentrations may be the cause.

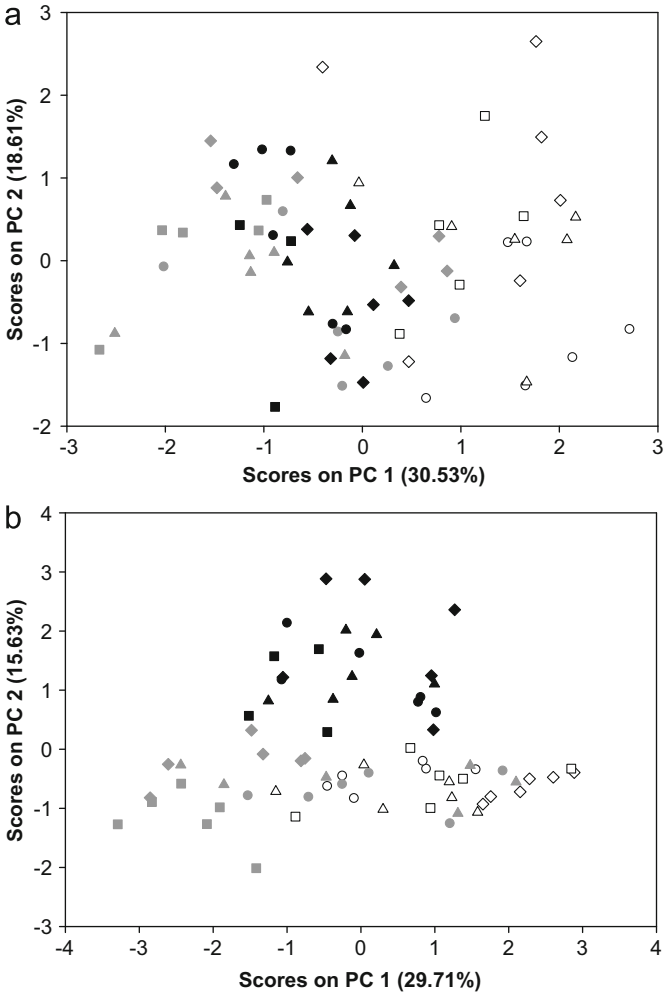

Fig. 1. PCA scores plot of the ^{1}H NMR spectra of skeletal muscle extracts from pre-smolts exposed for 96 h to the (a) WAF of PBCO (3 replicate experiments: WAF1, WAF2 and WAF3) and (b) CEWAF of PBCO (3 replicate experiments: CEWAF1, CEWAF2 and CEWAF3). All analyzes include an unexposed control. Controls from the 1st to 3rd experiments are ◇, ◈ and ◆. The three concentrations of WAF are characterized as 4.28 (○), 4.30 (Δ) and 11.24 (□) mg/L THC and the three concentrations of CEWAF are characterized as 13.1 (○), 20.4(Δ) and 62.5 (□) mg/L THC. The symbol shape represents a single dose and the symbol color follows the color used for the controls. Each data point represents one biological replicate.

Although the replicate tests are separated, there is no indication of a metabolic dose–response among any of the tests. Both WAF and CEWAF exposures display an overlap of metabolomes and lack separation whereas the PCA scores plots show no indication of dose–response within metabolic profiles although individual samples can be separated. Based on these PCA scores plots, there are no observable effects from short-term exposure on long-term (3 month) growth. Overall, the PCA plots represent a visual of the dose–response relationship for the individual fish, but in this study effects resulting from the treatments could not be determined strictly by the PCA plots. Therefore, resultant NMR spectra provide additional information capable of identifying the effects from physically and chemically dispersed oil exposures, where metabolic responses are a more accurate determination of treatment effects.

3.4. Metabolic changes in muscle tissue

NMR spectra were individually analyzed to avoid variation from changes in experimental conditions or the possibility of

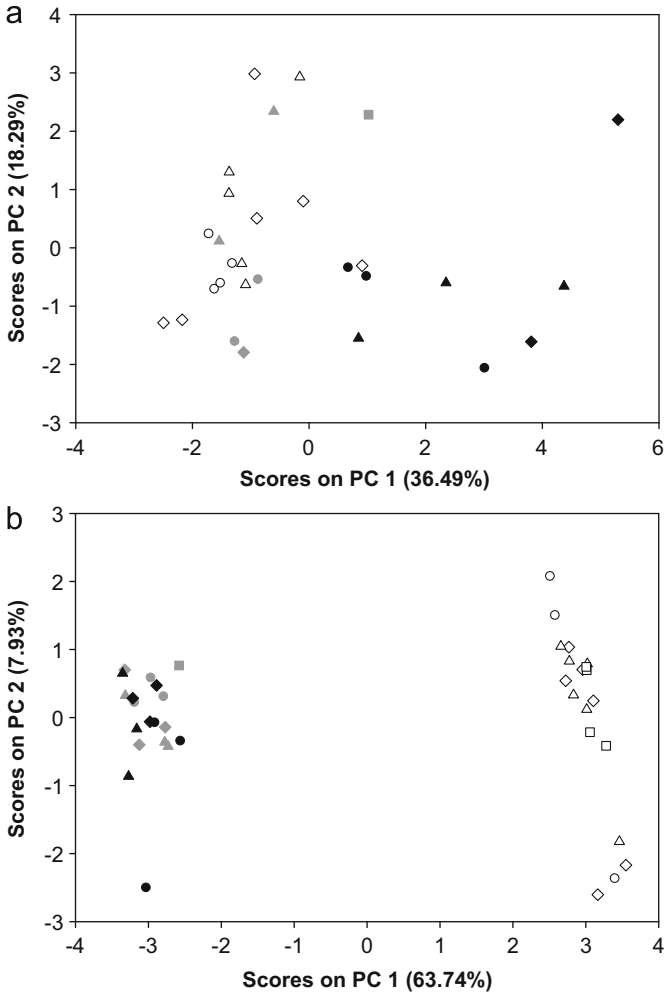

Fig. 2. PCA scores plot of the ^{1}H NMR spectra of skeletal muscle extracts from pre-smolts after 3 month following 96 h exposure to the (a) WAF of PBCO (3 replicate experiments: WAF1, WAF2 and WAF3) and (b) CEWAF of PBCO (3 replicate experiments: CEWAF1, CEWAF2 and CEWAF3). All analyzes include an unexposed control. Controls from the 1st to 3rd experiment are ◇, ◈ and ◆. The three concentrations of WAF are characterized as 4.28 (○), 4.30 (Δ) and 11.24 (□) mg/L THC and the three concentrations of CEWAF are characterized as 13.1 (○), 20.4(Δ) and 62.5 (□) mg/L THC. The symbol shape represents a single dose and the symbol color follows the color used for the controls. Each data point represents one biological replicate.

change in animal physiology between experiments. Well-resolved peaks were identified and integrated for comparison between experiments.

Concentration-dependent changes and results of one-way ANOVAs from identified metabolites are presented in Tables 3 and 4. Metabolite changes were calculated using the peak areas within the NMR spectra, specifically by dividing the average peak area of the treatment by that of the corresponding control. Therefore, a number below 100% represents a decrease in that metabolite within treated fish. Following 96 h exposures (Table 3), glutamate and taurine significantly increased ($p < 0.05$) and lactate significantly decreased from each WAF and CEWAF concentrations. Significant decreases for WAF included ATP, whereas for CEWAF decreases included alanine, phosphocreatine, glycine and histidine. Following 3 month (Table 4), taurine significantly increased for both WAF and CEWAF, whereas glutamate significantly increased for WAF. Overall, results indicate that metabolite responses were dependent on exposure time, concentration and WAF or CEWAF treatment.

4. Discussion

4.1. Toxicity of WAF and CEWAF

Dispersion of oil may increase contact and uptake of hydrocarbons by marine organisms, ultimately affecting their toxic actions, storage and detoxification (Mielbrecht et al., 2005). Hydrocarbon uptake and accumulation generally increases with increased water concentrations, but it is also influenced by water solubility and salinity. For instance, Ramachandran et al. (2006) note that an oil spill near estuaries can expose fish to PAH concentrations up to 60-fold greater in water with low salinity due to increased PAH solubility. However, the use of a dispersant can increase such exposure by 250 times when used in brackish or freshwater (Ramachandran et al., 2006).

In the results reported here, chemical dispersion (via Corexit 9500) increased the water's concentration of PAHs (e.g. naphthalenes and phenanthrene/anthracenes) when compared to physical dispersion (Tables 1 and 2). Although much higher oil loadings of PBCO were used for the WAF tests than the CEWAF tests, CEWAF contained different PAHs when analyzed by GC/MS. It was seen that WAF contained higher concentrations of benzene than CEWAF, whereas the overall THC concentrations were greatest

Table 3
Metabolite changes (%) in the muscle of pre-smolts exposed for 96 h to the WAF [a, b] or CEWAF [a, b] of PBCO.

Metabolites	WAF (mg/L)			(*p*)	CEWAF (mg/L)			(*p*)
	4.2	4.3	11.2		13.1	20.4	62.5	
Lactate	84.1	90.0	81.1	0.0177*	103	99.1	78.4	0.0436*
Alanine	100	96.7	96.4	0.365	121	115	93.2	0.0012*
Glutamate	118	139	145	0.0001*	114	120	127	< 0.0001*
Succinate	103	114	123	0.0747	97.5	94.0	96.3	0.715
Phosphocreatine	101	99.8	99.5	0.681	99.5	99.8	104	0.0397*
Taurine	109	105	122	0.0084*	110	113	117	0.0165*
Glycerophosphocholine	83.6	80.0	91.7	0.482	63.2	79.1	80.9	0.290
Glycine	102	113	95.4	0.239	99.1	90.4	58.8	0.0008*
AMP	94.5	102	116	0.0603	107	108	98.4	0.763
Histidine	76.0	87.7	89.8	0.258	73.2	53.8	47.0	0.0006*
ATP	112	90.3	58.7	0.0060*	181	184	157	0.882

[a] All spectra were normalized by total spectral area. Metabolite changes were derived via the ratio between the averages of the treatment and control peak areas. WAF and CEWAF were characterized by total hydrocarbon content (mg/L).

[b] Significant *p*-values are marked by *($p < 0.05$).

Table 4
Metabolite changes (%) in the muscle of pre-smolts after 3 month of growth following exposure to the WAF [a, b] or CEWAF [a, b] of PBCO.

Metabolites	WAF (mg/L)			(*p*)	CEWAF (mg/L)			(*p*)
	4.2	4.3	11.2		13.1	20.4	62.5	
Lactate	119	107	37.4	0.306	109	86.0	85.8	0.306
Alanine	109	91.3	131	0.823	99.5	118	88.4	0.624
Glutamate	88.3	73.2	121	0.0219*	103	118	137	0.0237*
Succinate	99.2	105	89.8	0.711	117	100	98.5	0.803
Phosphocreatine	107	112	119	0.402	97.7	100	96.2	0.402
Taurine	78.0	64.8	176	< 0.0001*	110	140	186	0.0008*
Glycerophosphocholine	103	73.2	44.5	0.604	102	105	169	0.619
Glycine	133	127	94.1	0.0590	95.2	106	90.3	0.0590
AMP	107	121	94.8	0.797	141	128	117	0.865
Histidine	83.7	88.0	123	0.485	110	103	87.5	0.485
ATP	169	186	201	0.183	58.1	88.6	89.3	0.707

[a] All spectra were normalized by total spectral area. Metabolite changes were derived via the ratio between the averages of the treatment and control peak areas. WAF and CEWAF were characterized by total hydrocarbon content (mg/L).
[b] Significant *p*-values are marked by *($p < 0.05$).

Table 5
Measured BTEX, total hydrocarbon content (THC) and total petroleum hydrocarbons TPH in WAF at time 0 (T_0).

WAF (g/L)	Benzene (ug/L)	Toluene (ug/L)	Ethylbenzene (ug/L)	Xylene-m/p (ug/L)	Xylene-o (ug/L)	THC (mg/L)	TPH (mg/L)
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.50	856	1660	184	510	234	4.28	0.83
1.00	1110	1610	149	387	194	4.30	0.85
2.00	3830	5000	292	815	403	11.2	0.91
4.00	5410	4430	293	814	398	12.4	1.03
8.00	6600	4970	336	933	458	14.5	1.22

Table 6
Measured BTEX, total hydrocarbon content (THC) and total petroleum hydrocarbons (TPH) in CEWAF at time 0 (T_0).

CEWAF (g/L)	Benzene (ug/L)	Toluene (ug/L)	Ethylbenzene (ug/L)	Xylene-m/p (ug/L)	Xylene-o (ug/L)	THC (mg/L)	TPH (mg/L)
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00
0.06	122	275	52.3	166	68.7	13.4	12.5
0.13	350	779	149	454	193	20.4	18.5
0.25	782	1730	314	990	396	62.5	58.3
0.50	2470	5400	890	2860	1140	118	105
1.00	4690	9220	1450	4740	1910	296	274

for CEWAF, but based on the THC content, the mean WAF LC_{50} (7.6 mg/L THC) was ca. 6-fold lower than that for CEWAF (48.6 mg/L THC), potentially indicating hydrocarbon bioavailability was reduced by Corexit 9500 application. For salmon smolts, Lin et al. (2009) reported that the mean 96 h LC_{50} for the WAF of PBCO (7.46 mg/L) was ca. 20-fold lower than CEWAF (155.93 mg/L), also suggesting that bioavailability may have been reduced under dispersed conditions. In comparison, pre-smolts were more sensitive to both WAF and CEWAF than smolts, based on LC_{50} values. Reduced bioavailability, in both studies, may be attributed to creation of dispersant-based micelles, which may impair absorption of hydrocarbons across membranes.

Membrane bi-layers contain different compositions of phospolipids, influencing the partitioning of foreign compounds and their biological actions (Sikkema et al., 1995). Hydrocarbons have been found to accumulate in the central aliphatic portion of lipid bi-layers, resulting in a dramatic change in membrane structure and function, including its role as a barrier to pathogens, energy transduction and matrix formation for both proteins and enzymes (Sikkema et al., 1995). In general, lipophilic compounds exert a non-specific toxicity (narcosis) towards lipid–lipid and lipid–protein interactions (Sikkema et al., 1995). Particularly, membrane functions are more sensitive to narcotic chemicals due to the disturbance of membrane lipids, where accumulation may result in a change in lipid fluidity, phospholipid structure, membrane thickness and activity of membrane-bound enzymes (van Wezel and Opperhuizen, 1995). Overall, this non-specific toxicity may possibly be overlooked when assessing toxic effects based on metabolic changes. PAHs are classified as Type I (non-polar) narcotics, with narcosis as the primary mode of toxicity (DiToro et al., 2000). Bradbury et al. (1989) suggest that there are multiple mechanisms/sites of action that narcotic chemicals target; therefore damage to membranes and their functions may justify the similar metabolic outcome for each of the acute toxicity tests.

4.2. Comparative metabolic actions of WAF and CEWAF

Oil toxicity depends on physical–chemical properties of the oil and an organism's physiology (NRC, 2005). Maximum hydrocarbon uptake via passive diffusion has been found to occur within the first few hours of an exposure (Wolfe et al., 2001). Following diffusion, PAHs may bind and activate the aryl hydrocarbon receptor (AhR), a cytosolic protein complex, which enters the nucleus and interacts with target genes, resulting in modulation of gene transcription and induction of CYP1A, which may produce toxic epoxides and other oxidized forms (Leaver,

1996). Long-term actions from the resultant genetic damage may include reduced fertility, impaired development and ultimately reduced recruitment. Kocan et al. (1996) also note that long-term genetic damage may result from mutations potentially being passed onto further generations. Over time, fish development would facilitate the biotransformation and depuration of accumulated residues, thus minimizing their long-term actions.

Based on the acute and long-term results, it was determined that for both time frames, as well as their respective controls, very similar metabolic profiles resulted. The main difference being that acute exposures produced more changes that were significant ($p < 0.05$; Table 3).

Lactate significantly decreased during acute exposure to both WAF and CEWAF. Its presence may indicate that skeletal muscle, from both exposures, was experiencing varied oxygen concentrations. Variations in oxygen can ultimately lead to a disruption of ATP production via the TCA cycle. Although lactate was present, its decrease indicates that it was possibly being consumed for ATP (via TCA; thus more efficient) or for the formation of glucose via gluconeogenesis. Results also indicate a significant decrease occurred in ATP from each WAF exposure, whereas ATP did not significantly increase from each CEWAF exposure. This insignificant change is unclear. Lack of energy substrates will affect the rate of intermediate metabolism and an organism's ability to repair cellular or protein damage resulting from hydrocarbon exposure or overall stress. Phosphocreatine, also dependent on ATP concentrations, decreased slightly after WAF exposure and significantly increased after CEWAF exposure. Since phosphocreatine concentrations are low within skeletal muscle, it is used as an emergency energy source, ultimately producing ATP via ADP phosphorylation (Boyer, 2006). The findings correlate with the change in ATP after exposures to either WAF or CEWAF.

Organic osmolytes such as taurine and glycerophosphocholine were also identified. Taurine significantly increased during acute exposure to both WAF and CEWAF (Table 3) and after 3 month following the exposures (Table 4), whereas glycerophosphocholine remained steady. During hyperosmolarity and cell shrinkage, cells accumulate osmolytes to adapt to changes in cell volume, but this process is metabolically expensive (Sperelakis, 1998). These two metabolites indicate cell volume alteration and the attempt to regulate natural cell osmosis. Taurine accumulation for WAF may be contributing to the significant decrease in ATP, due to energy being consumed to maintain cell balance.

Significant changes in amino acids were also observed (Tables 3 and 4). Histidine significantly decreased after CEWAF exposure, and although a decrease was observed for WAF it was not significant. Histidine is often catabolized into TCA cycle intermediates (i.e. α-ketoglutarate), aiding in energy production. Glutamate significantly increased after both exposure conditions and after 3 month of growth. As a non-essential amino acid and excitatory neurotransmitter, it participates in cellular metabolism and neuronal signal transmission (von Bohlen und Halbach, 2002). Increased glutamate concentrations may result from protein degradation leading to the catabolism of amino acids (e.g. alanine), or are available for the synthesis of neurotransmitive substrates (e.g. δ-aminobutyric acid; GABA), possibly indicating an affect on the nervous system (Boyer, 2006). Glycine, also a non-essential amino acid, participates in protein and nucleotide metabolism and contains neurotransmitter properties (von Bohlen und Halbach, 2002). Glycine decreased significantly after exposure to CEWAF, but an insignificant decrease was observed for higher concentrations of WAF.

Identified amino acids, osmolytes and energetic metabolites may be bio-indicators of protein synthesis and/or degradation, cellular repair and/or degradation, changes in cell structure or overall stress. Observed metabolic responses from both WAF and CEWAF appear to be similar and may indicate metabolic changes are dependent on treatments and/or exposure concentrations. However, it is unknown which individual hydrocarbons are influencing such metabolic responses. There was no indication of long-term effects arising from the 96 h exposures, to either type of oil, as observed by changes in growth rates or metabolic profiles.

5. Conclusion

The possibility of an oil spill occurring in San Francisco Bay during the seaward migration of salmon pre-smolts, potentially exposing them to toxic petroleum hydrocarbons, is real. Our results provide toxicological information that indicates use of a dispersant, such as Corexit 9500, may potentially reduce the bioavailable PAH fraction within the water column. Based on the LC_{50} values, the WAF possesses a greater potency than the CEWAF but, based on similar metabolite profiles, the operative dissolved hydrocarbon concentration may have been similar. Overall, the metabolic results provide clues to the potential mechanisms of toxicity that hydrocarbons pose to anadromous fish.

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Appendix A. Supporting Information

Supporting Information associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2010.03.001.

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