ACUTE AND CHRONIC EFFECTS OF CRUDE AND DISPERSED OIL ON CHINOOK SALMON PRE-SMOLTS (*ONCORHYNCHUS TSHAWYTSCHA*)

A Final Report Submitted to:
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Abstract

Due to the large maritime transport of crude oil from Alaska to California, there is significant potential for catastrophic spills that could seriously impact salmon populations during key periods of their migration, particularly when pre-smolts are migrating towards the San Francisco Bay from the San Pablo Bay and Suisun Bay areas. This study examined the toxic actions of the water-accommodated fraction (WAF) and chemically-dispersed fraction (CEWAF) of Prudhoe Bay Crude Oil (PBCO) on the pre-smolts of Chinook salmon (*Onchorhyncus tshawytscha*). A closed, flow-through test system for exposing salmon pre-smolts to declining concentrations of WAF or CEWAF (using the dispersant Corexit 9500) of PBCO was developed. The WAF and CEWAF were fully characterized for total hydrocarbons (THC) by gas chromatography-flame ionization detection. After exposure to various concentrations of oil or dispersed oil for 96 hours, the mean LC_{50} based on THC of the WAF tests was approximately six-fold lower than that of the CEWAF tests. The mortality results suggest that application of oil dispersants decreased the toxicity of oil. A subset of the surviving fish was then sacrificed to examine the metabolic impacts of WAF and CEWAF. After removing muscle and liver tissues from the pre-smolts, the tissues were flash frozen. The low molecular weight metabolites were extracted and analyzed using one-dimensional ^{1}H and projections of two-dimensional ^{1}H, ^{1}H J-resolved nuclear magnetic resonance (NMR) spectroscopy. Following spectral processing, the metabolites were examined using principal components analysis (PCA) following analysis of variance to identify the metabolic changes. Metabolomic analysis results for the 96 hour exposures showed an increase in amino acids and a decrease in important nucleotides. This suggests the breakdown of proteins for cellular repair and increased use of energy due to stress.

Keywords: metabolomics, Chinook salmon, pre-pre-smolts, toxicity, oil spill, dispersed oil

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1.0 Introduction

Currently all salmon species, as well as migratory steelhead trout, are classified as "threatened" under the Federal Endangered Species Act. While they are struggling to recover from the combined effects of over-fishing, habitat decline and pollution, there is serious concern that marine oil spills and associated response activities near rivers of spawning importance may impact pre-smolts entering the ocean. In particular, there is concern that oil dispersant application could increase the toxicity of crude oil to pre-smolts as they adapt to seawater.

A number of studies have investigated the toxicity of oil to salmon, particularly using embryos, alevins, and fry of the Alaskan pink salmon *Oncorhynchus gorbuscha* (Rice *et al.,* 1975; Swartz, 1985; Heintz *et al.,* 2000; Rice *et al.,* 2001) and coho salmon *O. kisutch* (Stickle *et al.,* 1982; Thomas *et al.,* 1987, 1989). However, there is currently little information in the scientific literature comparing the lethal and sublethal impacts of oil and dispersed oil on salmon pre-smolts. Due to extensive maritime transport of crude oil from Alaska to California, there is significant potential for a catastrophic spill, which could seriously impact the waters of San Francisco Bay. Shallow estuaries, such as the San Pablo Bay and Suisun Bay, which form the northern extension of San Francisco Bay, may also be impacted. Salmon pre-smolts develop in these estuaries after migrating from the Sacramento and San Joaquin River delta before continuing their migration pattern into San Francisco Bay. Due to maritime conditions, dispersants are the only response option available for central and northern California waters during 60% or more of the year (S. L. Ross, 2002). Information on the relative toxicity of dispersed and un-dispersed oil is therefore needed by resource agencies responsible for SF Bay spill response activities.

This study compared the toxic actions of the water-accommodated fraction (a naturally dispersed fraction; WAF) and chemically dispersed fraction (a chemically enhanced water-accommodated fraction; CEWAF) of Prudhoe Bay Crude Oil (PBCO) to the pre-pre-smolts of Chinook salmon (*O. tshawytscha*). The null hypothesis to be tested is: *the toxic impacts of crude oil on Chinook salmon pre-smolts are not increased by application of oil dispersants.*

2.0 Objectives

The primary objective of this project was to conduct short-term 96-h declining exposures to assess the relative toxicities of dispersed and un-dispersed PBCO to pre-smolt Chinook salmon. The secondary objective was to determine levels of oil causing metabolic stress using an advanced NMR-based metabolomic approach. The final objective was to culture surviving fish in clean (oil-free) seawater to determine the influence of the short-term exposures on long-term growth and viability of salmon as they undergo smoltification.

Chinook salmon pre-smolts (~6 cm), obtained from the California Department of Fish and Game (CDFG) Feather River Hatchery, were exposed for 96 h to various concentrations of either the WAF or CEWAF of PBCO using methods we have also standardized through Chemical Response to Oil Spills Environmental Research Forum (CROSERF) and a declining exposure protocol, also previously developed in our laboratory. The WAF and CEWAF were fully

characterized for total hydrocarbons by gas chromatography (GC-FID), and a subset of samples were characterized for polycyclic aromatic hydrocarbons (PAHs) using GC-mass spectrometry. Declining exposure concentrations will better simulate actual spill conditions, where both dilution and dispersion occur.

3.0 Methods

This project was conducted at three laboratories. All fish exposures to oil were conducted at the Marine Pollution Studies Laboratory (MPSL) at Granite Canyon, near Monterey, CA. Cooperative aquatic toxicology research is conducted at MPSL by the California Department of Fish and Game and UC Davis. Metabolomic analysis of tissues from exposed pre-smolts was conducted at the UC Davis NMR Facility and Department of Environmental Toxicology. Hydrocarbon analysis of oiled seawater was conducted at the CDFG Water Pollution Control Laboratory, Rancho Cordova, CA.

3.1 Exposure Materials

All methods followed Singer *et al.,* (1998), and recent standardized methods recommended in Singer *et al.,* (2000). All testing was conducted using PBCO obtained from Resource Technology Corporation (Laramie, WY, USA), and the dispersant Corexit 9500, obtained *gratis* from Nalco/Exxon Energy Chemicals, L.P. (Sugar Land, TX, USA). Chemical dispersion of oil was carried out at a nominal oil:dispersant ratio of 10:1 (v:v). Untreated oil testing was performed using the WAF of unweathered PBCO. Untreated oil WAFs were prepared using a standardized low-energy mixing method (Singer *et al.,* 2000), which involved layering a known mass of crude oil onto a standard volume (22 L) of laboratory well water in a 23-L polycarbonate carboy. Mixing energy was provided by magnetic stirrers at a rate of 110 ± 10 rpm (or such a speed that did not produce any discernable vortex) for 24 h; this mixing rate was sufficient to provide circulation of water throughout the bottle without creating a vortex. No particulate material was present, and therefore no settling time was necessary (Singer *et al.,* 1998).

Treated (chemically-dispersed) oil tests were performed with solutions prepared in much the same way as WAFs, employing the same carboys, stirrers, and volume of fresh water. These solutions were designated CEWAFs because dispersants chemically enhance the accommodation of bulk oil into water by increasing its functional solubility. Mixing energies used to prepare CEWAFs were increased to create a vortex 20–25% of water depth to provide sufficient mixing energy for dispersion. Once the vortex was established, known volumes of oil and dispersant were delivered in sequence into the center of the vortex using beakers and micropipettes (after Singer *et al.,* 1998). Exact masses of oil delivered were calculated by difference. Mixing lasted 18 h, followed by 6 h of settling time to allow the largest oil droplets to resurface (Singer *et al.,* 2000). The 18-h mix:6-h CEWAF settle regimen was used to match the 24-h total preparation time used for WAFs.

3.2 Gas Chromatography

Chromatographic measurement of total hydrocarbon content (TPH – C10–C36) was accomplished using a Hewlett-Packard 6890 gas chromatograph fitted with a flame ionization detector (FID). This instrument set-up typically allowed reliable resolution of compounds from methyl-cyclohexane up to approximately *n*-C36. A liquid-liquid triplicate extraction method was used with dichloromethane (DCM) as the solvent. After each extraction, the solvent phase was collected and combined, with the final extract made up to a standard volume. The extracts were not concentrated in order to retain as many volatile compounds as possible. Extracts were stored in the freezer until analysis, at which time an aliquot was transferred to a 2-mL screw-cap GC vial. Quantitation of test samples was performed against a set of PBCO standards (Payne, 1994).This was done in an attempt to better represent the number and relative proportions of the various DCM-soluble compounds contained in the oil. A stock solution was first prepared by adding a measured mass of oil directly into a sealed, septum-capped vial containing 4.5 mL DCM (also weighed for concentration calculation). This stock was then serially diluted volumetrically with DCM directly into septum-capped vials by means of gas-tight syringes.

Check standards were run with each batch to show that the instrument had not drifted and that the standard curve was still good. Samples were measured by summation of total resolved chromatogram peak area after subtraction of dispersant peaks when appropriate (Payne, 1994). These data were then quantified using the average response factor of the similarly integrated (total peak area) whole oil standards. While unresolved or non-chromatographable compounds were not directly measured by this method, their inclusion in the mass of oil used to prepare standards allowed them to be accounted for in response factor calculations (Payne, 1994). This technique did not allow for direct quantitation of individual hydrocarbons; however, it produced concentrations based on the total response of samples (corrected for background response of the seawater matrix), designated THC (C6–C36) (*i.e.,* total hydrocarbon content = BTEX C6–C9 compounds + TPH C10–C36) and thus was not biased by quantifying a specific set of target analytes (Girling *et al.,* 1994). Concentrations of the volatile hydrocarbons benzene, toluene, ethyl benzene and xylenes (BTEX) were analyzed using HP GC/MS 6890-5973 with a HP 7695 purge & trap concentrator and a Teledyne Tekman autosampler (US EPA Method 8260).

Extraction methods for analyses of PAHs were developed and validated by WPCL and are based on modifications of methods described in EPA Methods 3500B and 3510c from the EPA SW-846. One-liter samples [and the sample bottles themselves] were subjected to liquid/liquid extraction with methylene chloride three times after addition of deuterated surrogates, then dried with sodium sulfate and brought to a final volume of 1.0 mL in isooctane by Kuderna-Danish (K-D) apparatus and nitrogen evaporation. Internal standard was added 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 WPCL and is based on EPA Method 8270B.

3.3 Toxicity Test Procedures

Spiked-exposure 96-h toxicity tests were completed with salmon pre-smolts (*O. tshawytscha*) using established test procedures (Singer *et al.* 2000), modified to accommodate larger

organisms. Chinook salmon pre-smolts (~6 cm) were obtained from the CDFG Nimbus Hatchery. To ensure against infection, the fish were quarantined under the care of CDFG pathologist Tresa Veek, until needed for tests. These fish were fed antibiotic-treated salmon pellets and were dosed with formalin to limit ciliated protozoan infection (ich). Fish were transported from the hatchery in three separate trips beginning in April 2007. Once at MPSL, pre-smolts were held in 4 ft. tanks under a partially closed freshwater re-circulating system, and received periodic treatments with formalin to limit parasite infection.

Spiked exposures were conducted in sealed, 18-L polycarbonate flow-through exposure chambers. Tests involved six treatments: five WAF or CEWAF treatments and a fresh water control, with each treatment having three replicates. Treatments within individual tests in this study were derived from a range of oil loadings, with replicate tests employing equivalent loading ranges. Water temperature, DO, and pH were monitored daily during testing. Diluent was laboratory well water.

Treatment replicates within each test were aliquotted from three replicate carboys, each containing a single oil loading preparation. Upon completion of WAF or CEWAF preparation, approximately 6 L (33%) of test solution was drained from each of three replicate carboys directly into each of the three replicate exposure chambers, until each contained 18 L of test solution. Thus, test solutions in the three replicate exposure containers were comprised of composite solutions from the three test solution preparation carboys, to minimize between-replicate variability in hydrocarbon concentrations. Once the exposure containers were filled, eight salmon were added to each chamber by unsealing the chamber lid, introducing the animals by hand, and resealing the chamber. The tests were then initiated by immediate commencement of flushing of all chambers with clean, aerated fresh water at a rate of approximately 20 mL/min (Figure 1). Prior to conducting definitive experiments with pre-smolts, preliminary experiments were conducted with PBCO CEWAF to determine the optimal flushing rate to approximate hydrocarbon declines predicted by oil spill models designed for San Francisco Bay. The results showed that a flushing rate of 20 mL/min resulted in an approx. 88% decline in hydrocarbons after 24 h, which is comparable to the model predictions. This flush rate is 10% of that used in our previous experiments with salmon smolts (i.e., 200 mL/min; Tjeerdema *et al.* 2007). Smolt survival was acceptable in experiments using the lower flush rate. This flush rate was used in all experiments using pre-smolts. After flushing for 24 h, exposure chambers were aerated for the remainder of the test.

At the end of each 96-h test, two surviving fish from each of three replicates were dissected for metabolomic analyses (described below), and the remaining survivors were transferred to culture tanks for long-term growth studies. At the initiation of the grow-out phase, the fish were acclimated from freshwater to full seawater salinity (~33‰) by daily 5‰ incremental increases of salinity.

After test initiation, total carbon (TC) concentrations in all chambers were monitored every 2 h up to 8 h, and at 24 h to characterize flush rates. Total carbon analysis was conducted using high temperature combustion on a Teledyne Apollo 9000 TOC analyzer (Teledyne, Santa Clara, CA). In order to minimize loss of the lowest boiling-point fractions, TC samples were collected by gas-tight syringe directly from each chamber through the Teflon septum and immediately

analyzed.TC data were used to assess acceptability of oil decline rates (Singer *et al.,* 1998). Flush rates were also monitored by measuring exit flow of the individual chambers every 24 h.

3.4 Statistics

Variation within and among test populations was assessed by using three replicate exposure chambers within each test treatment and by running three replicate tests for each species/toxicant (WAF or CEWAF) combination. Median-effect concentrations (LC_{50}s) were estimated using the trimmed Spearman-Karber procedure (Hamilton *et al.,* 1977). Test acceptability considered 80% survival in controls to be acceptable.

3.5 Animal and Experimental Design for Metabolomics studies

Each complete experiment consisted of five concentrations of WAF or CEWAF and a control, each replicated three times (for a total of 18 chambers, each containing 8 fish). Experiments for metabolomic analyses were repeated 3 times for statistical validity. Two additional experiments conducted for the long-term grow study (one WAF and one CEWAF tests) were also analyzed. Therefore, a total of 4 WAF and 4 CEWAF tests were processed metabolic analysis. After the 96-h exposures, two surviving fish from each replicate tank were sacrificed. Muscle and liver tissues were immediately dissected, flash frozen in liquid N_2, and stored at -80^{o}C until extraction.

3.6 Metabolite Extraction

Extraction methods were taken from previous work (Lin *et al.*). Briefly, frozen muscle or liver samples from surviving fish was homogenized in a liquid N_2-cooled mortar and lyophilized overnight. The homogenous dry tissue powder was weighed, and then extracted with 20 mL/g (dry mass) of methanol/water (2/1). Samples were vortexed for 15 sec three times and put on ice in between. Following centrifugation (12,000 g, 10 min, 4°C), 0.60 mL of supernatant was removed and then lyophilized prior to NMR analyses.

3.7 1D ^{1}H and 2D –J-Resolved NMR Spectroscopy

Metabolomic analyses were performed as previously described, with slight modifications (Viant, 2003). First, all lyophilized extracts were resuspended with sodium phosphate buffer in D_2O (0.1 M, pH 7.4) containing sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate (TMSP), which served as an internal chemical shift standard. All NMR spectra were measured at 500.11 MHz using Avance DRX-500 spectrometers (Bruker, Fremont, CA; Figure 2). Acquisition parameters for the 1D NMR spectroscopy consisted of a 4.5-μs (60˚) pulse, 7-kHz spectral width, 2.5-s relaxation delay with pre-saturation of the residual water resonance, and 100 transients collected into 16k data points, requiring a 9-min total acquisition time. All data sets were zero-filled to 32k points, exponential line-broadenings of 0.3 Hz were applied before Fourier transformation, and the spectra were phase and baseline corrected, and calibrated (TMSP, 0.0 ppm) using XWIN-NMR and Topspin software (Version 3.1; Bruker).

2D-J-Resolved NMR spectra were acquired using 4 transients per increment for a total of 32 increments, which were collected into 16k data points using spectral widths of 8 kHz in F2

(chemical shift axis) and 32 Hz in F1 (spin-spin coupling constant axis). A 2.5-s relaxation delay, with pre-saturation of the residual water was employed, resulting in a total acquisition time of 15 min. Datasets were zero-filled to 128 points in F1, and both dimensions multiplied by qsine-bell window functions prior to Fourier transformation. The spectra were tilted by 45º, symmetrized about F1, calibrated (TMSP, 0.0 ppm), and the proton-decoupled skyline projections (p-JRES) obtained, all using XWIN-NMR and Topspin software.

3.8 NMR Spectral Pre-Processing and Statistical Analyses

Each spectrum was segmented into 1760 chemical shift bins between 0.2 and 10.0 ppm, corresponding to a bin width of 0.005 ppm (2.5 Hz), using custom-written *ProMetab* software (Version 1; Viant, 2003) in MATLAB (The MathWorks, Natick, MA). The area within each spectral bin was integrated to yield a 1 x 1760 vector containing intensity-based descriptors of the original spectrum. Bins representing the residual water peak (from 4.60 to 5.20 ppm) were removed. In some cases, groups of bins were compressed into a single bin in order to capture peaks with variable chemical shifts into a single bin. The total spectral area of the remaining bins was normalized to unity to facilitate comparison between the spectra. The binned data was subject to the generalized log transformation, and the columns were mean-centered before multivariate analysis.

Principal component analysis (PCA) of all the pre-processed NMR data was conducted using the PLS_Toolbox (Version 3.5; Eigenvector Research, Manson, WA) within MATLAB. Each data set was examined to identify potential outliers as well as similarities between the metabolic fingerprints, in an unsupervised manner. In this pattern recognition technique, the algorithm calculates the highest amount of correlated variation along PC1, with subsequent PCs containing correspondingly smaller amounts of variance. The loading vector for each PCA model was examined to identify the metabolites contributing to these clusters. One-way and two-way ANOVAs were conducted on the changes of the peak areas between the replicates and doses using the Sigma-Stat software; results of the two-way ANOVAs are presented.

4.0 Results

4.1 System Performance

Flush rates in the exposure aquaria were controlled using manual flow meters which were calibrated daily. Initially, flush rates were characterized using total carbon analyses to confirm that there were no differences in flush rates between replicate aquaria and between aquaria from different oil loading treatments. Total carbon measures from the first and second WAF and CEWAF tests are presented in Appendix I. Total carbon concentrations were consistent between replicate aquaria in the CEWAF tests. Because total carbon concentrations were low in the first two WAF tests, flow rates were also measured directly in the three WAF and CEWAF tests to assess between-replicate and between-treatment flush rates. These data also demonstrate minimal between replicate variability and between-treatment variability in flush rates in these tests (Appendix II).

As discussed above, we used measures of TC in CEWAF in a preliminary evaluation of flushing rates during our year 1 activities (in 2006). This analysis suggested that we achieved an 88% decline of TC after 24 h using a flushing rate of 20 mL/minute. For the 2007 tests, TPH concentrations at 24 h were compared to Time 0 TPH. The results show some variability in residual TPH after 24 h of flushing, with greater variability in residual TPH in the higher concentrations of the first two CEWAF tests. For example, 24 h TPH (as a percentage of the T0 concentration) ranged from 13% to 27% in the first WAF test, 19 to 26% in the second WAF test, and 14% to 30% in the third WAF test. In the third WAF test, the 24h TPH in the highest concentration deviated from the rest (Tables 1-3).

Greater TPH variability was observed at 24h in the first two CEWAF tests. The 24h TPH concentration (as a percentage of the T0 concentration) ranged from 34% to 74% on the first CEWAF test, with comparable flushing in the first 3 concentrations, and slower flushing in the final two concentrations (Table 4). Residual 24h TPH was comparable in all concentrations except one (0.5 mg/L loading) in the second CEWAF test (Table 5). Residual TPH at 24 h was comparable at all concentrations in the third CEWAF test (Table 6). Greater variability was observed in the CEWAF tests than in the WAF tests, and this appeared to be related to stratification of oil in the CEWAF exposure chambers, particularly in the first CEWAF test.

	WAF 1			WAF 2			WAF 3		
Oil Loading Concentration	T0	T24	% of TO at T24	T0	T24	% of TO at T24	T0	T24	% of TO at T24
0 g/L	0.000			0.000			0.000		
0.5 g/L	0.791	0.105	13	0.804	0.179	22	0.895	0.124	14
1 g/L	0.787	0.133	17	0.856	0.209	24	0.900	0.147	16
2 g/L	0.855	0.174	20	1.010	0.193	19	0.867	0.160	18
4 g/L	0.994	0.265	27	0.901	0.235	26	1.210	0.224	19
8 g/L	1.320	0.192	15	1.390	0.352	25	1.410	0.416	30

Table 1. WAF #1 decline rates based on TPH

Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					100
0.5 g/L	0.791	0.105	0.134	13	128	100
1 g/L	0.787	0.133	0.158	17	119	100
2 g/L	0.855	0.174	0.159	20	91	21
4 g/L	0.994	0.265	0.187	27	71	4
8 g/L	1.320	0.192		15		0

Table 2. WAF #2 decline rates based on TPH

Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					100

0.5 g/L	0.804	0.179	0.142	22	79	100
1 g/L	0.856	0.209	0.164	24	78	100
2 g/L	1.010	0.193	0.156	19	81	29
4 g/L	0.901	0.235	0.185	26	79	8
8 g/L	1.390	0.352		25		0

Table 3. WAF #3 decline rates based on TPH

Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					92
0.5 g/L	0.895	0.124	0.155	14	125	100
1 g/L	0.900	0.147	0.162	16	110	75
2 g/L	0.867	0.160	0.179	18	112	17
4 g/L	1.210	0.224		19		0
8 g/L	1.410	0.416		30		0

Table 4. CEWAF #1 decline rates based on TPH

Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					100
0.0625 g/L	11.3	3.87	1.88	34	49	100
0.125 g/L	19.9	7.18	3.27	36	46	100
0.25 g/L	61.6	19.4	6.17	32	32	54
0.5 g/L	100.4	71.6		71		0
1 g/L	220.1	163.7		74		0

Table 5. CEWAF #2 decline rates based on TPH

Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					100
0.0625 g/L	12.780	1.274	0.804	10	63	100
0.125 g/L	14.209	1.842	1.368	13	74	100
0.25 g/L	55.072	4.876	2.050	9	42	58
0.5 g/L	67.267	36.500		54		0
1 g/L	289.938	41.063		14		0

Table 6. CEWAF #3 decline rates based on TPH

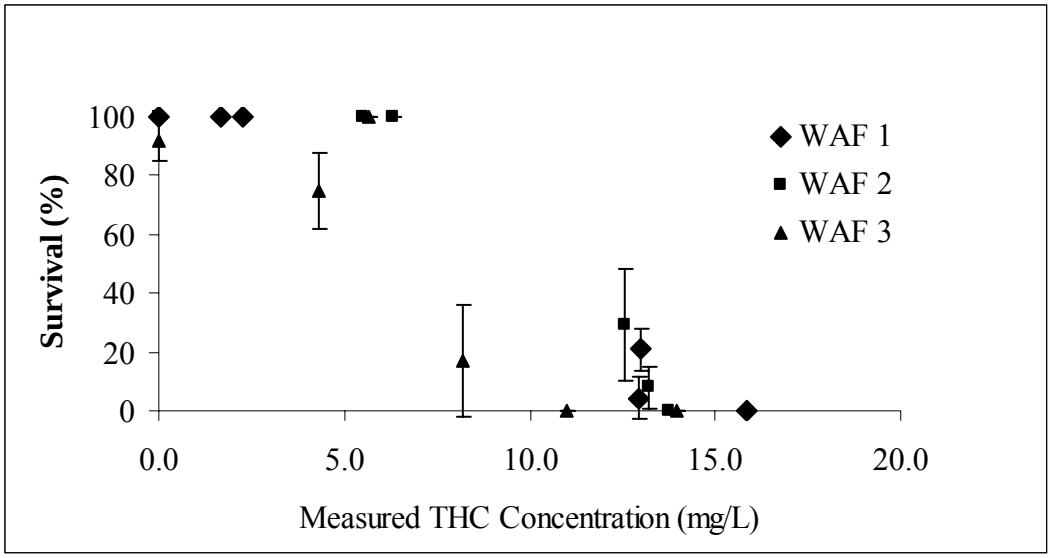
Oil Loading Concentration	T0	T24	T96	% of TO at T24	% of T24 at T96	Survival
0 g/L	0.000					92
0.0625 g/L	13.3	2.94	1.49	22	51	91
0.125 g/L	21.4	4.06	3.37	19	83	71
0.25 g/L	58.0	14.3	6.67	25	47	17
0.5 g/L	149	23.7		16		0
1 g/L	259	30.0		12		0

4.2 Acute Toxicity of WAF and CEWAF

THC ($C_6 - C_{36}$) in these experiments is defined as the sum of BTEX (C6–C9) plus total petroleum hydrocarbons (C_{10}–C_{36}; after Singer *et al.,* 1998). THC was quantified in Time 0 samples from all WAF and CEWAF tests and these were used to calculate 96-h LC_{50}s. Based on LC50s calculated using measures of THC in WAFs and CEWAFs, Chinook salmon pre-smolts were considerably more sensitive to WAFs of non-dispersed PBCO then to CEWAFs from oil treated with the dispersant Corexit 9500. The 96-h LC_{50}s for the three WAF tests were 6.5, 9.9 and 6.2 mg/L THC (mean WAF LC_{50} = 7.6 mg/L THC; Figure 1a). Figure 1b shows the relationship between pre-smolt survival and total PAH concentrations measured in the first and third WAF tests (see discussion below). The 96-h LC_{50}s for the three CEWAF tests were 60.5, 48.2 and 37.0 mg/L THC (mean CEWAF LC_{50} = 48.6 mg/L THC; Figure 2a.). Figure 2b shows the relationship between pre-smolt survival and total PAH concentrations measured in the first and third CEWAF tests (see discussion below).

Figure 1. a) Survival of Chinook salmon pre-smolts in 96-h WAF exposures relative to THC; b) Survival in WAF tests 1 and 3 relative to total PAH concentrations based on a summation of 13 parent PAH compounds.

a)



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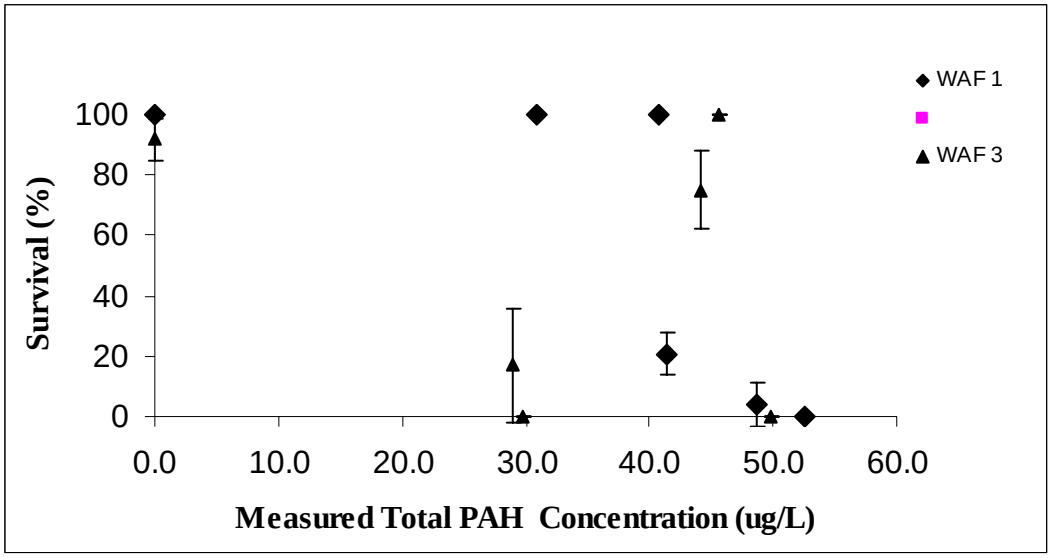
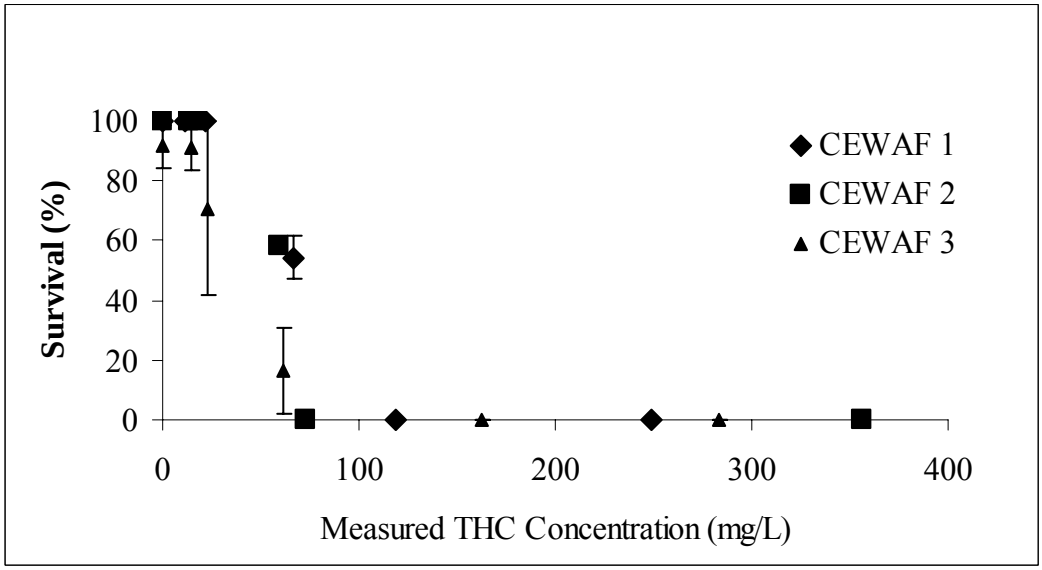


Figure 2. a) Survival of Chinook salmon pre-smolts in 96-h CEWAF exposures relative to THC; b) Survival in CEWAF tests 1 and 3 relative to total PAH concentrations based on a summation of 13 parent PAH compounds..

a)



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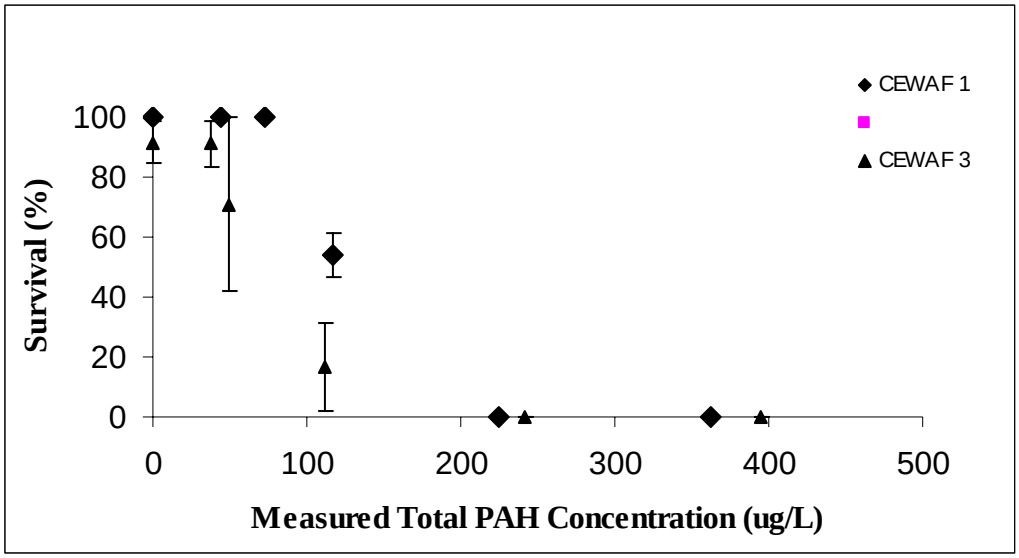
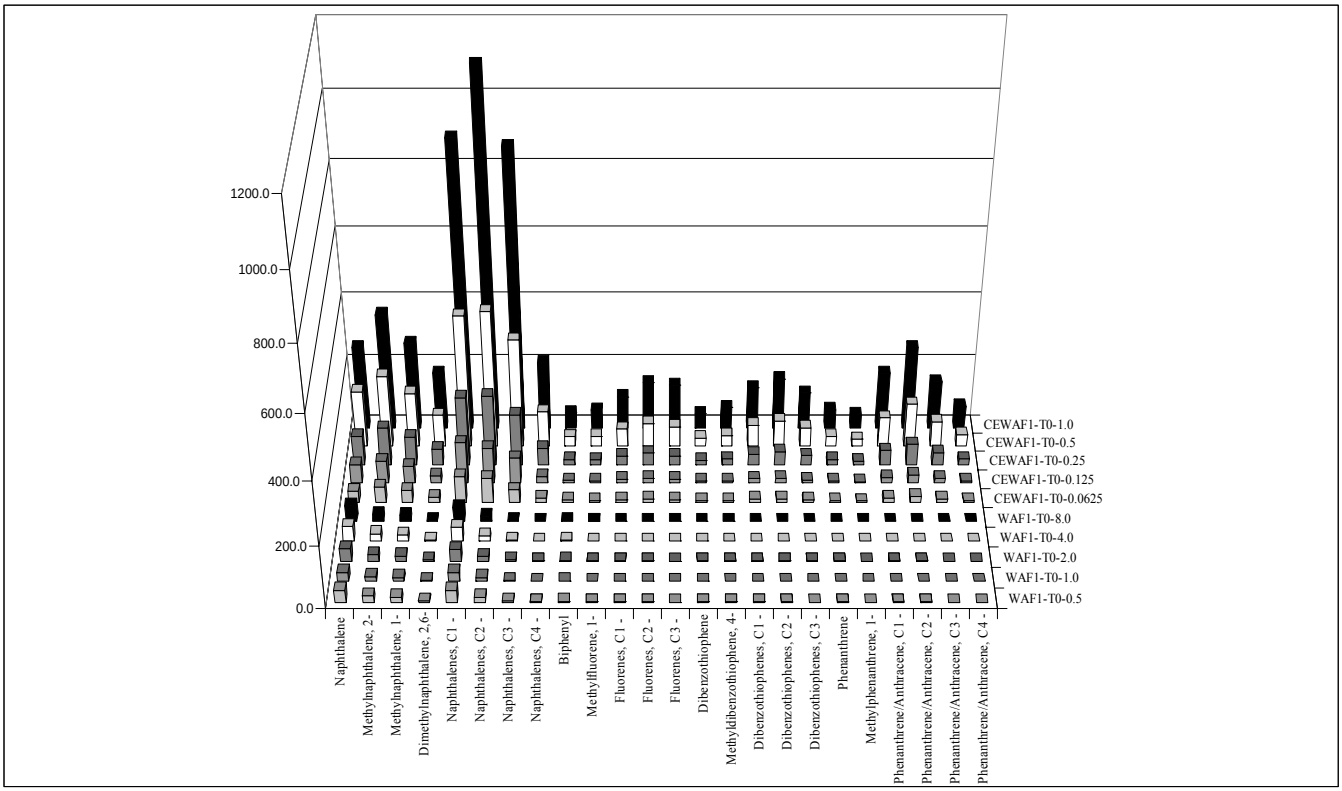


4.2.1 Relative Concentrations of PAHs in WAF and CEWAF Tests

Polynuclear aromatic hydrocarbons (PAHs) were measured in the first and third WAF and CEWAF experiments. Results for all PAH analyses are provided in Appendix III. As expected, much greater concentrations of PAHs were measured in the CEWAF solutions relative to the WAF solutions (Figure 3). The naphthalenes dominated the PAH totals in the CEWAF test, followed closely by the dibenzothiophenes and the phenanthrene/anthracenes.

Figure 3. Relative concentrations of selected PAHs in CEWAF test #1 relative to those in WAF test #1. PAHs in both experiments were measured in all 5 test PBCO loading concentrations at Time 0 (=T0). This figure depicts the dominant PAHs detected in these experiments; values for all measured PAHs are provided in Appendix III.



4.3 NMR Spectroscopy of Metabolites from Muscle Tissues

A representative ^{1}H spectrum of muscle extracts are presented in Figure 4. Major metabolites in all muscle tissue samples were assigned by comparison to tabulated chemical shifts and peak multiplicities in ^{1}H NMR spectra (Fan, 1996; Figure 12 and Table 7). Nucleotides (i.e. ATP) and several amino acids (i.e. alanine and histidine) were dominant in the spectra. Other metabolites included glycolytic products (i.e. lactate), citric acid cycle intermediates (i.e. succinate) and osmolytes (i.e. glycerophosphorylcholine).

Figure 4. Representative 1D ^{1}H NMR spectrum of pre-smolt muscle extracts.

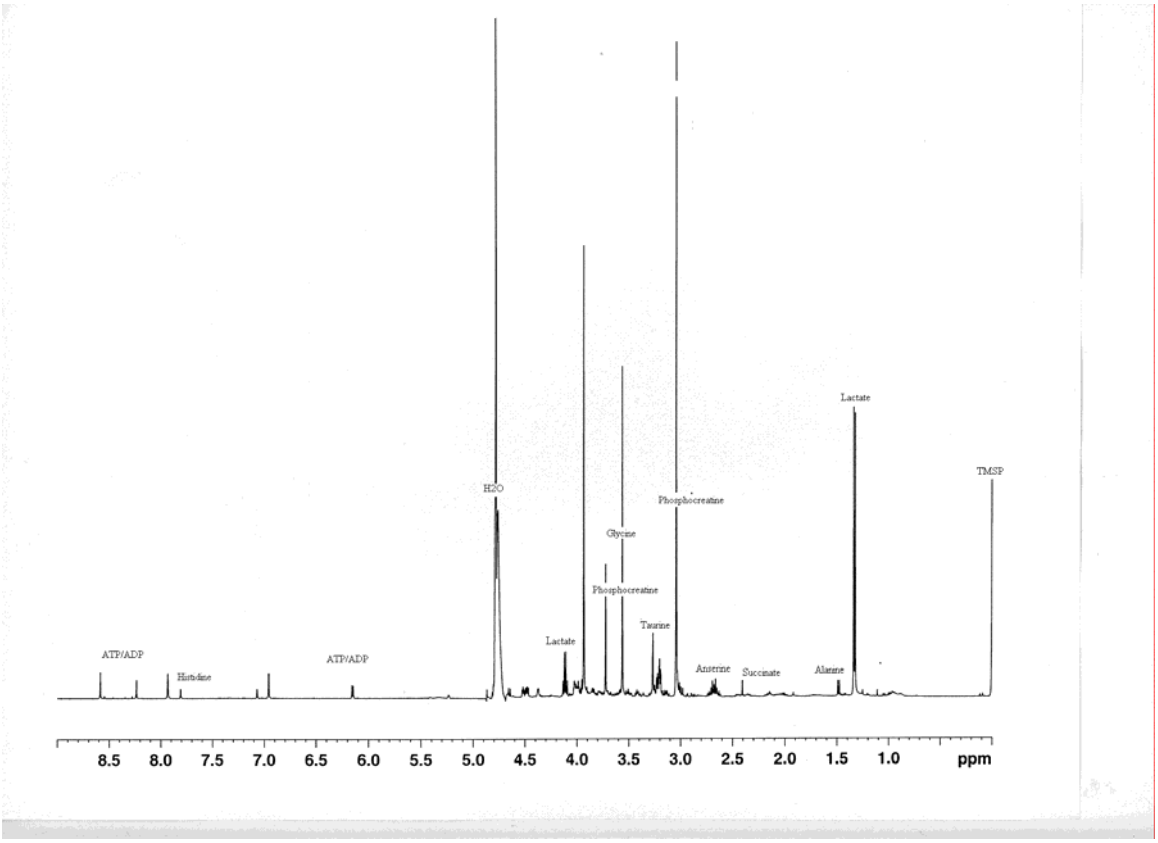


Table 7. Metabolites identified by NMR of muscle from salmon pre-smolts (a).

Metabolites	^{1}H NMR peak assignment (ppm) (b)
Lactate	1.33* (d), 4.115 (q)
Alanine	1.485* (d)
Glutamate	2.07 (m), 2.36* (t)
Succinate	2.41* (s)
Anserine	2.685 (m), 3.75 (s), 8.07 (s)
Phosphocreatine	3.04* (s), 3.935 (s)
Taurine	3.27 (t), 3.425* (t)
Glycerophosphorylcholine	3.36* (s)
Glycine	3.565* (s)
AMP	4.50 (m), 6.16 (d), 8.24 (s), 8.59* (s)
Histidine	7.09 (s), 7.87* (s)
ATP/ ADP	6.16 (d), 8.275 (s), 8.545* (s)

* Peaks are taken to quantify and compared between treatments.

(a) Metabolites have been identified to illustrate the range of metabolite classes detected by ^{1}H NMR.

(b) Peaks observed as a singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m)

4.4 NMR Spectroscopy of Metabolites from Liver Tissues

The ^{1}H NMR spectra of the liver samples contained many congested and overlapping peak areas. This may be caused by many macromolecules and lipids within the liver. These congested areas can bury many significant peaks and interfere with their peak assignments and integration. Tabulated chemical shifts, peak multiplicities and data from previous studies (Fan, 1996) were used to identify well resolved peaks within the ^{1}H and p-JRES spectra (Figure 5; Table 8). Metabolites such as lactate, alanine and taurine were abundant in the liver tissues. Other major metabolites identified included amino acids (glycine), nucleotides (ATP and NAD+) and carbohydrates (α-glucose).

Figure 5. Representative 2D-JRES spectrum of pre-smolt muscle extracts.

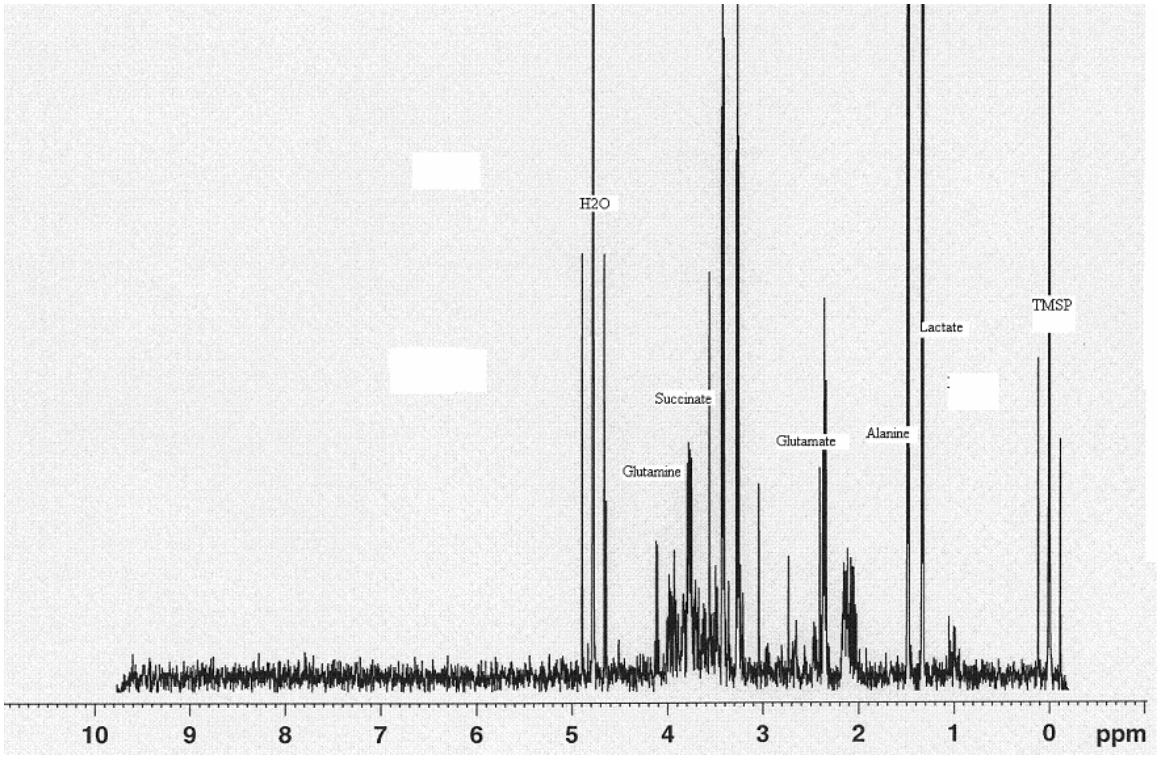


Table 8. Metabolites identified by NMR of liver from salmon pre-smolts (a).

Metabolites	^{1}H NMR peak assignment (ppm) (b)	p-JRES assignment (ppm)
Lactate	1.33* (d)	1.33 (d), 4.112 (q)
Alanine	1.485* (d)	1.48 (d)
Succinate	2.405* (s)	2.405 (s)
Glutamate		2.355 (t)
Phosphocreatine	3.04* (s), 3.935 (s)	3.04 (s)
Taurine	3.265 (t), 3.42* (t)	3.265 (t), 3.42 (t)
Glycerophosphorylcholine	3.36* (s)	3.359 (s)
Glycine	3.56* (s)	3.56 (s)
Glutamine		3.77 (m)
AMP	8.59* (s)	
α-glucose	5.25 (d)	
ATP/ ADP	8.28 (s), 8.55* (s)	
Formate	8.43* (s)	
NAD+/NADP+	8.84* (d), 9.15 (d), 9.34 (d)	

* Peaks are taken to quantify and compared between treatments.
(a) Metabolites have been identified to illustrate the range of metabolite classes detected by ^{1}H NMR.
(b) Peaks observed as a singlet (s), doublet (d), triplet (t), quartet (q), multiplet (m)

4.5 Dose-Response of Metabolic Profiles in Muscles of Pre-Smolts Exposed to WAF or CEWAF

The metabolic fingerprints provided sufficient information for determining metabolic effects. The ^{1}H NMR spectra of 3 WAF tests were analyzed by PCA, as it provides a graphical output, comprising of each sample, which summarizes the similarity of the NMR spectra. Each test (control and doses) was analyzed individually to avoid variation from experimental conditions and physical condition of the animal. Figure 6 provides examples of scores plots, showing metabolic dose-response information, for individual WAF tests.

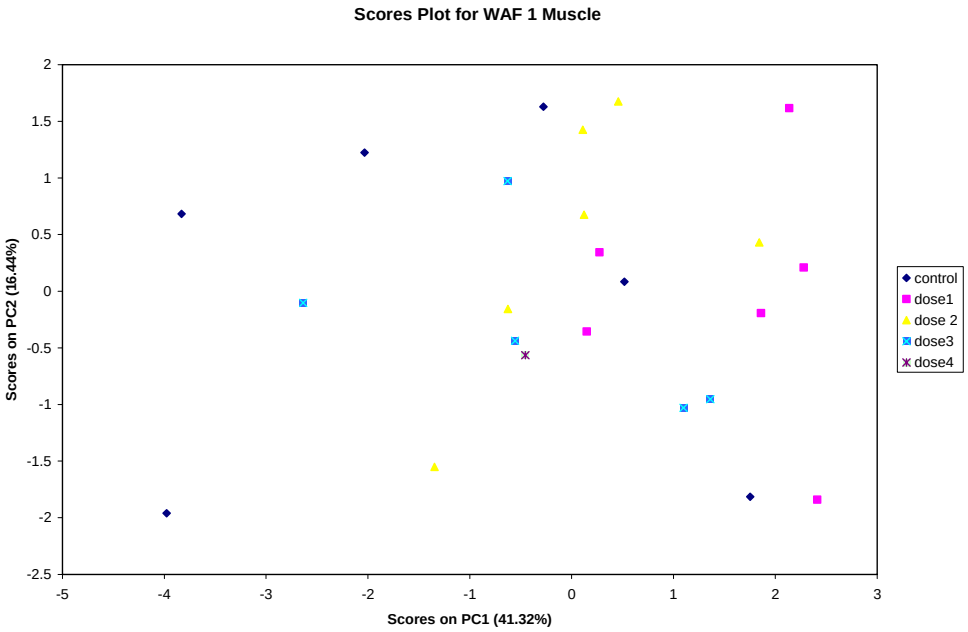
In the WAF1 test, there in not a good separation of metabolic profiles between the high and low doses. The doses, other than the control, tend to cluster together with positive PC1 scores. Animals treated with higher doses are separated from the lower doses in WAF2 and WAF3, along PC1 (41.55% and 40.03%). In WAF2, the animals treated with the higher doses cluster together with a negative PC1 score, while the control and animals treated with low doses have a positive PC1 score. In WAF3, there is not a good separation between metabolic profiles between the high and low doses along PC1, but along PC2, there is a separation. In WAF3, the control has a positive PC2 score, the high dose has a negative PC2 score, and the remaining doses lie closely to a PC2 score of zero.

The ^{1}H NMR spectra of 3 CEWAF tests were analyzed by PCA. Each test was analyzed individually to avoid any variation from the experiments and the physical state of the animal. Figure 7 provides the scores plots for the individual CEWAF tests. CEWAF1 and CEWAF2 have a significantly different dose-response in the metabolic profiles compared to CEWAF3. In

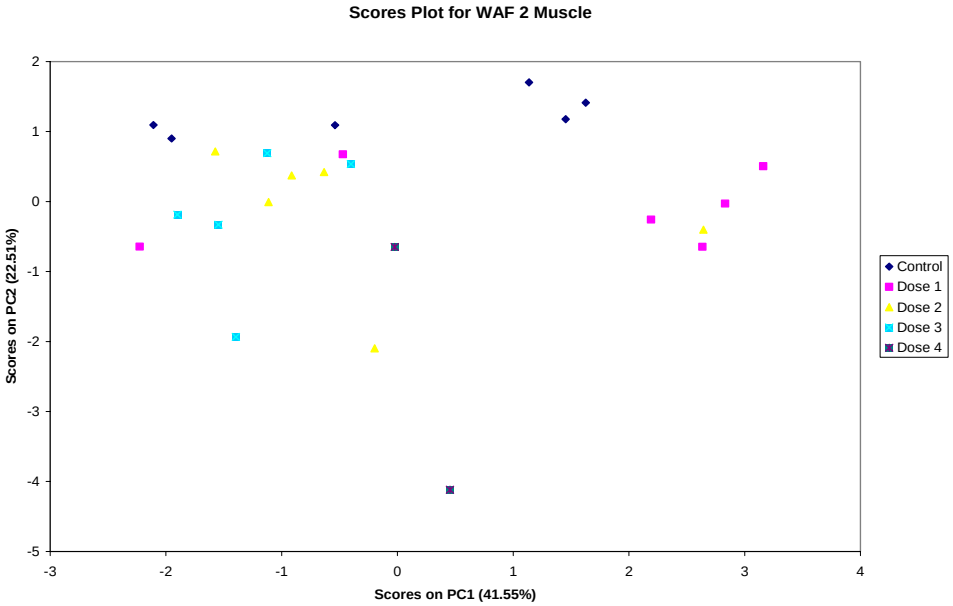
CEWAF1 the control scores cluster together, while in CEWAF2, all of the scores cluster together between a PC2 score of zero and negative one. For the individual tests, while the dose (in THC) increased, the PC1 scores decreased and the PC2 scores increased.

Figure 6. PCA scores plots from the analysis of the 1D ^{1}H NMR spectra of muscle from 3 individual WAF tests (a, b, c).

a.



b.



c.

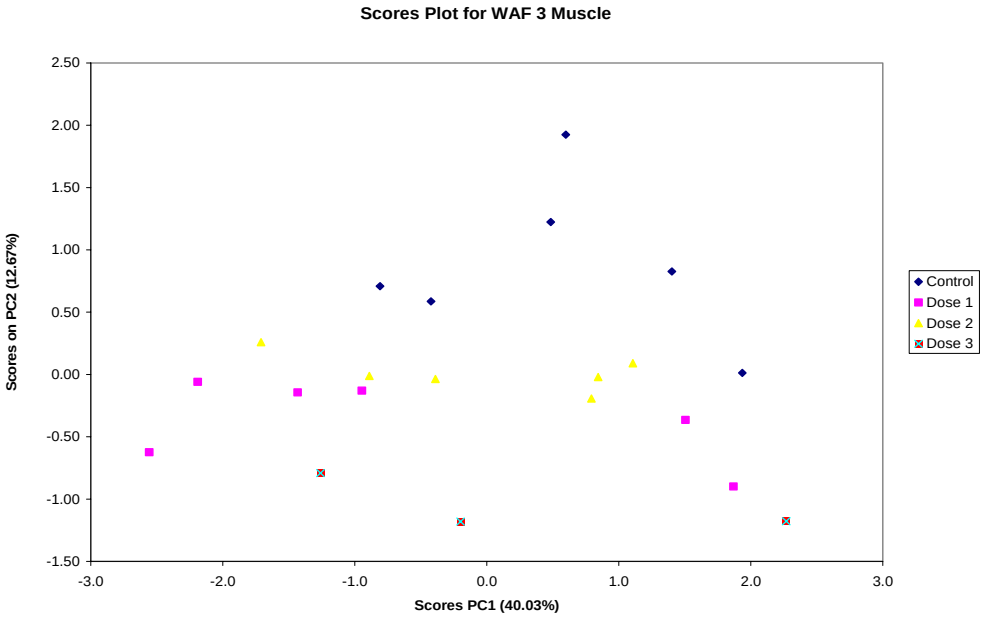
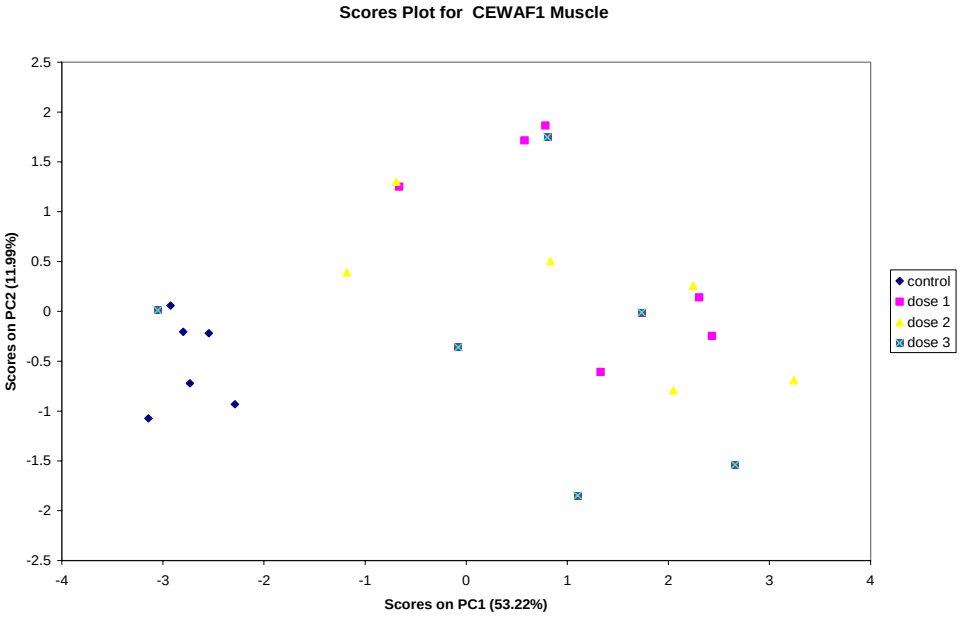
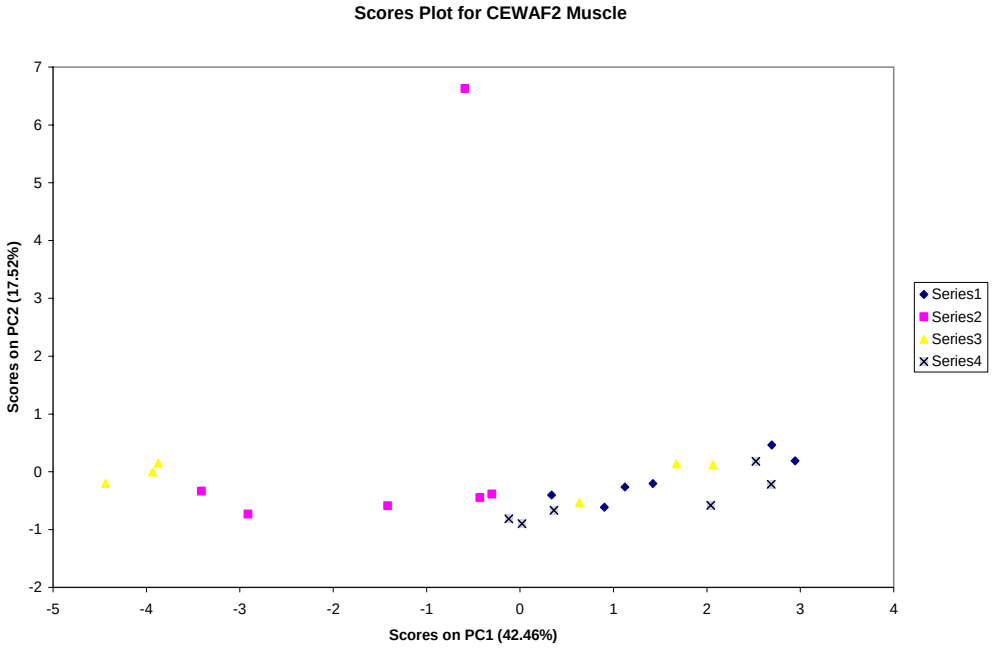


Figure 7. PCA scores plots from the analysis of the 1D ^{1}H NMR spectra of muscle from 3 individual CEWAF tests (a, b, c).

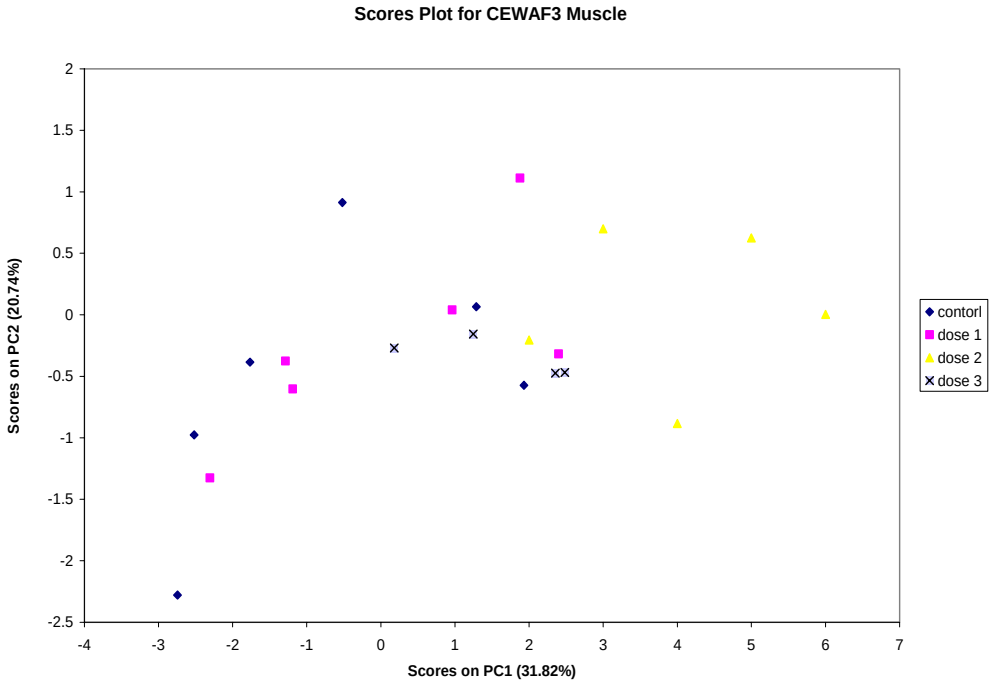
a.



b.


Scores Plot for CEWAF2 Muscle
Scores on PC2 (17.52%)
Scores on PC1 (42.46%)
Series1
Series2
Series3
Series4

c.

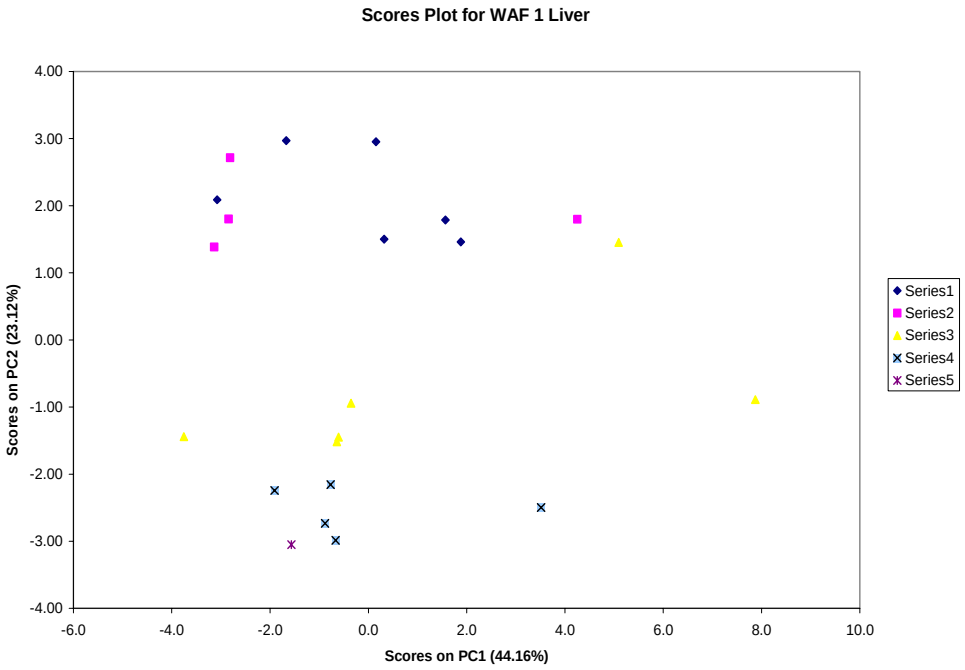

Scores Plot for CEWAF3 Muscle
Scores on PC2 (20.74%)
Scores on PC1 (31.82%)
contorl
dose 1
dose 2
dose 3

4.6 Dose-Response of Metabolic Profiles in Livers of Pre-smolts Exposed to WAF or CEWAF

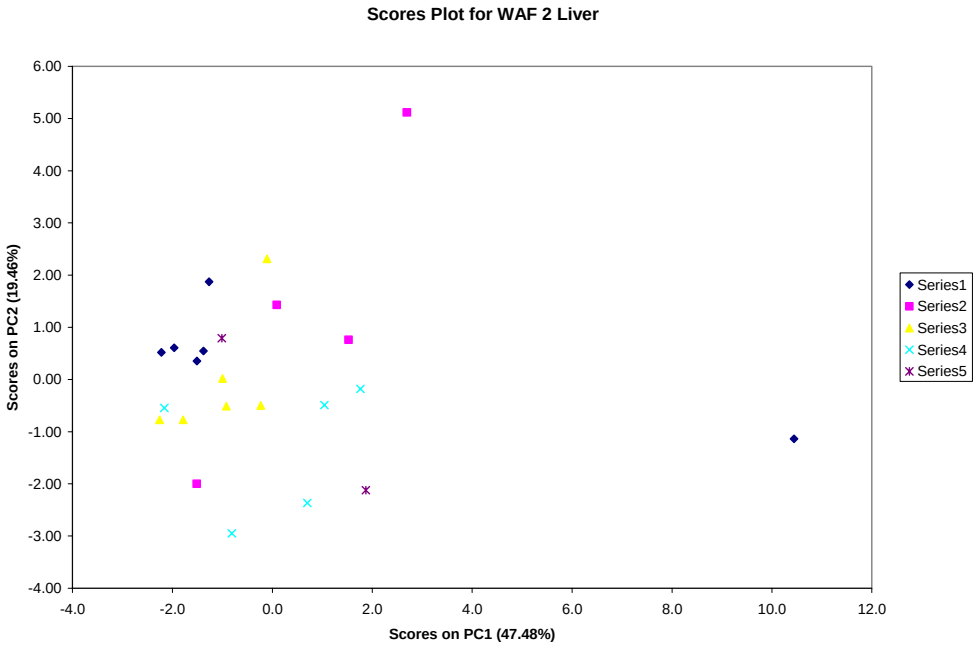
Since the liver tissue was relatively small, 1D and 2D JRES NMR was conducted. Although p-JRES spectra provide flatter baselines and better resolved peaks, the metabolic profiles from the 1D NMR spectra were analyzed using the same PCA method as the muscle samples. Major identified peaks in the 1D spectra were integrated and compared between each WAF dose (3 tests) and each CEWAF dose (3 tests). Figure 8 provides examples of scores plots, showing metabolic dose-response information, for individual WAF1 and WAF2 tests

Figure 8. PCA scores plots from the analysis of the 1D ^{1}H NMR spectra of liver from 3 individual WAF tests (a and b).

a.



b.



In the WAF1 test, there is a definite trend between doses. The control and low dose samples all cluster together with a positive PC2, while the higher doses cluster together with a negative PC2. For the WAF2 test, the control and all of the doses all cluster together. The metabolic profiles of animals treated with high doses are not comparably separate from those treated with low doses.

The ^{1}H NMR spectra of 3 CEWAF tests were analyzed by PCA. Each test was analyzed individually to avoid any variation from the experiments and the physical state of the animal. For all three tests, the PCA scores plots separate the metabolic profiles, but there is no trend of dose-response for the liver muscle exposed to CEWAF.

4.7 Specific Metabolic Changes in Muscles after Acute Exposures

PCA scores plots for individual WAF and CEWAF exposure showed metabolic dose-response. PCA loadings plots (Figure 9 and Figure 10) were constructed to identify peaks within the spectra that contributed to the variation in corresponding principle components. Peaks with a positive loading correspond to metabolites with higher levels within the control and peaks with negative loadings have higher levels within the treatment. Peaks which displayed the greatest change in the loadings plots were identified. The identified peaks within the loadings plots may be beneficial in determining possible biomarker development. Well resolved peaks were selected for further integration and compared between doses of 3 WAF and 3 CEWAF tests.

Results of the two-way ANOVA analysis are presented in the tables containing the metabolic changes (Tables 9-16). These tables summarize the metabolic changes and p values of identified metabolites in muscle and liver tissues, according to the doses for each WAF and CEWAF test.

The changes in specific metabolites were calculated by taking the ratio of the average peak area of the treatment over the average peak area of the control for each test (Table 9 and Table 10). The metabolic changes indicate that an increase or decrease of a metabolite, within the muscle tissue, may be dose, treatment and metabolite dependent. A summary of metabolic changes in muscle tissues due to increasing WAF and CEWAF doses is provided in Figure11. Specific changes include the increase of glutamate and taurine after WAF exposure and the decrease of alanine and glycine after CEWAF exposure. This indicates that the metabolic changes among doses are metabolite dependent. Statistically significant changes, within muscle tissue, were observed at all doses of WAF for glutamate ($p<0.05$), at low doses (i.e. lactate $p<0.05$), and intermediate doses (i.e. succinate, taurine and ATP/ADP $p<0.05$). Significant changes were also observed at low doses of CEWAF (i.e. alanine $p<0.05$), intermediate doses (i.e. taurine $p<0.05$) and high doses (i.e. lactate $p<0.05$). The change in taurine is significant in both the intermediate doses of WAF and CEWAF.

Figure 9. PC1 loadings plot from the analysis of the 1D ^{1}H NMR of muscle tissue from pre-smolts exposed to WAF. Metabolic assignments: 1. AMP, 2. ATP/ADP, 3. histidine, 4. taurine, 5. phosphocreatine. 6. succinate, 7. glutamtate, 8. alanine, 9. lactate.

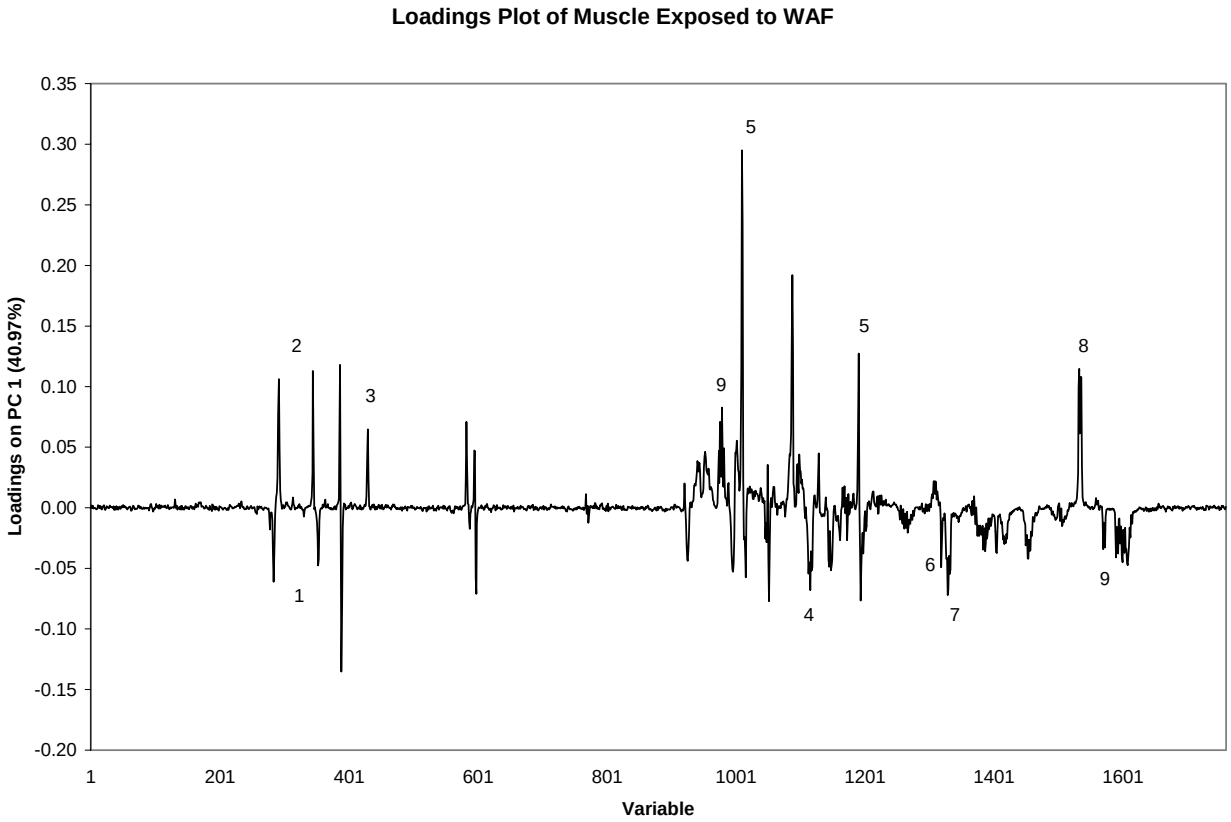


Figure 10. PC1 loadings plot from the analysis of the 1D ^{1}H NMR of muscle tissue from pre-smolts exposed to CEWAF. Metabolic assignments: 1. AMP, 2. ATP/ADP, 3. histidine, 4. taurine, 5. phosphocreatine. 6. succinate, 7. glutamtate, 8. alanine, 9. lactate

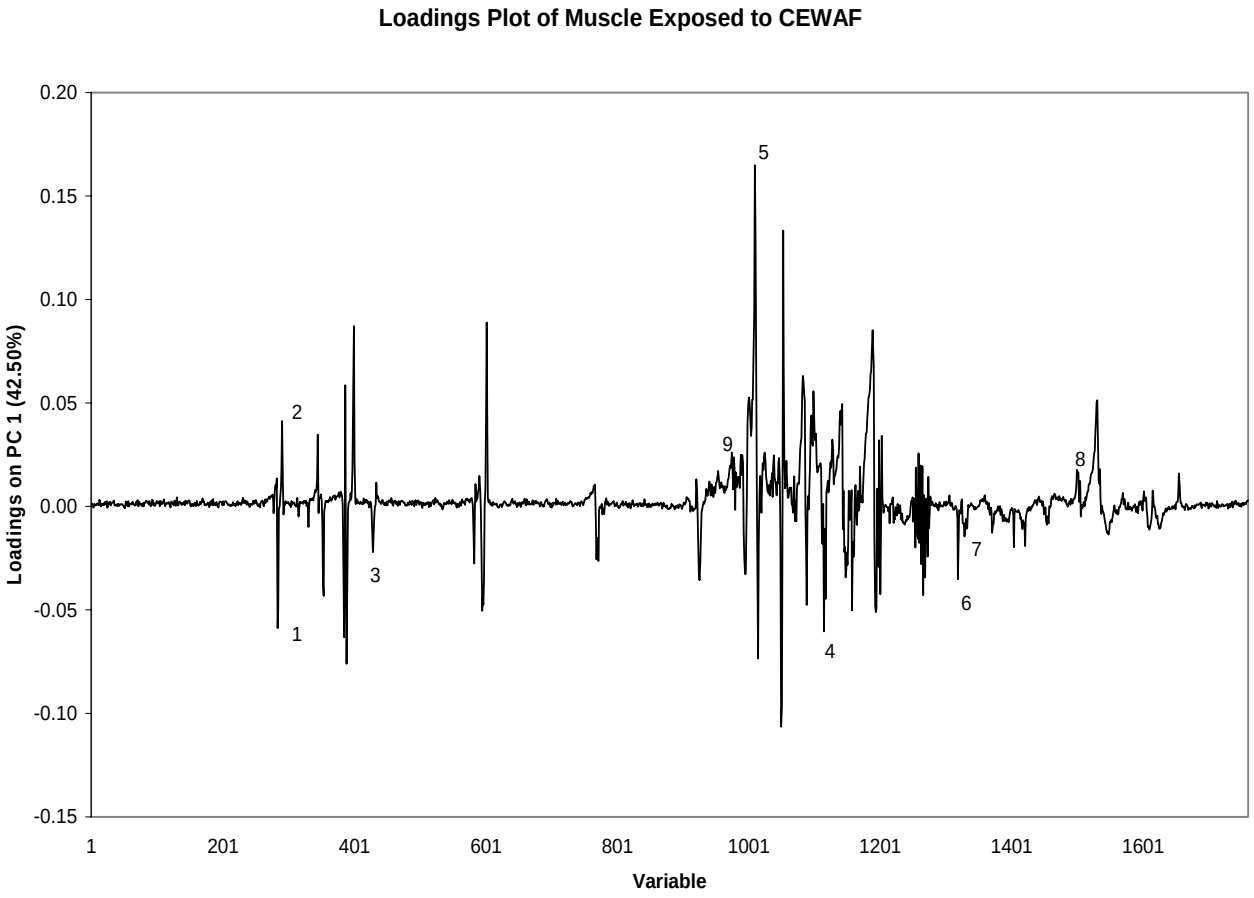


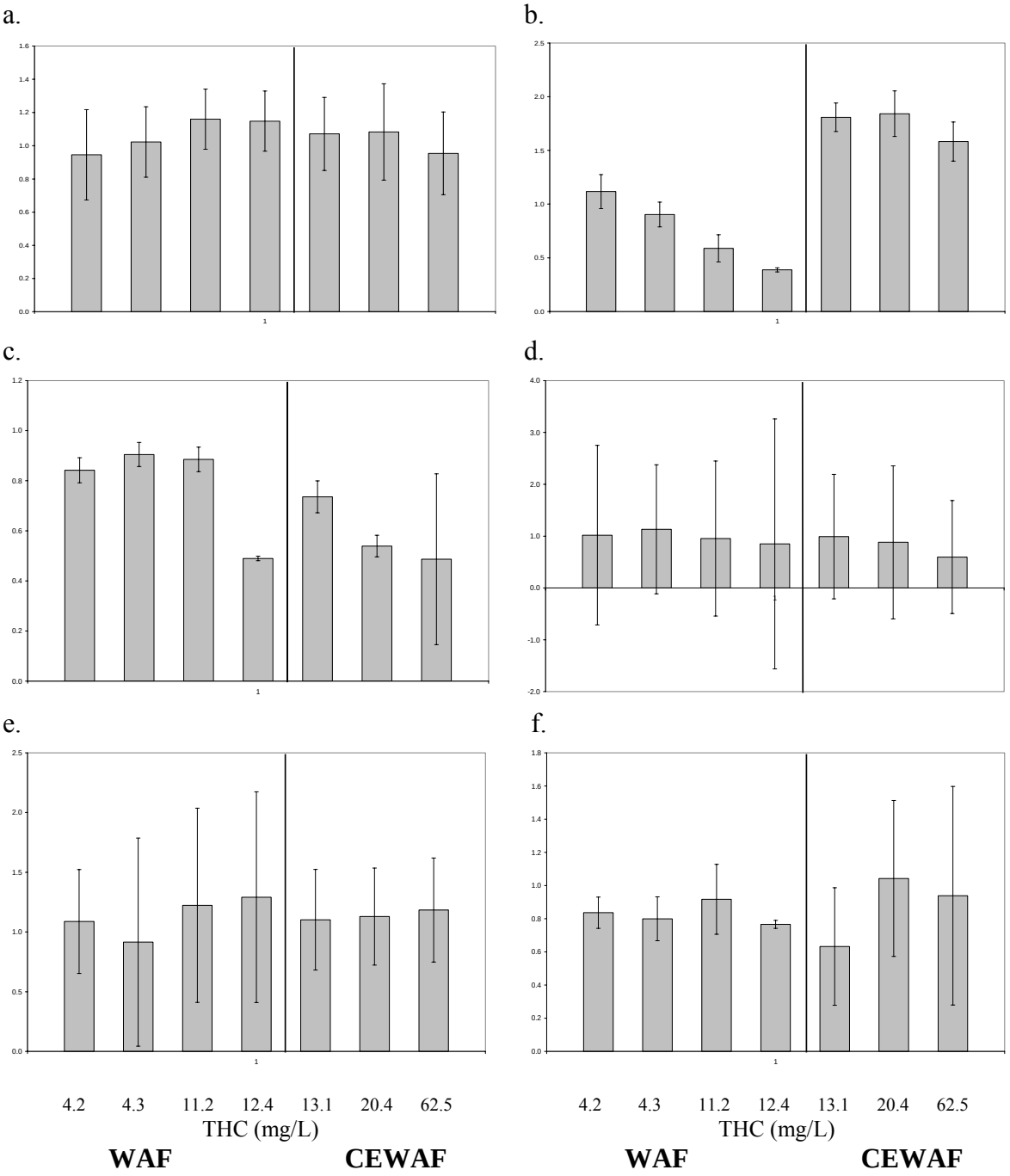
Table 9. Metabolic Changes in the muscle of pre-smolts exposed to WAF for 96-h including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	4.276 mg/L (p)	4.296 mg/L (p)	11.243 mg/L (p)	12.379 mg/L (p)
Lactate	1.33* (d)	0.94511 (0.012)	1.02266 (0.085)	1.15970 (0.041)	1.14753 (0.111)
Alanine	1.485* (d)	1.11670 (0.864)	0.90298 (0.325)	0.58748 (0.331)	0.38799 (0.124)
Glutamate	2.36* (t)	0.84192 (0.004)	0.90480 (<0.001)	0.88545 (<0.001)	0.49003 (0.013)
Succinate	2.41* (s)	1.01852 (0.502)	1.13030 (0.012)	0.95374 (0.003)	0.84998 (0.722)
Phosphocreatine	3.04* (s)	1.08733 (0.543)	0.91564 (0.850)	1.22373 (0.791)	1.29116 (0.275)
Taurine	3.425* (t)	0.83626 (0.077)	0.79953 (0.558)	0.91690 (0.002)	0.76614 (0.075)
Glycerophosphoryl-choline	3.36* (s)	1.01203 (0.145)	0.99641 (0.095)	0.99385 (0.557)	0.94224 (0.421)
Glycine	3.565* (s)	1.02658 (0.918)	1.14418 (0.112)	1.23278 (0.504)	0.97026 (0.372)
AMP	8.59* (s)	1.17836 (0.566)	1.38191 (0.764)	1.43642 (0.105)	1.60674 (0.554)
Histidine	7.87* (s)	1.00209 (0.322)	0.96656 (0.530)	0.96395 (0.579)	0.85597 (0.159)
ATP/ ADP	8.545* (s)	0.90027 (0.688)	1.00032 (0.372)	0.89664 (0.007)	0.74089 (0.134)

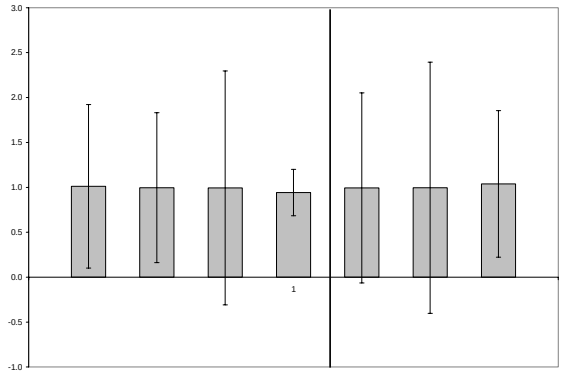
Table 10. Metabolic Changes in the muscle of pre-smolts exposed to CEWAF for 96-h including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	13.142 mg/L (p)	20.421 mg/L (p)	62.463 mg/L (p)
Lactate	1.33* (d)	1.07099 (0.911)	1.08270 (0.465)	0.95367 (0.007)
Alanine	1.485* (d)	1.80847 (0.001)	1.84173 (0.061)	1.58276 (0.151)
Glutamate	2.36* (t)	0.73589 (0.063)	0.53930 (<0.001)	0.48677 (0.002)
Succinate	2.41* (s)	0.99085 (0.543)	0.88055 (0.103)	0.59516 (0.407)
Phosphocreatine	3.04* (s)	1.10216 (0.507)	1.12916 (0.684)	1.18339 (0.014)
Taurine	3.425* (t)	0.63220 (0.061)	1.04262 (0.013)	0.93848 (0.003)
Glycerophosphoryl-choline	3.36* (s)	0.99376 (0.047)	0.99548 (0.176)	1.03813 (0.242)
Glycine	3.565* (s)	0.97522 (0.906)	0.98293 (0.230)	1.01157 (<0.001)
AMP	8.59* (s)	1.13088 (0.251)	1.18922 (0.364)	1.24935 (0.325)
Histidine	7.87* (s)	1.20640 (0.045)	1.14800 (<0.001)	0.93057 (0.814)
ATP/ ADP	8.545* (s)	0.91101 (0.676)	0.85457 (0.561)	0.82605 (0.828)

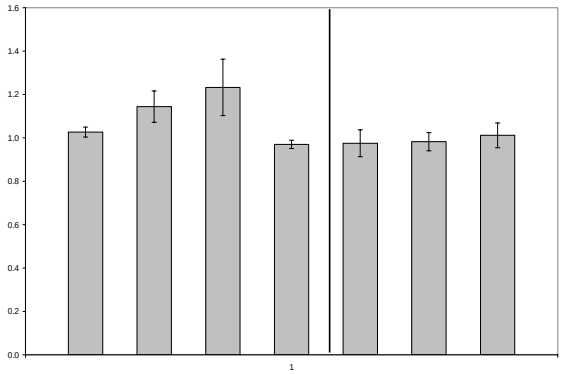
Figure 11. Changes in specific metabolites, in muscle tissue, after 96-h WAF and CEWAF exposure, include AMP (a), ATP/ADP (b), histidine (c), glycine (d), taurine (e), glycerophosphorylcholine (f), phosphocreatine (g), succinate (h), glutamtate (i), alanine (j), lactate (k).



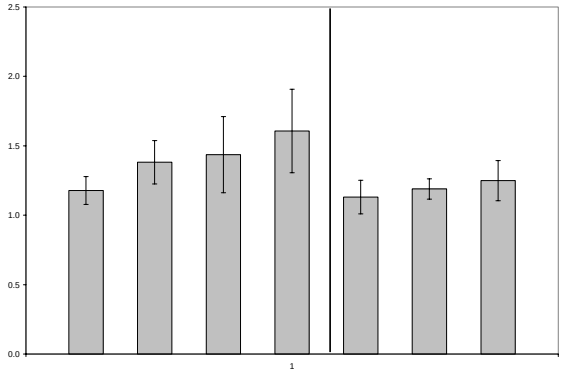
g.



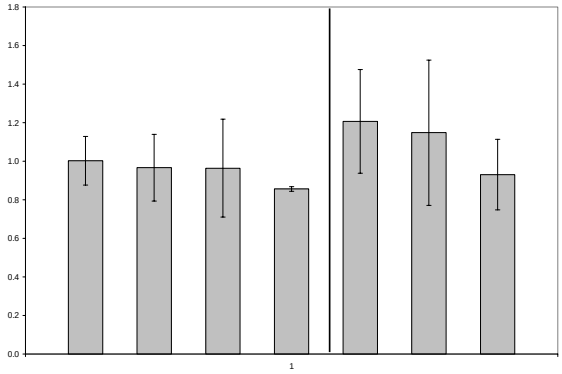
h.



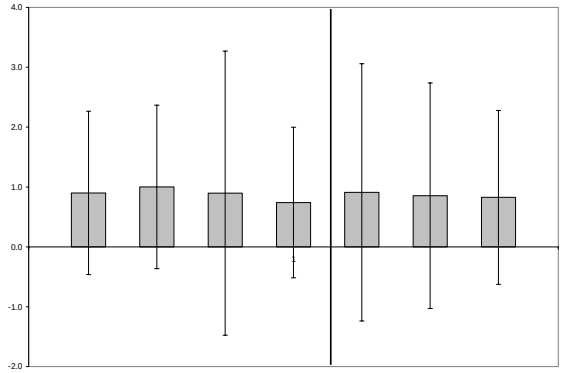
i.



j.



k.



4.2 4.3 11.2 12.4 13.1 20.4 62.5

THC (mg/L)

WAF **CEWAF**

4.2 4.3 11.2 12.4 13.1 20.4 62.5

THC (mg/L)

WAF **CEWAF**

4.8 Specific Metabolic Changes in Liver after Acute Exposures

PCA scores and loadings plots were constructed to identify samples and peaks within the spectra that contributed to the variation in corresponding principle components. Peaks which displayed the greatest change in the loadings plots were identified. The changes in specific metabolites

were calculated by taking the ratio of the average peak area of the treatment over the average peak area of the control for each test (Tables 11 and 12).

A summary of metabolic changes in liver tissues due to increasing WAF and CEWAF doses is provided in Figure12. Specific changes include the increase of formate and succinate after WAF exposure and the decrease of lactate and glycine after CEWAF exposure. This indicates that the metabolic changes among doses are metabolite dependent.

Statistically significant changes, within liver tissue, were observed at the low dose of WAF (i.e. NAD+/ NADP+ $p<0.05$), intermediate doses (i.e. formate $p<0.05$) and high dose (i.e. glycerophosphorylcholine $p<0.05$). Significant changes were also observed at low doses of CEWAF (i.e. alanine $p<0.05$), intermediate doses (i.e. succinate $p<0.05$) and high doses (i.e. phosphocreatine $p<0.05$). The change in formate is significant in both the intermediate and high doses of WAF and CEWAF.

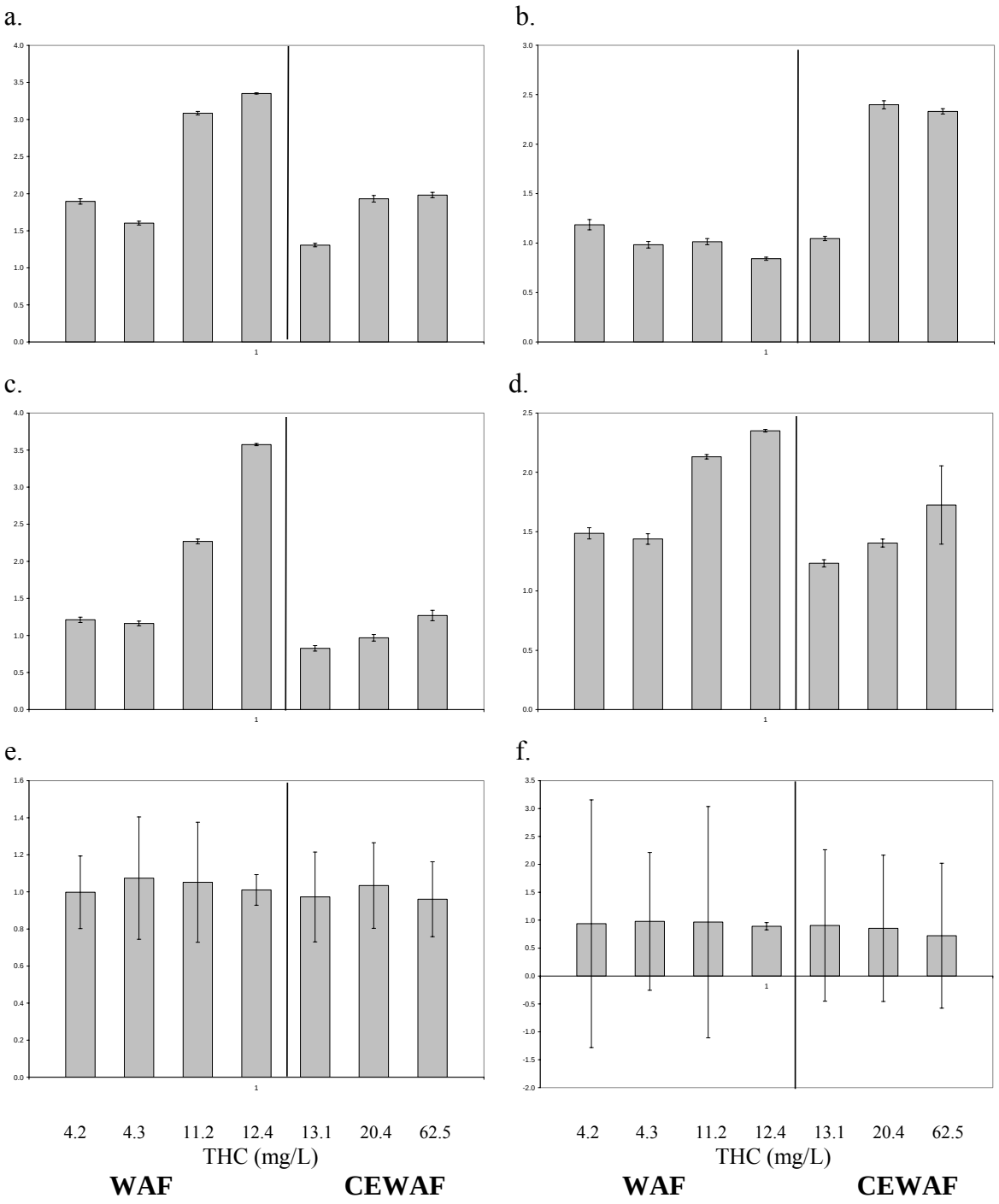
Table 11. Metabolic Changes in the liver of pre-smolts exposed to WAF for 96-h including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	4.276 mg/L (p)	4.296 mg/L (p)	11.243 mg/L (p)	12.379 mg/L (p)
Lactate	1.33* (d)	1.89559 (0.611)	1.60310 (0.094)	3.08472 (0.087)	3.35157 (0.258)
Alanine	1.485* (d)	1.18535 (0.941)	0.98381 (0.361)	1.01349 (0.225)	0.8416 (0.823)
Succinate	2.41* (s)	1.21143 (0.887)	1.16247 (0.573)	2.26805 (0.148)	3.57384 (0.317)
Phosphocreatine	3.04* (s)	1.48555 (0.359)	1.43839 (0.876)	2.13100 (0.256)	2.35026 (0.157)
Taurine	3.425* (t)	0.99801 (0.143)	1.07450 (0.838)	1.05191 (0.578)	1.01049 (0.480)
Glycerophosphoryl-choline	3.36* (s)	0.93682 (0.372)	0.98013 (0.174)	0.96564 (0.887)	0.89159 (0.001)
Glycine	3.565* (s)	1.26139 (0.915)	2.05956 (0.302)	1.04823 (0.463)	2.10072 (0.860)
AMP	8.59* (s)	1.08666 (0.333)	1.00535 (0.592)	1.08384 (0.808)	1.18680 (0.689)
Formate	8.43* (s)	1.01442 (0.082)	1.02682 (0.046)	1.12624 (<0.001)	1.22898 (0.060)
ATP/ ADP	8.545* (s)	1.02236 (0.824)	0.89880 (0.440)	0.84822 (0.001)	0.96124 (0.002)
NAD+/NADP+	8.84* (d)	1.03727 (0.036)	0.82634 (0.032)	0.76133 (<0.001)	0.65506 (0.093)

Table 12. Metabolic Changes in the liver of pre-smolts exposed to CEWAF for 96-h including two-way ANOVA p values (significant, $p<0.05$).

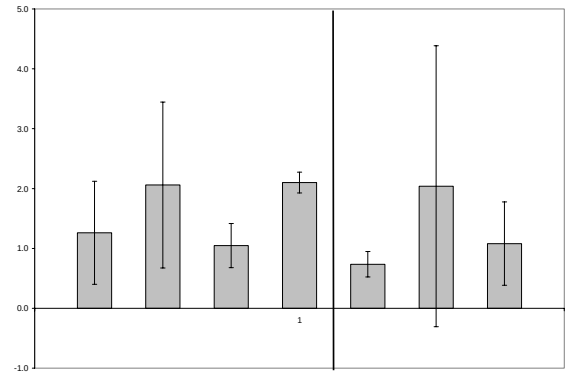
Metabolites	Chemical shift (ppm)b	13.142 mg/L (p)	20.421 mg/L (p)	62.463 mg/L (p)
Lactate	1.33* (d)	1.30660 (0.560)	1.93117 (0.165)	1.98126 (0.164)
Alanine	1.485* (d)	1.04568 (0.032)	2.39834 (0.023)	2.33158 (0.215)
Succinate	2.36* (t)	0.82675 (0.759)	0.96813 (0.032)	1.26885 (0.293)
Phosphocreatine	2.41* (s)	1.23339 (0.005)	1.40406 (0.151)	1.72469 (0.003)
Taurine	3.04* (s)	0.97284 (0.570)	1.03436 (0.108)	0.96043 (<0.001)
Glycerophosphoryl-choline	3.425* (t)	0.90534 (0.008)	0.85454 (0.441)	0.72114 (0.500)
Glycine	3.36* (s)	0.73553 (0.990)	2.03807 (0.745)	1.08090 (0.647)
AMP	3.565* (s)	1.10996 (0.852)	1.06848 (0.013)	1.15902 (0.005)
Formate	8.59* (s)	1.07407 (0.582)	1.11152 (0.061)	0.69033 (<0.001)
ATP/ ADP	7.87* (s)	1.23599 (0.589)	1.19976 (0.990)	1.05334 (0.061)
NAD+/NADP+	8.545* (s)	0.98832 (0.888)	0.98333 (0.036)	1.08251 (0.005)

Figure 12 Changes in specific metabolites in liver tissue, after 96-h exposure to WAF and CEWAF, include NAD+/ NADP+ (a), AMP (b), ATP/ADP (c), formate (d), glycine (e), taurine (f), glycerophosphorylcholine (g), phosphocreatine (h), succinate (i), alanine (j), lactate (k).



g.

h.



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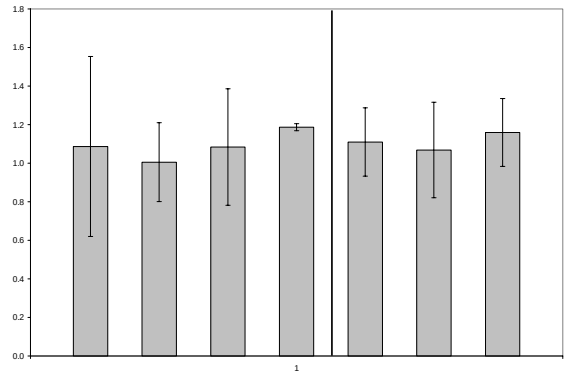

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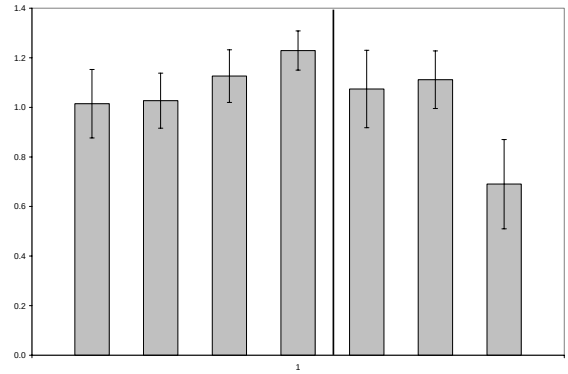

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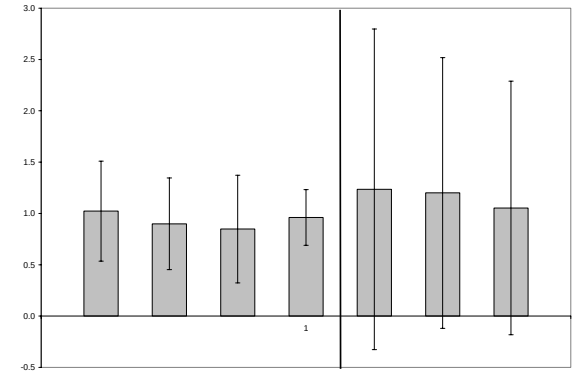

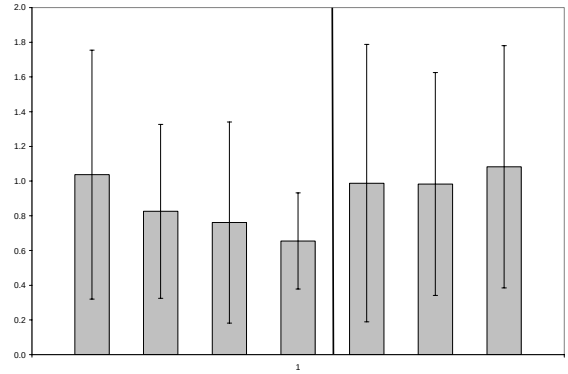

4.2 4.3 11.2 12.4 13.1 20.4 62.5

THC (mg/L)

WAF **CEWAF**

4.9 Specific Metabolic Changes in Muscles after Long-term Growth

Muscle tissue from the long-term growth fish were analyzed in the same manner as for the acute exposures. PCA scores plots and loadings plots were constructed and peaks which displayed the greatest change in the loadings plots were identified. Metabolic changes were also determined by calculating the ratio of the average peak area of the treatment over the average peak area of the control for each test (Tables 13 and 14). The metabolic changes indicate that an increase or

decrease of a metabolite, within the muscle tissue, may be dose, treatment and metabolite dependent. A summary of metabolic changes in muscle tissues due to increasing WAF and CEWAF doses is provided in Figure13. Specific changes include the increase of histidine, and taurine after WAF exposure and the decrease of succinate after CEWAF exposure

Statistically-significant changes, within muscle tissue, were observed at the low dose of WAF (i.e. glycine $p<0.05$), intermediate doses (i.e glutamate $p<0.05$) and high dose (i.e. taurine $p<0.05$). Significant changes were also observed at the low dose of CEWAF (i.e. AMP $p<0.05$) and intermediate dose (i.e. taurine $p<0.05$). The change in taurine is significant in both the intermediate and high doses of WAF and CEWAF.

Table 13. Metabolic Changes in the muscle of long-term grow out pre-smolts after being exposed to WAF for 96-h including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	4.276 mg/L (p)	4.296 mg/L (p)	11.243 mg/L (p)	12.379 mg/L (p)
Lactate	1.33* (d)	1.10914 (0.415)	1.27696 (0.972)	1.12029 (0.043)	1.04479 (0.247)
Alanine	1.485* (d)	1.48218 (0.627)	1.55321 (0.136)	1.36315 (0.757)	0.51935 (0.196)
Glutamate	2.36* (t)	0.82834 (0.048)	0.96500 (0.026)	1.06796 (0.841)	0.69355 (0.449)
Succinate	2.41* (s)	1.23715 (0.791)	1.18694 (0.407)	0.94061 (0.275)	0.70989 (0.431)
Phosphocreatine	3.04* (s)	0.82607 (0.345)	0.68983 (0.125)	1.27011 (0.449)	1.64964 (0.841)
Taurine	3.425* (t)	0.84637 (0.106)	0.62927 (0.134)	0.48550 (0.293)	0.99750 (0.048)
Glycerophosphoryl-choline	3.36* (s)	1.04433 (0.512)	1.07541 (0.246)	1.08010 (0.245)	1.00003 (0.267)
Glycine	3.565* (s)	1.02459 (0.016)	1.10329 (0.116)	1.03191 (0.403)	0.89992 (0.136)
AMP	8.59* (s)	0.90729 (0.615)	0.78670 (0.141)	1.10514 (0.287)	0.88814 (0.397)
Histidine	7.87* (s)	0.99481 (0.076)	0.86680 (0.820)	1.13665 (0.885)	0.68107 (0.177)
ATP/ ADP	8.545* (s)	1.19934 (0.327)	1.15663 (0.668)	0.93306 (0.203)	0.99886 (0.457)

Table 14. Metabolic Changes in the muscle of long-term grow out pre-smolts after being exposed to CEWAF for 96-h, including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	13.142 mg/L (p)	20.421 mg/L (p)	62.463 mg/L (p)
Lactate	1.33* (d)	1.34409 (0.984)	1.39022 (0.927)	1.32782 (0.201)
Alanine	1.485* (d)	0.65630 (0.721)	0.81273 (0.862)	0.75797 (0.939)
Glutamate	2.36* (t)	1.16822 (0.655)	1.15164 (0.803)	1.19421 (0.321)
Succinate	2.41* (s)	0.91138 (0.203)	1.02300 (0.829)	0.90882 (0.246)
Phosphocreatine	3.04* (s)	1.24778 (0.940)	1.24789 (0.885)	1.51751 (0.325)
Taurine	3.425* (t)	0.87344 (0.094)	0.87108 (0.029)	1.28732 (0.620)
Glycerophosphoryl-choline	3.36* (s)	0.99779 (0.085)	1.00568 (0.022)	0.96487 (0.707)
Glycine	3.565* (s)	1.12487 (0.108)	1.00730 (0.888)	1.03863 (0.609)
AMP	8.59* (s)	1.03933 (0.020)	1.01584 (0.034)	1.15807 (0.281)
Histidine	7.87* (s)	0.96231 (0.568)	1.05972 (0.075)	0.94195 (0.205)
ATP/ ADP	8.545* (s)	0.99947 (0.011)	1.02242 (0.107)	0.99356 (0.166)

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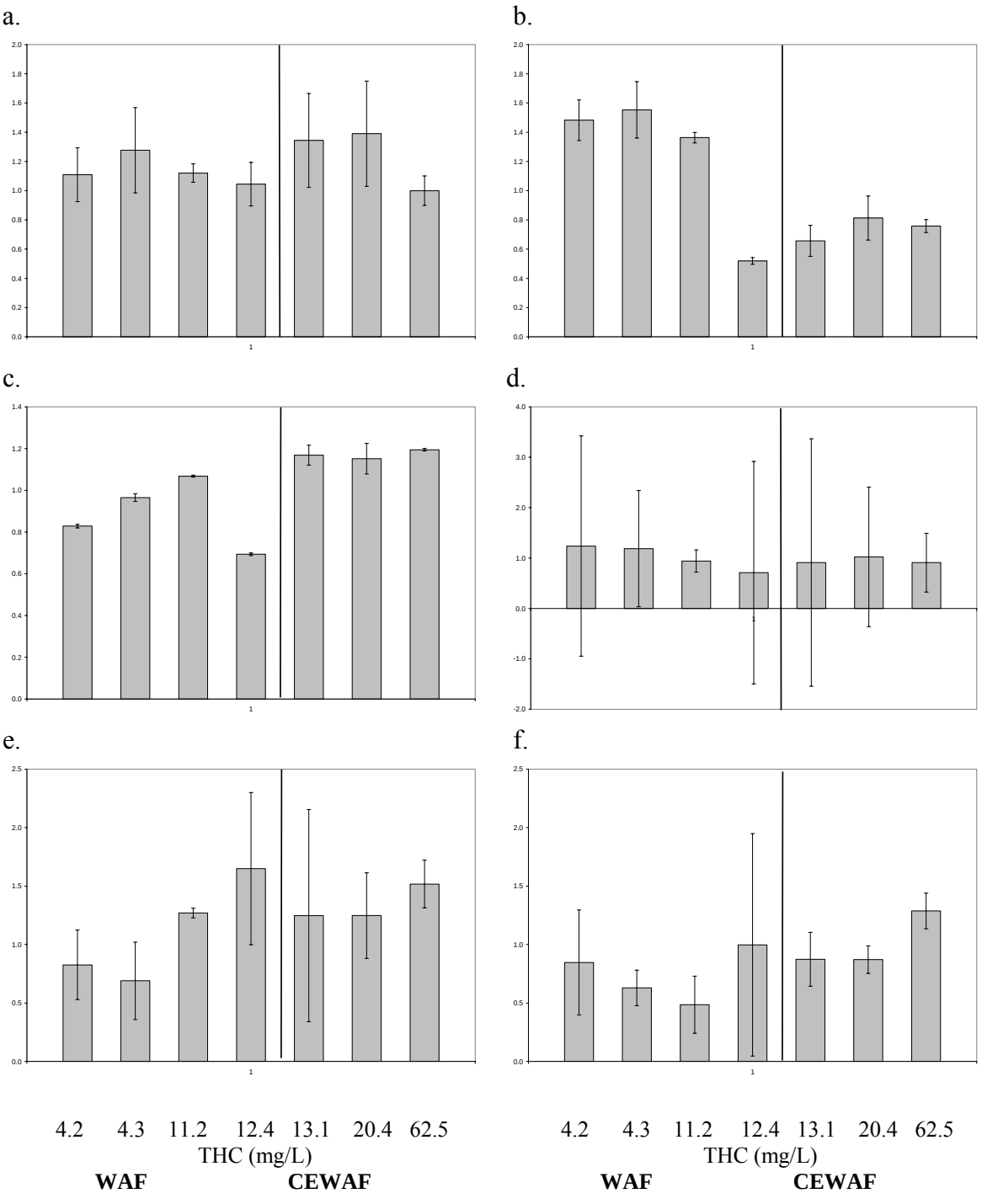
Figure 13. Changes in metabolites in muscle tissue after long-term grow out include AMP (a), ATP/ADP (b), histidine (c), glycine (d), taurine (e), glycerophosphorylcholine (f), phosphocreatine (g), succinate (h), glutamtate (i), alanine (j), lactate (k).



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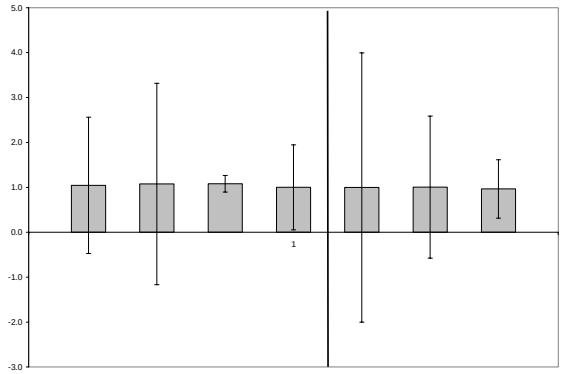

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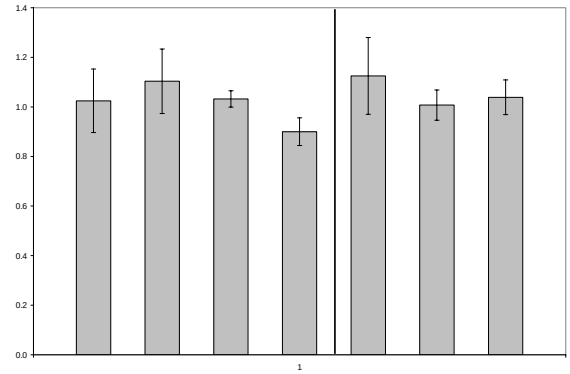

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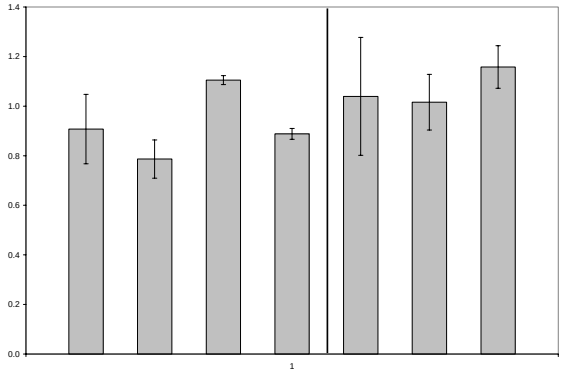
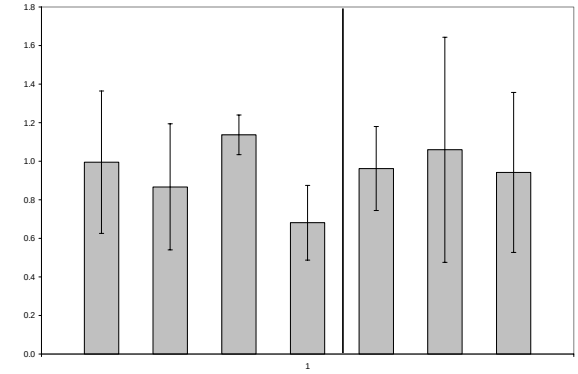


j.



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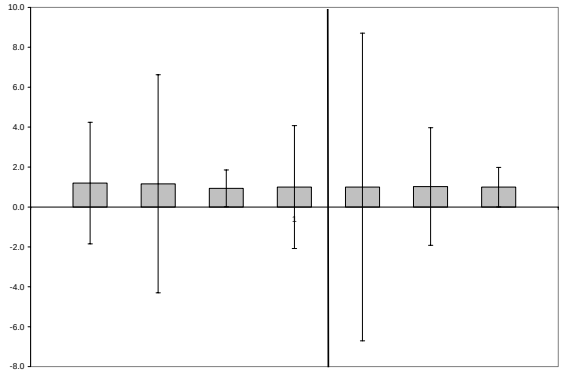


4.2 4.3 11.2 12.4 13.1 20.4 62.5

THC (mg/L)

WAF **CEWAF**

4.10 Specific Metabolic Changes in Liver after Long-Term Growth

Liver tissue from the long-term growth fish were analyzed in the same manner as for the acute exposures. PCA scores plots and loadings plots were constructed and metabolic changes were determined by calculating the ratio of the average peak area of the treatment over the average peak area of the control for each test (Tables 15 and 16). A summary of metabolic changes in

liver tissues due to increasing WAF and CEWAF doses are provided in Figure14. Specific changes include the increase of glycerophosphorylcholine and phosphocreatine after WAF exposure and the decrease of glycerophosphorylcholine after CEWAF exposure.

Statistically-significant changes, within liver tissue, were observed at the intermediate doses of WAF (i.e. glycerophosphorylcholine and taurine, $p<0.05$). Significant changes were also observed at the intermediate dose of CEWAF (i.e. AMP, $p<0.05$) and high dose (i.e. succinate, $p<0.05$). The change in taurine was significant in the intermediate dose of WAF, but was not significant in any of the CEWAF doses.

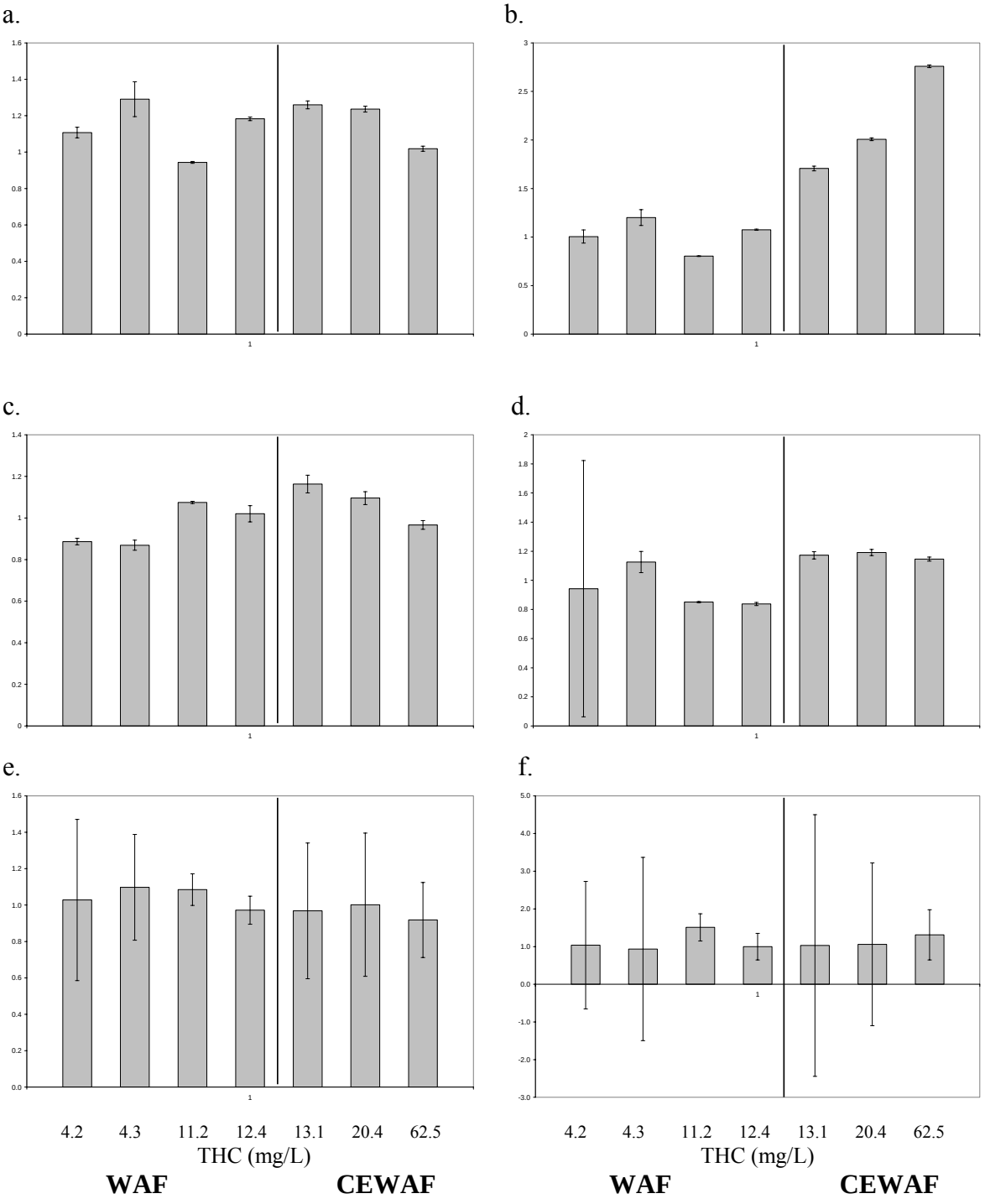
Table 15. Metabolic Changes in the liver of long-term grow out pre-smolts after being exposed to WAF for 96-h, including two-way ANOVA p values (significant, $p<0.05$).

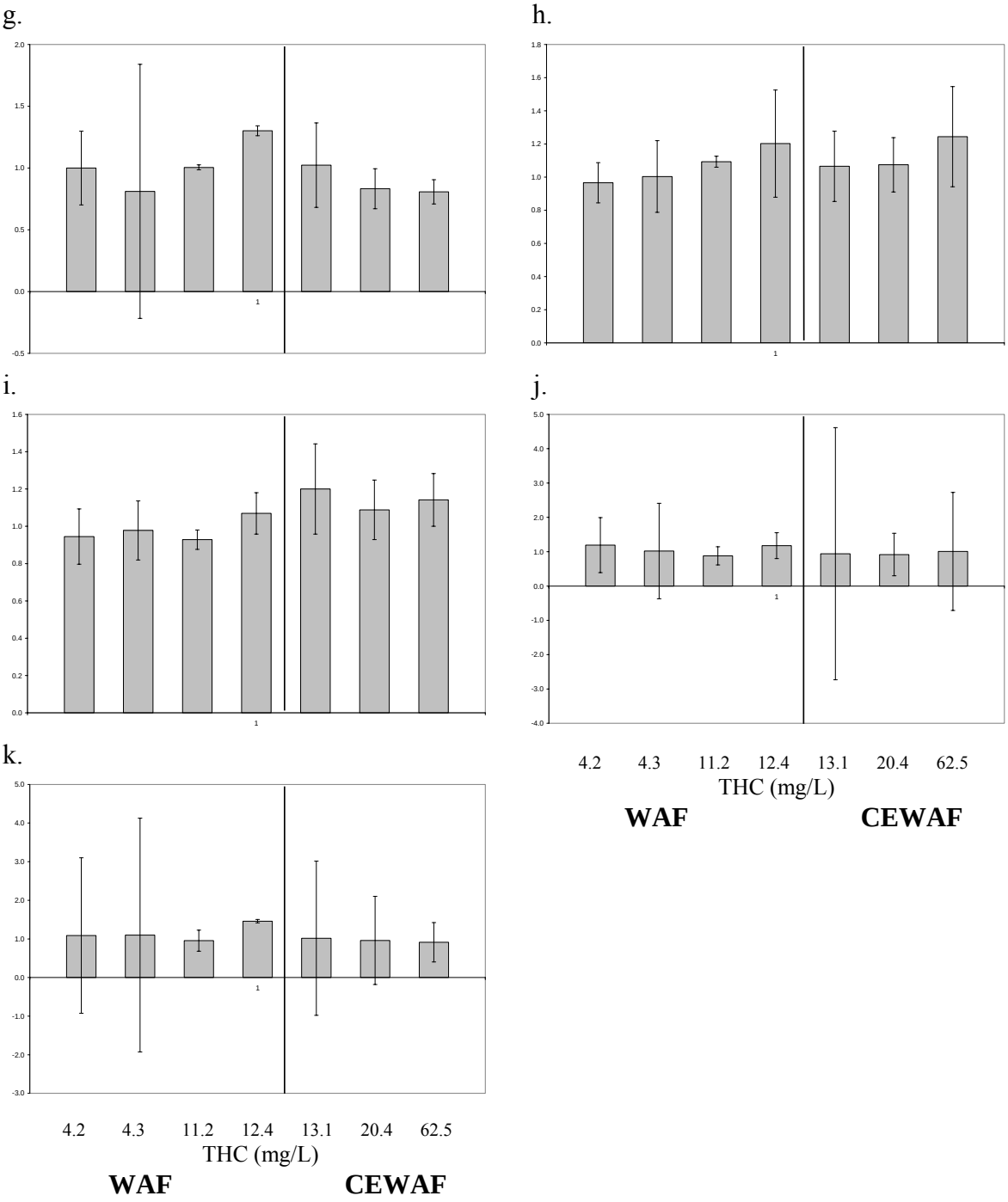
Metabolites	Chemical shift (ppm)b	4.276 mg/L (p)	4.296 mg/L (p)	11.243 mg/L (p)	12.379 mg/L
Lactate	1.33* (d)	1.10726 (0.260)	1.29102 (0.448)	0.94348 (0.547)	1.18294
Alanine	1.485* (d)	1.00528 (0.079)	1.20089 (0.920)	0.80321 (0.297)	1.07522
Succinate	2.41* (s)	0.88646 (0.478)	0.86885 (0.776)	1.07454 (0.498)	1.02040
Phosphocreatine	3.04* (s)	0.94234 (0.377)	1.12596 (0.933)	0.85075 (0.091)	0.83778
Taurine	3.425* (t)	1.02785 (0.889)	1.09758 (0.553)	1.08525 (0.008)	0.97216
Glycerophosphoryl-choline	3.36* (s)	1.03764 (0.820)	0.93548 (0.040)	1.51017 (0.742)	0.99805
Glycine	3.565* (s)	0.99999 (0.752)	0.81114 (0.109)	1.00490 (0.403)	1.30190
AMP	8.59* (s)	0.96617 (0.898)	1.00382 (0.350)	1.09291 (0.475)	1.20239
Formate	8.43* (s)	0.94506 (0.590)	0.97789 (0.242)	0.92845 (0.254)	1.06946
ATP/ ADP	8.545* (s)	1.19135 (0.280)	1.01869 (0.131)	0.87830 (0.531)	1.17551
NAD+/NADP+	8.84* (d)	1.08773 (0.440)	1.09880 (0.127)	0.95370 (0.769)	1.45926

Table 16. Metabolic changes in the liver of long-term grow out pre-smolts after being exposed to CEWAF for 96-h, including two-way ANOVA p values (significant, $p<0.05$).

Metabolites	Chemical shift (ppm)b	13.142 mg/L (p)	20.421 mg/L (p)	62.463 mg/L (p)
Lactate	1.33* (d)	1.25951 (0.931)	1.23646 (0.434)	1.01894 (0.275)
Alanine	1.485* (d)	1.70660 (0.442)	2.00759 (0.163)	2.75893 (0.988)
Succinate	2.36* (t)	1.16309 (0.081)	1.09549 (0.176)	0.96661 (0.015)
Phosphocreatine	2.41* (s)	1.17219 (0.339)	1.19126 (0.116)	1.14603 (0.436)
Taurine	3.04* (s)	0.96835 (0.814)	1.00214 (0.261)	0.91817 (0.290)
Glycerophosphoryl-choline	3.425* (t)	1.02963 (0.856)	1.06060 (0.002)	1.31067 (0.553)
Glycine	3.36* (s)	1.02394 (0.790)	0.83264 (0.627)	0.80769 (0.338)
AMP	3.565* (s)	1.06541 (0.217)	1.07465 (0.004)	1.24384 (0.042)
Formate	8.59* (s)	1.20039 (0.583)	1.08851 (0.223)	1.14155 (0.477)
ATP/ ADP	7.87* (s)	0.93994 (0.496)	0.91570 (0.305)	1.00725 (0.350)
NAD+/NADP+	8.545* (s)	1.01774 (0.401)	0.96135 (0.303)	0.91330 (0.896)

Figure 14. Changes in metabolites in liver tissue after long-term grow out include NAD+/NADP+ (a), AMP (b), ATP/ ADP (c), formate (d), glycine (e), taurine (f), glycerophosphorulcholine (g), phosphocreatine (h), succinate (i), alanine (j), lactate (k).





4.11 Long-Term Growth

One goal of this study was to assess impacts of short-term exposures to WAF and CEWAF on long-term growth of salmon pre-smolts. Surviving pre-smolts from all WAF and CEWAF tests were weighed upon test termination and placed in 3-ft diameter culture tanks for long-term growth and metabolomic analyses. Final growth measurements were completed on all WAF and CEWAF cultures as of November 2, 2007; these data are presented in Figures 4-9. Despite the

mortality associated with chronic bacterial and pathogen infections in these cultures, sufficient fish remained to allow growth measures over approx. 90 days. As described above, these fish required continued treatments with antibiotics and formalin until the final month of culture, when survival rates stabilized.

Continual growth (as wet weight) was observed in all control fish from all tests, except for control fish from the third WAF test (Figure 17). The reason for the lack of control growth in this test is not clear; like all of the cultures, these fish were fed by hand using the same feed and feeding rates as the other cultures. Growth data from the three WAF tests do not indicate any consistent residual effects of short-term WAF exposure on long-term growth of salmon smolts (Figures 15-17). Surviving fish from 2 g/L oil loading treatment from WAF #2 did not grow (Figure 16), but there were insufficient fish in this culture to draw conclusions regarding oil effects. The majority of fish from the CEWAF tests also grew at rates roughly similar to the control fish (Figure 18-20). The one exception was lower growth observed in fish from the 0.25 g/L loading in the CEWAF #2 (Figure 19).

Figure 15. Growth of smolts 99 days after 96-h exposure of pre-smolts to WAF #1.

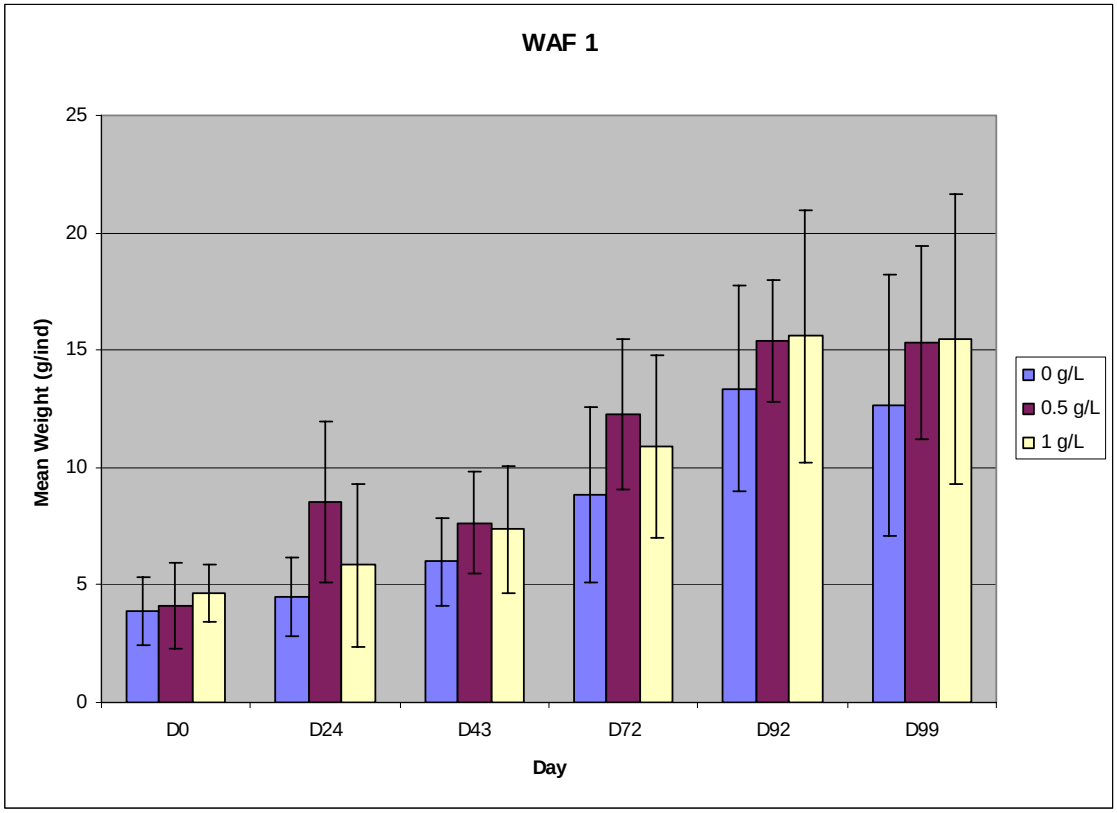


Figure 16. Growth of smolts 94 days after 96-h exposure of pre-smolts to WAF #2.

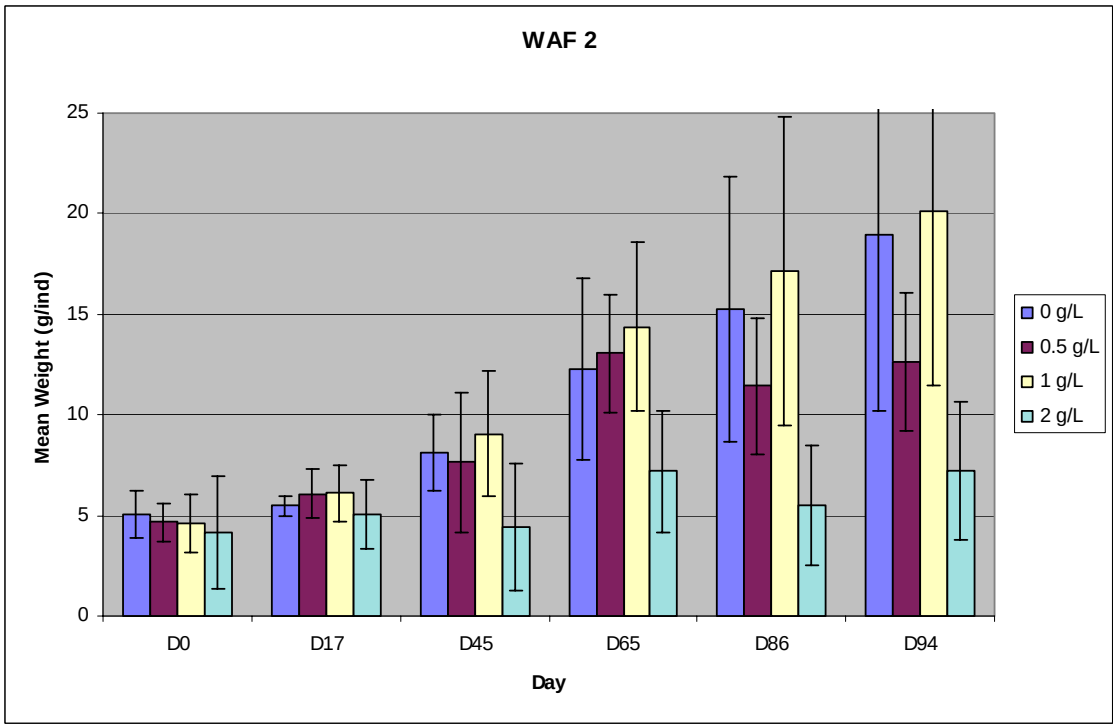


Figure 17. Growth of smolts 94 days after 96-h exposure of pre-smolts to WAF #3.

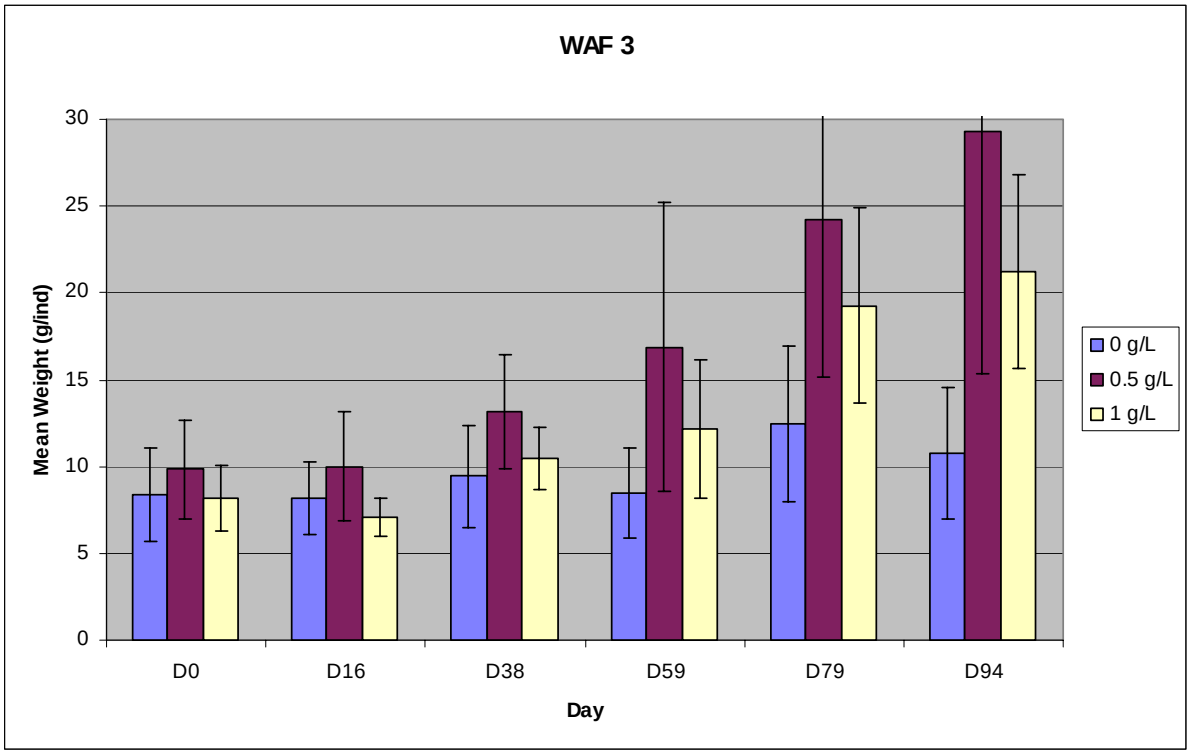


Figure 18. Growth of smolts 97 days after 96-h exposure of pre-smolts to CEWAF #1.

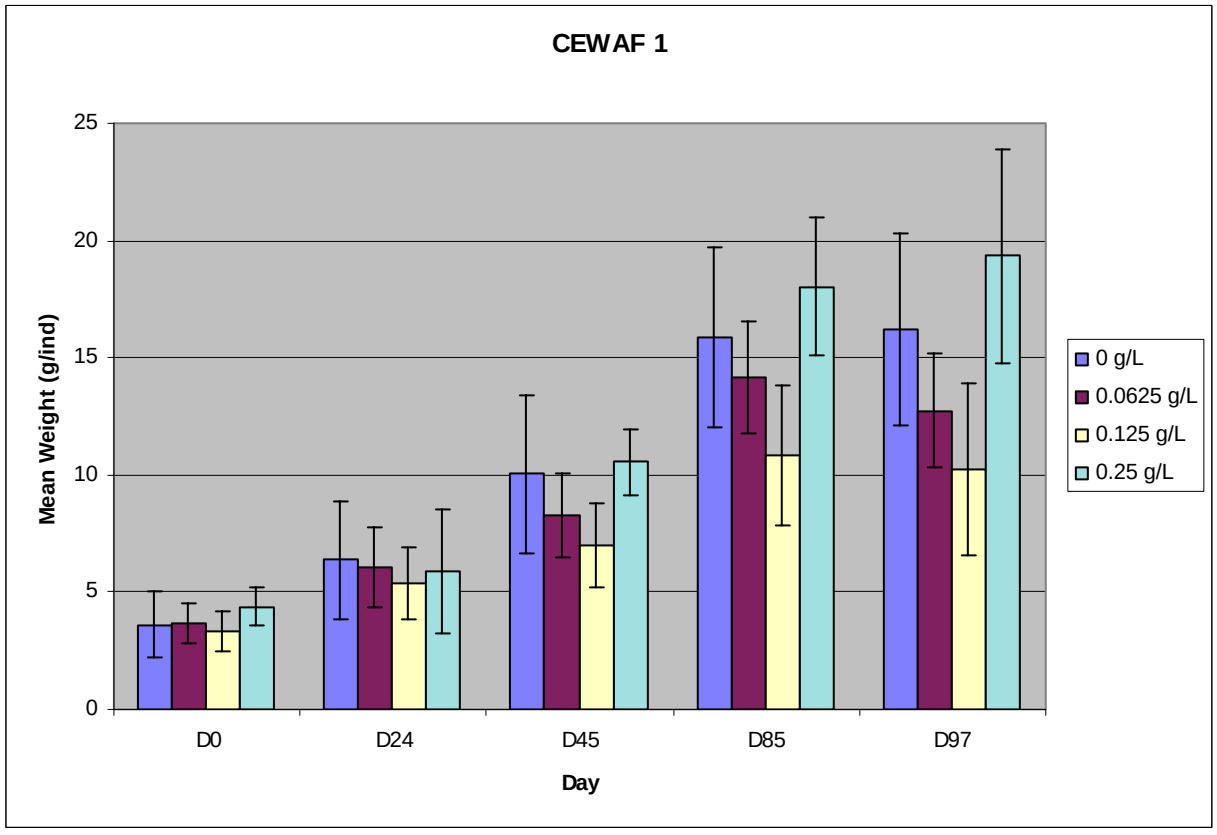


Figure 19. Growth of smolts 87 days after 96-h exposure of pre-smolts to CEWAF #2.

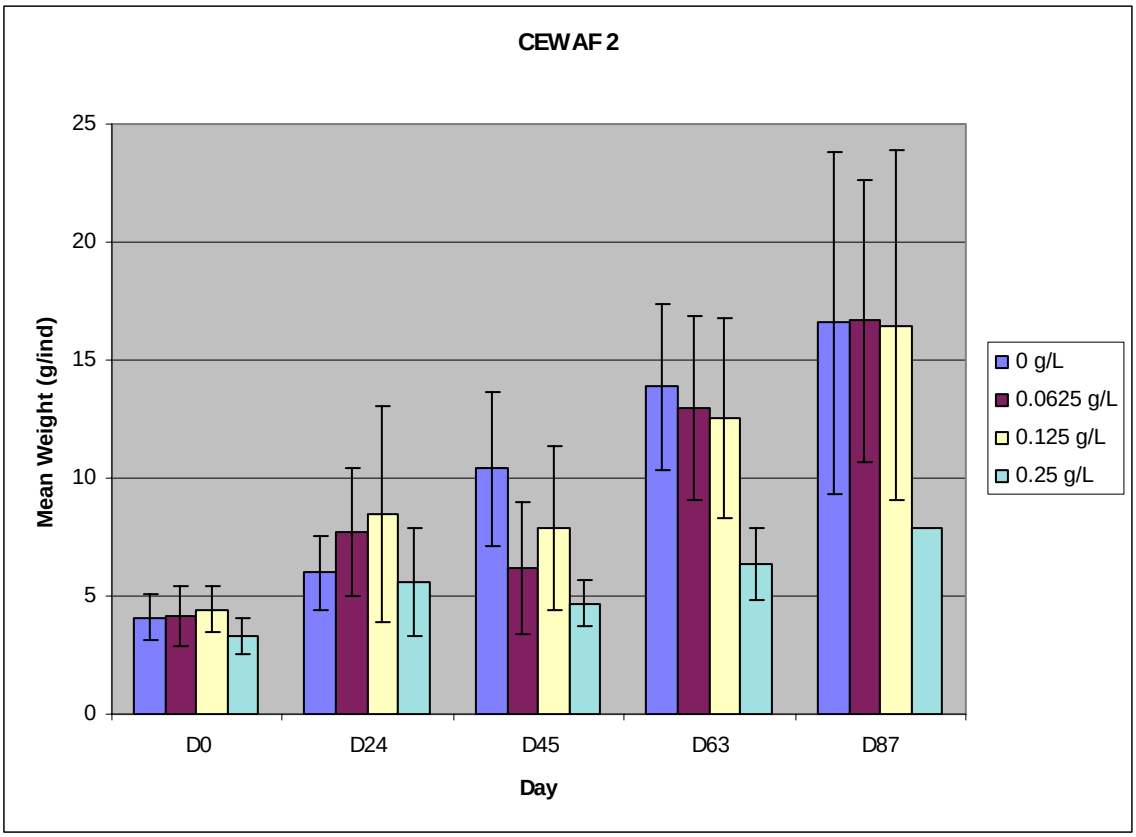
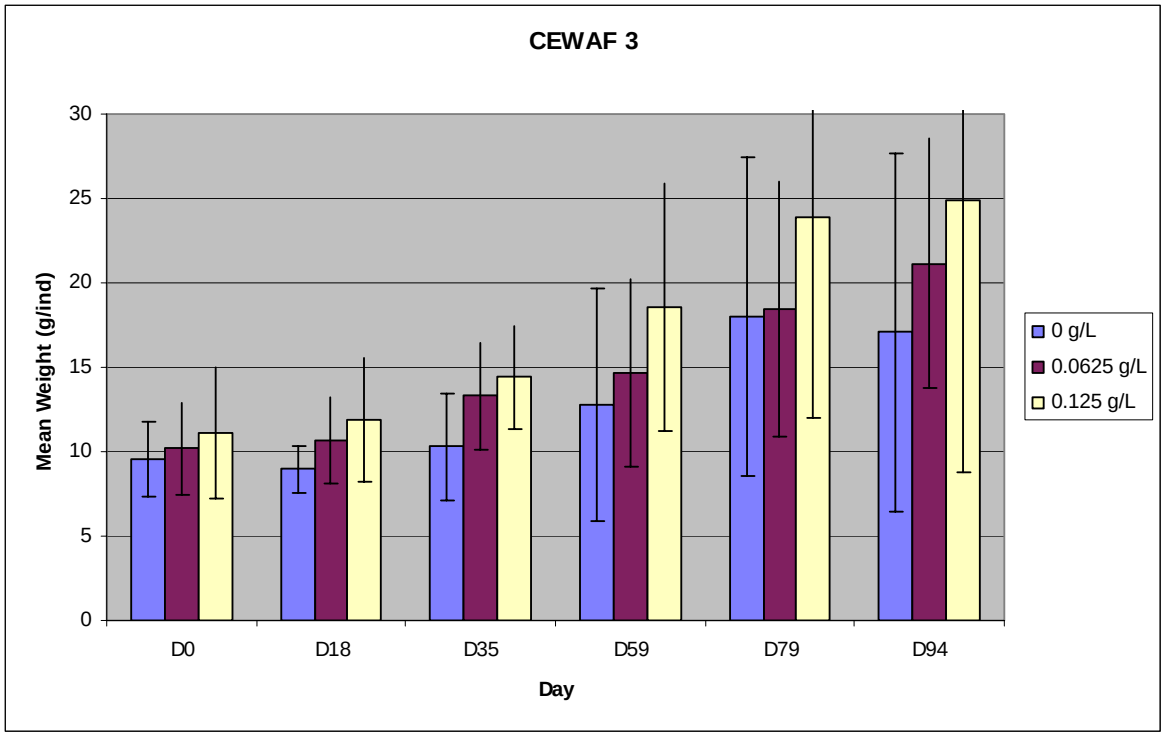


Figure 20. Growth of smolts 94 days after 96-h exposure of pre-smolts to CEWAF #3.



4.11.1 Effects of WAF and CEWAF on Long-Term Growth of Smolts

Because of poor survival in the previous (2005) experiments with seawater-acclimated smolts, two experiments were repeated as part of the current pre-smolt project. Two experiments were conducted with seawater-acclimated salmon smolts to assess effects of WAF and CEWAF on long-term growth. Water samples from these experiments were analyzed for TPH and BTEX to allow calculation of THC. Based on THC, LC50s from the summer 2007 WAF and CEWAF smolt exposures were comparable to those reported in the previous project (9.7 mg/L THC and 90.9 mg/L THC for WAF and CEWAF, respectively).

Continual growth was observed in surviving fish from both WAF and CEWAF exposures. No effect on long-term growth was observed after 96-h exposures to either WAF (Figure 21) or CEWAF (Figure 22).

Figure 21. Growth of salmon smolts 94 days after 96-h exposure of smolts to WAF #1.

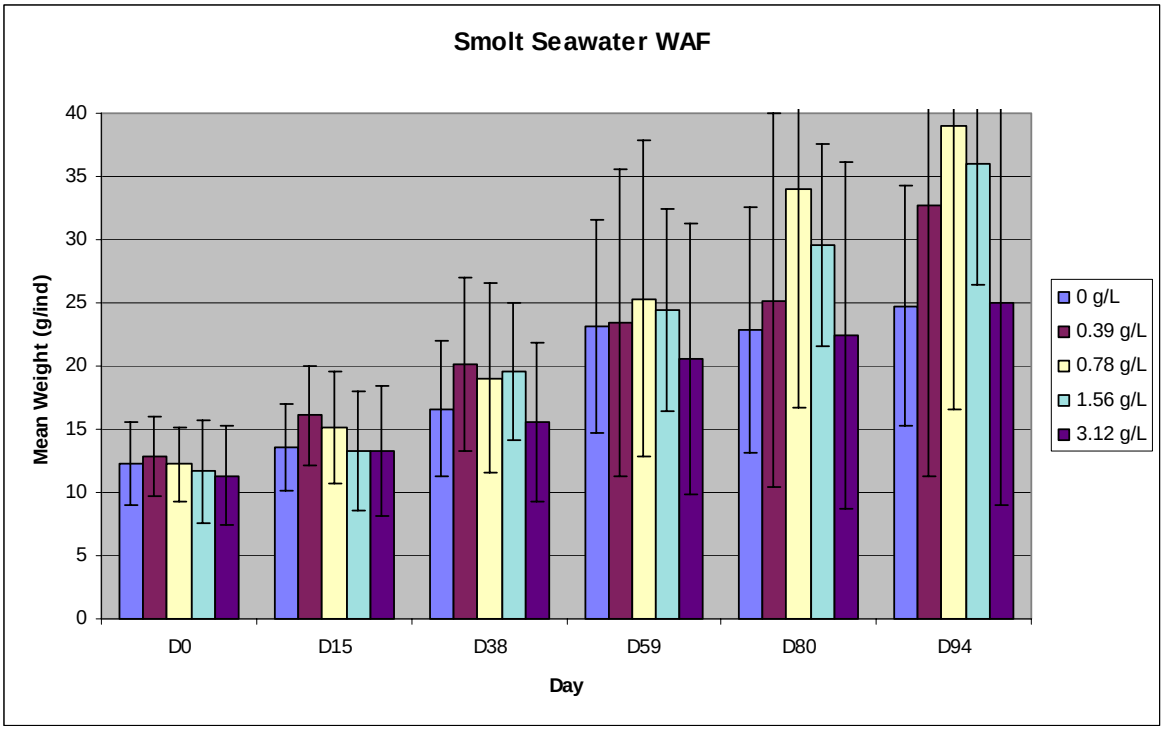
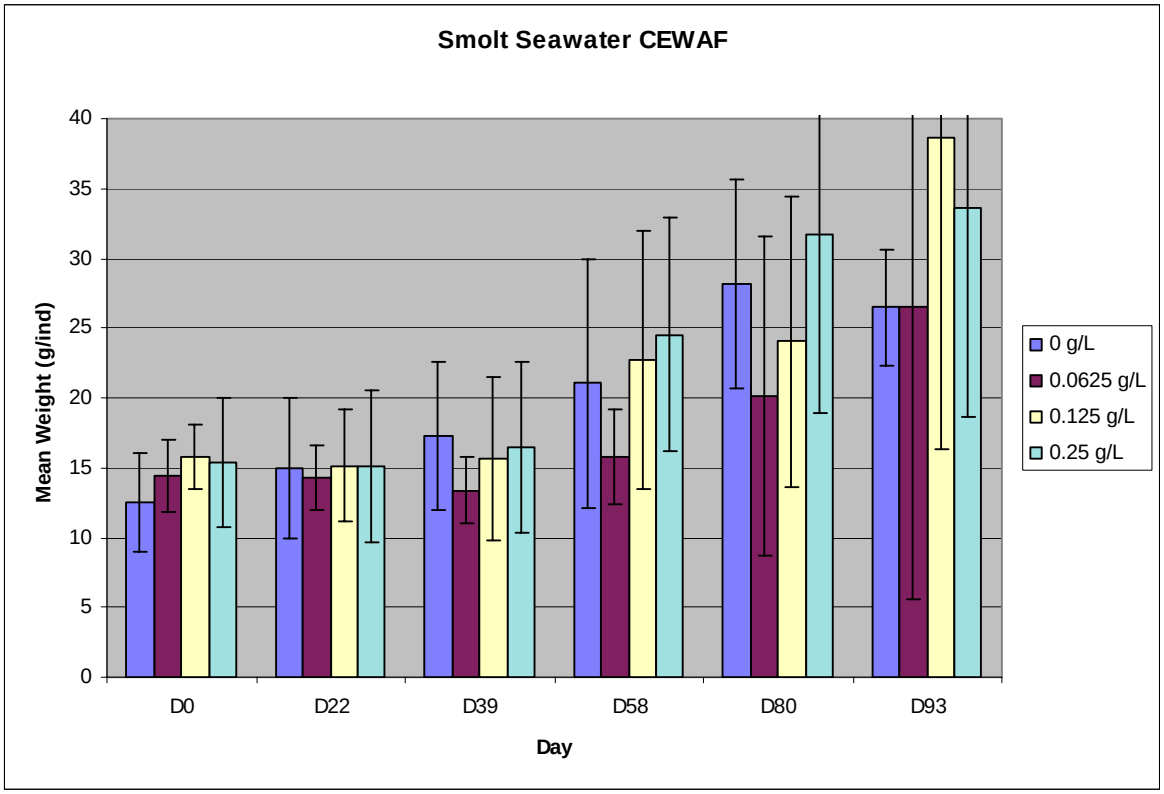


Figure 22. Growth of salmon smolts 93 days after 96-h exposure of smolts to CEWAF #1.



4.11.2 Specific Metabolic Changes in Muscles after Long-Term Growth of Smolts Exposed to WAF and CEWAF

Muscle and liver tissue from the long-term growth fish were analyzed in the same manor as for the salmon pre-smolts. PCA scores plots and loadings plots were constructed and peaks which displayed the greatest change in the loadings plots were identified. Metabolic changes were also determined by calculating the ratio of the average peak area of the treatment over the average peak area of the control for each test (Table 17 and Table 18). The metabolic changes indicate that an increase or decrease of a metabolite, within the muscle tissue, may be dose, treatment and metabolite dependent. A summary of metabolic changes in muscle tissues due to increasing WAF and CEWAF doses is provided in Figure 23. Specific changes include the increase of glutamate and the decrease of alanine after WAF exposure.

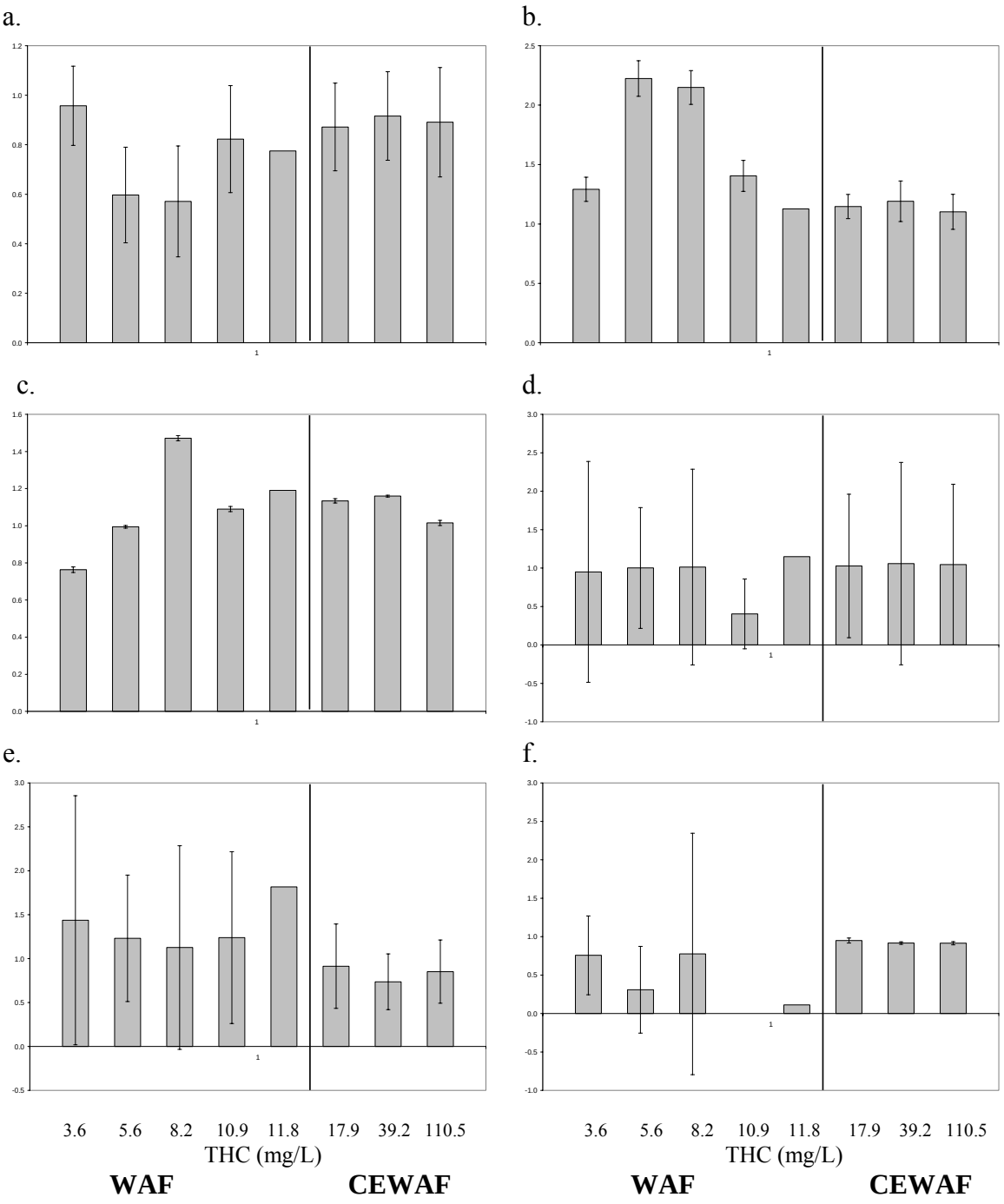
Table 17. Metabolic changes in the muscle of long-term grow out smolts after 96-h WAF exposure.

Metabolites	Chemical shift (ppm)b	3.574 mg/L	5.586 mg/L	8.171 mg/L	10.869 mg/L	11.82 mg/L
Lactate	1.33* (d)	0.95750	0.59705	0.57118	0.82278	0.77480
Alanine	1.485* (d)	1.29203	2.22446	2.14831	1.40468	1.12683
Succinate	2.41* (s)	0.76298	0.99430	1.47159	1.08951	1.19042
Phosphocreatine	3.04* (s)	0.94967	1.00169	1.01317	0.40363	1.14834
Taurine	3.425* (t)	1.43708	1.23047	1.12654	1.23929	1.81631
Glycerophosphoryl-choline	3.36* (s)	0.75736	0.30945	0.77505	0.00046	0.11150
Glycine	3.565* (s)	0.98835	1.05755	1.04793	1.02008	0.98371
AMP	8.59* (s)	0.87434	0.99948	0.91539	0.44286	1.05588
Formate	8.43* (s)	1.15968	1.22240	1.20188	1.46345	1.82179
ATP/ ADP	8.545* (s)	1.13561	0.97530	0.92106	0.86125	0.59303
NAD+/NADP+	8.84* (d)	0.90528	0.75176	0.77037	0.63658	0.81699

Table 18. Metabolic changes in the muscle of long-term grow out smolts after 96-h CEWAF exposure.

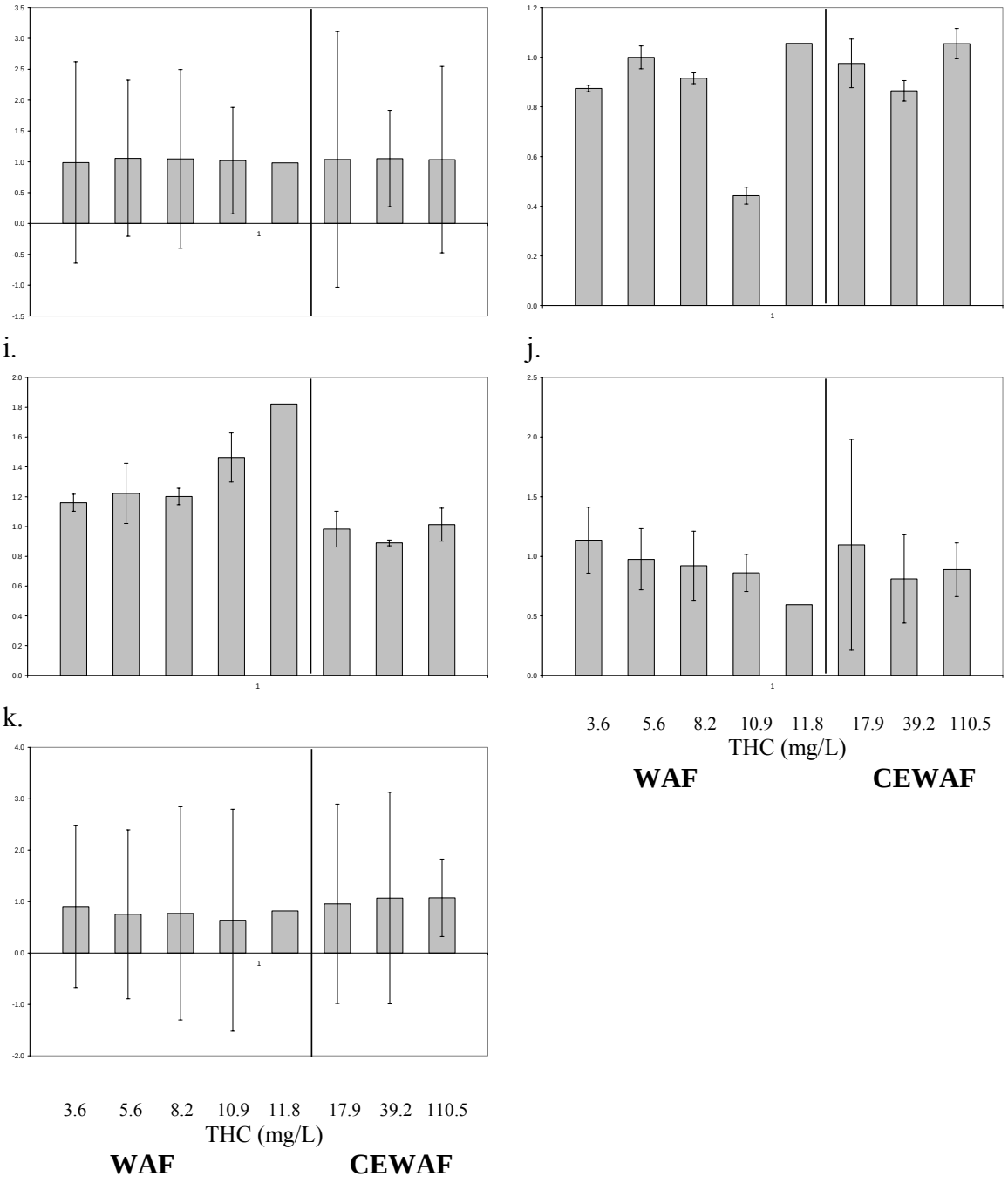
Metabolites	Chemical shift (ppm)b	17.958 mg/L	39.182 mg/L	110.539 mg/L
Lactate	1.33* (d)	0.87176	0.91619	0.89113
Alanine	1.485* (d)	1.14672	1.19135	1.10226
Succinate	2.36* (t)	1.13410	1.15942	1.01498
Phosphocreatine	2.41* (s)	1.02701	1.05788	1.04532
Taurine	3.04* (s)	0.91375	0.73597	0.85172
Glycerophosphoryl-choline	3.425* (t)	0.94921	0.91692	0.91481
Glycine	3.36* (s)	1.03969	1.05273	1.03506
AMP	3.565* (s)	0.97517	0.86463	1.05485
Formate	8.59* (s)	0.98291	0.89049	1.01297
ATP/ ADP	7.87* (s)	1.09650	0.81069	0.88812
NAD+/NADP+	8.545* (s)	0.95805	1.06875	1.07196

Figure 23. Changes in metabolites from muscle tissue after long-term grow out of smolts include AMP (a), ATP/ ADP (b), histidine (c), glycine (d), taurine (e), glycerophosphorylcholine (f), phosphocreatine (g), succinate (h), glutamtate (i), alanine (j), lactate (k).



g.

h.



5.0 Discussion

5.1 Relative Toxicity of Dispersed and Non-Dispersed Oil

Our results show that based on THC, the mean LC_{50} of the WAF tests (LC_{50} = 7.56 mg/L THC) was approximately six-fold lower than that of the CEWAF tests (LC_{50} = 48.6 mg/L THC). This suggests that although there were much higher concentrations of total hydrocarbons present in the CEWAF solutions, hydrocarbon bioavailability to salmon pre-smolts was lower under

dispersed conditions. The current results were similar to those observed in the previous project exposing Chinook salmon smolts to WAF and CEWAF. There was a greater disparity in response of smolts to WAF and CEWAF compared to pre-smolts; the mean LC_{50} of the smolt WAF tests (LC_{50} = 7.46 mg/L THC) was approximately 20-fold lower than that of the CEWAF tests (LC_{50} = 155.93 mg/L THC). Smolt tests conducted as part of the current study had a similar range of LC_{50}s (9.7 mg/L THC and 90.9 mg/L THC for WAF and CEWAF, respectively).

Reduced hydrocarbon bioavailability in pre-smolt CEWAFs might be attributed to several factors. Addition of dispersant to oil in water results in a multiphase system consisting of dissolved hydrocarbons, dissolved surfactants, micelles, and particulate bulk-oil (Singer *et el.,* 1998). The majority of these constituents are less bioavailable than the lighter hydrocarbon fractions present in WAFs, particularly when compared to the un-weathered PBCO WAF used in the present study.

Measures of individual PAHs in these experiments show higher concentrations of PAH constituents in the CEWAF tests, including naphthalenes and the phenanthrene/anthracenes. In comparisons of WAF and CEWAF toxicity using topsmelt larvae (*Atherinops affinis*), Singer *et al.* (1998) also found this species was more sensitive to PBCO WAF than to CEWAF. Their studies included PAH characterizations in both WAF and CEWAF solutions, and they found that WAF solutions of PBCO dispersed with Corexit 9500 were composed of an average of 96% volatiles (compounds chromatographing earlier than naphthalene), whereas CEWAFs contained only 67% volatiles. Singer *et al.* (1998) have noted that CEWAF solution chromatographs are more comparable to bulk oil than those from WAF solutions.

5.2 Application of Metabolomics

NMR-based metabolomics provides a sensitive assessment of the metabolic health of whole organisms. In this study, metabolic changes were characterized at various concentrations of WAF and CEWAF. Large changes in metabolites were often seen before the LC50 values. This approach provides a sensitive indicator of environmental stress and may be used as an early indicator of the health of aquatic organisms when exposed to oil spills.

The research on metabolic responses of salmon pre-smolts to oil provides information on the potential mechanisms of oil toxicity. These experiments, both WAF and CEWAF, appear to result in similar metabolic effects at different concentrations. Exposure to both WAF and CEWAF resulted in an increase of amino acid production, while the production of important fuel molecules decreased within muscle tissue. Amino acids are particularly important for protein synthesis and cellular repair. The imbalance of the supply of amino acids and their use may affect development, growth, reproduction and overall adjustment to stress. These experiments also indicate that metabolic changes are organ specific.

5.3 Dose-Dependent Metabolic Changes

To summarize the metabolic fingerprints and determine the most variance, PCA, an unsupervised analytical method, was employed. Since these experiments were conducted at different times, due to the limitation of equipment, it is likely that the physical state of the fish had changed

during the tests. This may have contributed to the variation among samples, resulting in background noise and the metabolic responses of the fish to be different. Other studies have found age (Plumb *et al.,* 2005), strain and gender (Plumb *et al.,* 2003) to contribute to metabolic variation. It is also possible that experimental conditions may have contributed to the metabolic effects.

To illustrate the similarities between the metabolic profiles, PCA scores plots were constructed. The PCA scores plots of muscle tissue from fish exposed to WAF showed a dose-response, particularly for WAF #3. However, the greatest responses occurred at the middle doses. Although this method is can resolve changes in profiles between doses, it is not able to identify any dose-response trends for each metabolite.

Results from the PCA of liver tissue do not identify any trend any dose-response trends. Although the PCA scores plots separate the metabolic profiles and have no trend, there may be specific metabolites containing a dose-response relationship.

5.4 Role of Endogenous Metabolites

In the experiments of both the WAF and CEWAF acute and long-term grow out; of muscle tissue, the identified metabolites and the metabolic effects appear to be similar. An increase of amino acids may have resulted from protein breakdown to repair cell injury, or the formation of carbon backbones necessary for the synthesis of important substrates such as succinate. The imbalance of the supply of amino acids and their use may affect development, growth, reproduction and overall adjustment to stress.

Metabolites that were abundant within the muscle tissue, ATP/ ADP, AMP, phosphocreatine, glycerophosphorylcholine, and succinate play specific roles during metabolism.

The levels of energy compounds ATP, ADP, and AMP all resulted in changes relative to one another. Since these compounds differ by one phosphate group, it is obvious that the increase in AMP and the decrease of ATP/ADP for WAF are related. For CEWAF, there is a definite decrease in AMP, where ATP/ADP levels out. This indicates that the ATP/ ADP are not being overexerted as an energy source.

Phosphocreatine decreased in the acute study when exposed to WAF, while it increased when exposed to CEWAF. A significant change in phosphocreatine was only observed at the high dose of CEWAF. This metabolite is important in buffering the production of ATP. Phosphocreatine will donate its phosphate group to ADP in order to produce more ATP for energy consumption. In the muscle tissue, the relationship between phosphocreatine and ATP is apparent. Both substrates decrease, after being exposed to WAF for both experimental studies. A decrease in phosphocreatine was also observed in medaka embryos that were exposed to trichloroethylene (Viant *et al.,* 2005a). Other studies observing a decrease in phosphocreatine include eyed eggs of Chinook salmon exposed to pesticides (Viant *et al.,* 2006) and juvenile steelhead trout subjected to heat stress (Viant *et al.,* 2003a). These studies also indicate the relationship between phosphocreatine and ATP. For the CEWAF exposure, ATP levels out while phosphocreatine

increases. This suggests that there may be another source of ATP when the use of phosphocreatine is not necessary.

Glycerophosphorylcholine, an osmolyte, is found within cells and maintains both cellular volume and fluid balance. A decrease of this metabolite in the acute WAF study and an increase in the long-term CEWAF study was observed. For the CEWAF acute test, a significant change was observed at the lowest dose. When this metabolite decreases within cells, the total osmolyte concentration is affected and may impact osmotic stress and increase the need for cellular repair.

Succinate, an important citric acid cycle intermediate, increased after acute WAF exposure and decreased after acute and long-term CEWAF exposures. The reason for this difference in responses between WAF and CEWAF is unclear. At the intermediate doses of WAF, succinate changed significantly. This metabolite shows that the WAF and CEWAF do alter the citric acid cycle.

5.5 Potential Biomarkers of WAF or CEWAF Exposures

Several endogenous metabolites identified within muscle and liver tissue may serve as potential biomarkers. Some of the metabolites show consistent changes at various doses which suggest these metabolites can be used as endpoints for WAF and CEWAF toxicity.

Glycerophosphorylcholine, present in both the muscle and liver tissue, could potentially serve as a biomarker for the toxicity of WAF or CEWAF.

5.6 Concluding Remarks

Response decisions regarding the use of dispersants depend on the complex interaction of oil release scenarios with numerous environmental factors that are unique to each oil spill (NRC, 2005). Our results with Chinook salmon pre-smolts provide important toxicological information for spill response decision makers, and suggest that use of dispersant, in this case Corexit 9500, may not result in increased toxicity to migrating salmon. In our study, THC-normalized LC50s from WAF tests were six-fold lower than those from CEWAF tests. Because these experiments used unweathered PBCO, these results likely represent a worse-case scenario where dispersant is applied shortly after a spill. In the majority of real-world spill situations, a considerable amount of weathering would be expected before dispersant application. Because lighter hydrocarbon fractions would be the first to volatilize, these fractions would rapidly decrease in most spill situations. Therefore, under field conditions we would expect less of a disparity between toxicities of treated and untreated oil, relative to the results of the current study.

Our results also illustrate the utility of metabolomic analyses for assessing sublethal effects of hydrocarbons on salmon pre-smolts. Metabolomics provide a cost-effective tool for determining subtle impacts of oil on migrating salmon, and may be used to investigate effects of non-lethal hydrocarbon concentrations. Additional work needs to be conducted to identify appropriate metabolic biomarkers which can be linked to long-term impacts of oil exposure.

Our results do not suggest that short-term oil exposures result in long-term growth effects.

The results from the metabolomic analysis do not indicate that the short-term exposures led to any long-term growth effects. After long-term growth was complete, it appears that the metabolic profiles contained the same metabolites as the acute study. There was a definite increase in fuel metabolites, a decrease in glycerophorylcholine and a similar change in succinate as the acute exposures.

6.0 Technology Transfer

Currently, there is limited information available on the effects of oil or chemically-dispersed oil on the pre-smolts of anadromous fishes. The results from this project will provide resource managers and spill responders with basic information on the acute and chronic effects of oil spills on migrating salmon and also provide data to support decisions regarding the advisability of applying dispersants under spill conditions where migrating salmon are present. We are in the process of finalizing papers to be published in peer-reviewed international scientific journals. Results from this project will also be distributed to other agencies that are involved in oil spill response, such as California Department of Fish & Game (CDFG) – Office of Spill Prevention and Response (OSPR), and the UC Davis Oiled Wildlife Care Network (OWCN); both also have provided matching funds for the above project.

7.0 Achievement and Dissemination

7.1 Publications

Lin CY, Viant MR and Tjeerdema RS, 2006. Metabolomics: Methodologies and applications in the environmental sciences. *J. Pestic. Sci.* 31, 245–251 (invited).

Lin CY, Wu H, Tjeerdema RS and Viant MR, 2007. Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics. *Metabolomics* 3, 55–67.

Tjeerdema RS, Lin CY, Anderson BS, Viant MR, Crane DL and Sowby ML, 2007. Effects of oil on the smolts of salmon (*Oncorhynchus tschawytscha*) – preliminary muscle results. *Proceedings of the International Effects of Oil on Wildlife Conference* 9, 220–228 (invited).

Tjeerdema RS, 2008. Application of NMR-based techniques in aquatic toxicology: Brief examples. *Mar. Pollut. Bull.* 57, 275–279 (invited).

Anderson, BS, Arenella-Parkerson D, Phillips BM, Crane DL and Tjeerdema RS. Effects of dispersed and un-dispersed oil on developing topsmelt embryos (*Atherinops affinis*). *Environ. Pollut.* (in press).

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Lin CY, Anderson B, Phillips BM, Peng AC, Hsieh F, Viant MR and Tjeerdema RS. Comparative actions of crude and dispersed oil in Chinook salmon smolts as characterized by NMR-based metabolomics. *Metabolomics* (in preparation).

Viant MR, Bearden D, Bundy JG, Burton I., Collette T., Ekman D., Karakach T, Lin CY, Rochfort S, de Ropp J, Teng Q, Tjeerdema RS, Walter J. and Wu H. International NMR-based environmental metabolomics intercomparison exercise. *Environ. Sci. Technol.*(in preparation).

7.2 Conference Presentations

Tjeerdema RS., MViant MR and Lin CY, 2008. Application of NMR-Based Techniques in Aquatic Toxicology. *Proceedings of the Workshop on Metabolomics and Environmental Biotechnology.* Environmental Biotechnology Working Group, European Commission – US Task Force on Biotechnology Research, Palma de Mallorca, Spain (invited).

Van Scoy AR, Lin CY, Anderson BS, Phillips BM, Viant MR and Tjeerdema RS, 2008. Impacts of Crude Versus Dispersed Oil in Salmon as Characterized by NMR-Based Metabolomics**.** *Proceedings of the Workshop on Metabolomics and Environmental Biotechnology.* Environmental Biotechnology Working Group, European Commission – US Task Force on Biotechnology Research, Palma de Mallorca, Spain (invited).

Tjeerdema RS, Viant MR and Lin CY, 2007. Application of NMR-Based Techniques in Aquatic Toxicology. *Proceedings of the Fifth International Conference on Marine Pollution and Ecotoxicology.* Hong Kong, People's Republic of China (invited keynote address).

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Figure 24 . 20-L polycarbonate carboys used for WAF and CEWAF preparation (above) and 18-L polycarbonate aquaria used for smolt exposures (below).

Figure 25. Avance DRX-500 NMR spectrometer.

APPENDIX I – Total Carbon Measurements from WAF and CEWAF Experiments

WAF 1 051707

Concentration	Rep	T0	Mean	SD	T2	Mean	SD
0	1	31.7778	34.58	2.54	30.8716	35.61	6.00
	2	36.7525			33.6126		
	3	35.1957			42.3566		
0.5	1	34.8707	33.74	1.05	33.1363	45.35	10.69
	2	33.5578			49.8884		
	3	32.7867			53.0208		
1	1	35.1733	34.78	1.22	43.6639	50.47	9.88
	2	33.4123			45.9508		
	3	35.7608			61.8011		
2	1	35.8999	36.43	1.02	35.4981	42.79	6.56
	2	35.7825			44.6728		
	3	37.605			48.2098		
4	1	39.0728	43.42	9.66	37.5795	37.01	2.92
	2	36.6898			33.8519		
	3	54.4831			39.599		
8	1	36.8525	36.11	1.48	42.9614	40.18	3.32
	2	34.4113			36.5055		
	3	37.069			41.0775		

WAF 2 061407

Concentration	Rep	T0	Mean	SD	T2	Mean	SD
0	1	35.8859	36.40	3.20	33.0663	39.52	7.73
	2	33.4835			48.0939		
	3	39.8282			37.4138		
0.5	1	48.6366	38.97	8.45	52.5918	45.09	6.52
	2	35.2871			41.9388		
	3	32.9813			40.7463		
1	1	44.8478	38.25	5.99	45.2397	50.88	4.95
	2	33.1565			54.4902		
	3	36.7535			52.9065		
2	1	37.3357	40.13	2.44	51.66	48.81	2.52
	2	41.8742			46.9054		
	3	41.1663			47.8561		
4	1	36.1619	46.23	10.28	45.8448	52.31	5.63
	2	56.7102			56.1746		
	3	45.8301			54.9076		
8	1	56.9597	44.01	11.43	52.6522	53.54	4.30
	2	35.3339			49.7512		
	3	39.73			58.2141		

CEWAF 1 052507

Concentration	Rep	T0	Mean	SD	T2	T4	T6	T8	T24	Mean	SD
0	1	32.14	30.29	2.42	33.02	40.54	37.41	36.13	34.68	35.96	1.23
	2	27.55			30.86	33.88	40.43	36.18	36.07		
	3	31.17			33.41	54.29	34.95	36.46	37.12		
0.0625	1	44.40	42.33	2.11	43.45	41.96	42.87	44.75	40.31	41.80	1.48
	2	42.40			51.26	42.85	49.49	50.90	43.26		
	3	40.19			35.21	40.49	42.86	43.25	41.84		
0.125	1	50.53	53.96	3.31	56.94	47.22	47.78	49.30	43.06	45.02	3.27
	2	57.14			50.74	57.63	52.09	46.81	43.21		
	3	54.23			53.97	51.64	49.83	53.14	48.80		
0.25	1	107.01	105.09	3.39	97.37	78.48	76.97	65.28	45.75	48.65	2.79
	2	107.09			91.74	72.32	69.70	71.39	51.31		
	3	101.17			111.28	71.68	71.11	68.76	48.89		
0.5	1	150.52	154.58	5.54	132.57	108.47	90.26	105.71	55.42	61.15	8.45

	2	152.33			140.28	127.09	98.51	105.61	70.85		
	3	160.89			138.55	112.66	96.22	100.65	57.17		
1	1	428.77	369.35	54.28	279.61	269.26	256.05	230.46	86.18	133.81	55.98
	2	356.91			299.08	265.94	296.08	273.97	195.47		
	3	322.37			303.07	261.85	230.88	268.30	119.78		

CEWAF 2 060707

Concentration	Rep	T0	Mean	SD	T2	T4	T6	T8	T24	Mean	SD
0	1	35.5923	34.60	0.88	35.4717	35.4198	34.316	32.27	36.0622	35.57	0.70
	2	34.2671			34.3925	34.0742	34.4881	35.7823			
	3	33.9271			32.4245	33.7596	37.4699	32.6141	35.077		
0.0625	1	40.4557	42.25	1.66	56.2521	42.3322	39.0472	40.4243	36.8176	36.26	0.49
	2	43.7228			43.4014	46.7661	54.9452	58.6376	35.9013		
	3	42.5691			39.7857	40.9518	40.5906	38.2293	36.0489		
0.125	1	54.4181	56.61	2.61	64.7333	54.087	44.5337	55.1362		38.10	1.00
	2	59.5036			71.4843	49.6731	46.1987	44.7935	38.808		
	3	55.9049			42.8776	66.0107	43.0699	40.3673	37.3923		
0.25	1	96.9004	95.63	4.22	82.6572	68.9362	63.2528	54.0519	41.007	39.62	1.74
	2	99.0793			74.6237	64.7379	61.3731	54.5438	37.667		
	3	90.9207			63.0406	73.6054	59.6808	54.3391	40.188		
0.5	1	122.1822	119.17	3.44	85.8442	91.2094	79.7095	63.8493	51.1566	48.85	3.62
	2	119.9138			85.936	68.3652	68.2251	65.5742	44.6828		
	3	115.4191			90.0143	82.1094	77.9545	69.6248	50.7238		
1	1	462.8598	455.46	17.75	360.4155	352.9763	242.2233	224.7328	91.7389	86.54	5.07
	2	435.2085			413.2661	329.8379	284.375	227.1081	81.6004		
	3	468.3151			352.6314	305.7301	275.5396	246.1674	86.2941		

APPENDIX II – Flow Measurements from Individual WAF and CEWAF Experiments

WAF 1 051707

Tank	T6.5	Mean	SD	T24	Mean	SD
1	22	21.00	1.00	24	23.00	1.73
2	20			24		
3	21			21		
4	22	21.33	1.15	32	24.67	7.51
5	20			25		
6	22			17		
7	20	20.00	1.00	13	18.33	4.73
8	21			20		
9	19			22		
10	22	19.67	2.08	17	23.67	5.77
11	19			27		
12	18			27		
13	19	19.67	1.15	17	20.00	2.65
14	19			22		
15	21			21		
16	22	22.00	1.00	25	23.33	2.08
17	21			21		
18	23			24		

WAF 2 061407

Tank	6/12/2007	Mean	SD	6/13/2007	Mean	SD	6/14/2007	Mean	SD	6/15/2007	Mean
1	20	21.00	1.00	19	20.33	1.53	20	20.00	2.00	18	18.00
2	22			22			22			20	
3	21			20			18			16	
4	18	19.67	2.08	18	20.00	1.73	20	20.00	2.00	16	22.67
5	19			21			18			20	
6	22			21			22			32	
7	19	19.33	0.58	21	22.00	2.65	22	21.33	1.15	25	22.67
8	19			25			22			25	
9	20			20			20			18	
10	18	19.67	2.08	20	20.33	2.52	19	20.33	2.31	17	18.67
11	22			23			23			22	
12	19			18			19			17	
13	23	21.67	1.53	22	21.00	1.73	21	20.33	1.15	20	22.67
14	20			19			19			19	
15	22			22			21			29	
16	21	21.00	2.00	22	21.67	2.52	21	21.00	1.00	21	20.33
17	23			24			22			22	
18	19			19			20			18	

WAF 3 062807

Tank	6/27/2007	Mean	SD	6/28/2007	Mean	SD	6/29/2007	Mean	SD
1	22	20.00	2.00	250	97.00	132.50	24	20.67	3.51
2	20			20			21		
3	18			21			17		
4	18	21.00	2.65	24	26.33	4.93	26	25.00	1.73
5	22			23			23		
6	23			32			26		
7	21	19.67	1.53	22	20.67	3.21	22	20.67	2.31
8	20			23			22		
9	18			17			18		
10	18	18.67	0.58	21	21.33	0.58	20	21.00	1.00
11	19			21			22		

12	19			22			21		
13	21	19.67	1.53	22	20.67	1.53	22	21.33	3.06
14	18			21			24		
15	20			19			18		
16	18	20.00	2.00	22	21.00	2.65	16	22.67	7.02
17	20			18			30		
18	22			23			22		

CEWAF 1 052407

Tank	T4-6	Mean	SD	T6-8	Mean	SD
1	20	20.00	0.00	20	21.00	1.73
2	20			20		
3	20			23		
4	20	19.67	1.53	23	21.00	2.65
5	21			22		
6	18			18		
7	22	19.67	2.08	18	19.00	1.73
8	19			21		
9	18			18		
10	23	21.00	2.65	23	20.67	2.08
11	22			20		
12	18			19		
13	19	19.67	2.08	19	19.00	1.00
14	18			18		
15	22			20		
16	21	20.67	2.52	21	19.67	2.31
17	18			17		
18	23			21		

CEWAF 2 060707

Tank	6/4/2007	Mean	SD	6/5/2007	Mean	SD	6/7/2007	Mean	SD	6/8/2007	Mean
1	23	20.33	2.52	23	21.33	2.89	21	21.33	0.58	21	20.00
2	20			23			21			19	
3	18			18			22			20	
4	23	22.00	1.73	23	22.33	2.08	24	24.00	0.00	22	20.00
5	23			24			24			21	
6	20			20			24			17	
7	18	20.00	1.73	18	20.33	2.08	22	21.67	1.53	16	17.33
8	21			22			23			16	
9	21			21			20			20	
10	21	21.67	1.15	22	22.67	0.58	21	22.67	1.53	20	20.67
11	23			23			24			24	
12	21			23			23			18	
13	18	20.33	2.08	18	20.67	2.31	17	20.00	6.08	17	17.67
14	21			22			27			22	
15	22			22			16			14	
16	18	19.67	2.89	18	20.67	3.79	16	19.00	5.20	16	20.00
17	23			25			25			27	
18	18			19			16			17	

CEWAF 3 070507

Tank	6/4/2007	Mean	SD	6/5/2007	Mean	SD	6/7/2007	Mean	SD	6/8/2007	Mean
1	23	20.33	2.52	23	21.33	2.89	21	21.33	0.58	21	20.00
2	20			23			21			19	
3	18			18			22			20	
4	23	22.00	1.73	23	22.33	2.08	24	24.00	0.00	22	20.00
5	23			24			24			21	

6	20			20			24			17	
7	18	20.00	1.73	18	20.33	2.08	22	21.67	1.53	16	17.33
8	21			22			23			16	
9	21			21			20			20	
10	21	21.67	1.15	22	22.67	0.58	21	22.67	1.53	20	20.67
11	23			23			24			24	
12	21			23			23			18	
13	18	20.33	2.08	18	20.67	2.31	17	20.00	6.08	17	17.67
14	21			22			27			22	
15	22			22			16			14	
16	18	19.67	2.89	18	20.67	3.79	16	19.00	5.20	16	20.00
17	23			25			25			27	
18	18			19			16			17	

APPENDIX III – PAHs Measured in WAF and CEWAF Solutions

PAHs in CEWAF Test #1

CEWAF #1	T0-0.0625 (ppb)	T0-0.125 (ppb)	T0-0.25 (ppb)	T0-0.5 (ppb)	T0-1.0 (ppb)	T24-0.0625 (ppb)	T24-0.125 (ppb)	T24-0.25 (ppb)	T24-0.5 (ppb)	T24-1.0 (ppb)	T96-0.0625 (ppb)	T96-0.125 (ppb)	T96-0.25 (ppb)
Naphthalene	34.7	60.3	92.7	178	261	6.23	14.3	18.3	45.6	54.4	<RL	<RL	<RL
Methylnaphthalene, 2-	49.2	72.3	119	228	367	1.76	1.76	8.6	61.7	97.1	<RL	<RL	<RL
Methylnaphthalene, 1-	38.1	55.4	90.5	173	276	5.85	13.0	15.3	52.2	76.7	<RL	<RL	<RL
Dimethylnaphthalene, 2,6-	16.6	23.1	50.2	101	181	1.91	4.87	7.79	42.0	60.8	<RL	<RL	<RL
Trimethylnaphthalene, 2,3,5-	4.02	6.64	16.3	31.3	60.6	0.799	1.86	3.74	21.9	24.1	<RL	<RL	<RL
Naphthalenes, C1 -	84.7	132	217	417	894	8.07	15.7	25.0	118	181	<RL	<RL	<RL
Naphthalenes, C2 -	77.3	114	222	431	1098	10.0	22.8	35.3	190	265	<RL	<RL	<RL
Naphthalenes, C3 -	40.1	82.0	163	343	869	4.94	16.8	36.3	199	250	0.0578	<RL	1.39
Naphthalenes, C4 -	14.3	20.7	53.6	113	214	2.47	5.39	0.981	66.5	83.7	0.186	<RL	3.06
Biphenyl	6.61	8.81	15.8	30.6	50.7	1.08	2.39	2.78	11.1	16.2	<RL	<RL	<RL
Acenaphthylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Acenaphthene	0.719	1.03	<RL	<RL	8.96	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	2.78	3.72	7.4	14.6	25.8	<RL	0.859	1.53	7.17	9.27	<RL	<RL	<RL
Methylfluorene, 1-	4.79	6.89	15.4	31.7	59.7	0.629	1.54	4.12	18.9	22.7	<RL	<RL	0.758
Fluorenes, C1 -	8.46	12.0	27.4	56.0	103	1.10	2.70	7.05	32.9	39.6	<RL	<RL	0.857
Fluorenes, C2 -	10.3	15.4	37.5	74.5	149	1.90	3.91	11.9	50.9	54.8	0.261	<RL	2.87
Fluorenes, C3 -	8.57	13.0	28.2	62.9	141	2.02	3.83	11.8	41.7	46.5	0.370	<RL	2.78
Dibenzothiophene	4.61	6.20	13.1	26.4	47.4	0.824	1.46	3.07	14.5	18.0	1.06	1.69	1.86
Methyldibenzothiophene, 4-	4.94	7.13	17.3	35.6	67.1	1.07	2.02	5.29	22.3	26.3	0.445	0.962	1.89
Dibenzothiophenes, C1 -	9.83	14.2	34.1	68.7	132	2.16	4.10	10.4	42.9	51.7	0.729	1.78	3.43
Dibenzothiophenes, C2 -	10.9	16.3	41.2	83.8	162	2.65	5.12	14.6	54.4	65.1	0.592	1.22	4.61
Dibenzothiophenes, C3 -	8.14	10.7	28.6	60.0	116	2.11	4.02	11.7	42.6	49.9	0.653	1.35	3.83
Phenanthrene	5.49	7.48	16.2	32.0	60.4	0.598	1.39	3.92	19.2	22.5	<RL	<RL	<RL
Methylphenanthrene, 1-	3.22	4.62	11.2	22.6	44.0	<RL	1.18	3.45	14.2	16.9	<RL	<RL	<RL
Dimethylphenanthrene, 3,6-	1.11	1.69	<RL	8.51	15.9	<RL	<RL	1.37	5.53	5.23	<RL	<RL	<RL
Phenanthrene/Anthracene, C1 -	13.0	18.8	46.3	92.8	179	2.10	4.92	14.1	58.4	70.0	<RL	<RL	<RL
Phenanthrene/Anthracene, C2 -	19.9	26.9	65.9	138	262	4.10	8.02	22.9	94.9	115.6	0.575	1.37	6.20
Phenanthrene/Anthracene, C3 -	11.6	15.2	39.8	80.1	153	2.86	5.03	14.7	56.9	68.8	0.796	1.47	4.97
Phenanthrene/Anthracene, C4 -	4.55	7.97	18.0	37.7	71.5	1.54	2.41	7.57	24.9	33.3	0.541	0.953	2.46
Anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluoranthene, 2-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene/Pyrenes, C1 -	2.10	3.05	7.34	15.3	29.6	0.549	0.895	2.73	11.1	13.1	0.117	<RL	0.858
Pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

Benz[a]anthracene	<RL	0.582	<RL	<RL	5.84	<RL	<RL	0.602	<RL	<RL	<RL	<RL	<RL
Chrysene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysenes, C1 -	1.00	1.46	<RL	8.25	15.3	<RL	0.538	1.47	5.45	6.47	0.107	<RL	<RL
Chrysenes, C2 -	1.07	1.68	<RL	8.43	16.1	<RL	0.573	1.71	6.09	7.01	0.144	<RL	0.612
Chrysenes, C3 -	0.712	1.13	<RL	<RL	10.9	<RL	<RL	1.15	<RL	<RL	0.109	<RL	<RL
Benzo(b)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(k)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(e)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(a)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Indeno(1,2,3-c,d)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Dibenz(a,h)anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(g,h,i)perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

PAHs in WAF test #1

WAF Test#1 ng/ml (ppb)	T0-0.5 ppb	T0-1.0 ppb	T0-2.0 ppb	T0-4.0 ppb	T0-8.0 ppb	T24-0.5 ppb	T24-1.0 ppb	T24-2.0 ppb	T24-4.0 ppb	T24-8.0 ppb	T96-0.5 ppb	T96-1.0 ppb	T96-2.0 ppb	T96-4.0 ppb
Naphthalene	39.2	29.5	39.9	46.9	50.8	0.0335	0.0316	0.130	0.0211	0.0402	0.0341	0.0176	0.0191	0.0164
Methylnaphthalene, 2-	20.3	16.0	20.3	24.0	24.1	0.0180	0.0199	0.0175	0.0340	0.0455	0.0832	0.0252	0.0179	0.0155
Methylnaphthalene, 1-	16.4	12.9	16.2	19.3	19.4	0.00739	0.00751	0.00788	0.0179	0.0210	0.0443	0.0166	0.00851	0.00733
Dimethylnaphthalene, 2,6-	3.06	2.47	3.03	3.58	3.50	<RL	0.00596	0.0212	0.0111	0.0231	0.0565	0.0113	0.00512	<RL
Trimethylnaphthalene, 2,3,5-	0.493	0.372	0.455	0.529	0.497	0.0111	0.0137	0.0356	0.0306	0.0406	0.00840	<RL	<RL	0.00910
Naphthalenes, C1 -	38.3	30.0	38.0	45.1	45.2	0.0252	0.0277	0.0246	0.0531	0.0674	0.131	0.0422	0.0268	0.0229
Naphthalenes, C2 -	15.1	12.5	14.6	17.8	17.2	0.0521	0.0910	0.227	0.0618	0.128	0.198	0.0444	0.0198	0.0180
Naphthalenes, C3 -	3.30	2.82	3.41	4.10	3.94	0.0811	0.112	0.273	0.144	0.212	0.105	0.0368	0.0509	0.0668
Naphthalenes, C4 -	0.465	0.411	0.573	0.658	0.672	0.0194	0.0335	0.0589	0.104	0.107	0.0312	0.0146	0.0147	0.00831
Biphenyl	2.47	2.03	2.46	2.92	2.90	0.119	0.147	0.266	<RL	0.0754	0.00563	<RL	<RL	<RL
Acenaphthylene	0.0668	0.0649	0.0672	0.0765	0.0801	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Acenaphthene	0.150	0.136	0.157	0.188	0.173	<RL	0.00628	0.0158	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	0.632	0.561	0.623	0.734	0.724	<RL	0.00617	0.0286	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluorene, 1-	0.436	0.410	0.447	0.527	0.510	0.0107	0.0125	0.0423	0.0429	0.0457	<RL	<RL	<RL	<RL
Fluorenes, C1 -	0.760	0.714	0.804	0.940	0.912	0.0180	0.0210	0.0640	0.0617	0.0628	<RL	<RL	0.00712	0.00702
Fluorenes, C2 -	0.308	0.309	0.380	0.446	0.371	0.0184	0.0224	0.0495	0.0708	0.0546	0.0136	0.0126	0.0256	0.0300
Fluorenes, C3 -	<RL	0.136	0.152	0.163	0.173	0.0110	0.0132	0.0229	0.0353	0.0281	<RL	0.00954	0.0159	0.0191
Dibenzothiophene	0.669	0.632	0.704	0.770	0.760	0.0879	0.100	0.0344	0.0219	0.0113	0.183	0.202	0.0994	0.0641
Methyldibenzothiophene, 4-	0.211	0.209	0.254	0.250	0.245	0.0265	0.0311	0.0251	0.0133	0.00818	0.0259	0.0277	0.0242	0.0216
Dibenzothiophenes, C1 -	0.623	0.577	0.619	0.693	0.651	0.0241	0.0298	0.0253	0.0161	0.0106	0.0227	0.0310	0.0277	0.0220
Dibenzothiophenes, C2 -	0.363	0.460	0.495	0.627	0.575	0.0661	0.102	0.134	0.187	0.0993	0.0489	0.0711	0.112	0.125
Dibenzothiophenes, C3 -	0.145	0.247	0.336	0.401	0.415	0.0472	0.0858	0.117	0.157	0.0749	0.0250	0.0501	0.0885	0.102
Phenanthrene	0.664	0.658	0.756	0.787	0.782	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylphenanthrene, 1-	0.143	0.137	0.161	0.179	0.168	0.00580	0.00973	0.0237	0.0181	0.0111	<RL	0.00588	0.00953	0.0146
Dimethylphenanthrene, 3,6-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Phenanthrene/Anthracene, C1 -	0.585	0.566	0.757	0.830	0.778	0.00857	0.0173	0.0332	0.0264	0.0149	0.00519	0.00914	0.0139	0.0119
Phenanthrene/Anthracene, C2 -	0.424	0.418	0.550	0.563	0.457	0.0261	0.0345	0.0661	0.0833	0.0519	0.0144	0.0191	0.0340	0.0438
Phenanthrene/Anthracene, C3 -	0.142	0.154	0.185	0.205	0.169	0.00977	0.0167	0.0238	0.0380	0.0202	<RL	0.00736	0.0107	0.0215
Phenanthrene/Anthracene, C4 -	<RL	<RL	<RL	0.0626	<RL	<RL	0.00836	0.0111	0.0213	0.0099	<RL	<RL	0.00630	0.0111
Anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluoranthene, 2-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene/Pyrenes, C1 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benz[a]anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysenes, C1 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

Chrysenes, C2 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysenes, C3 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(b)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(k)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(e)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(a)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Indeno(1,2,3-c,d)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Dibenz(a,h)anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(g,h,i)perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

PAHs in CEWAF test #3

CEWAF Test #3	T0-0.0625 (ppb)	T0-0.125 (ppb)	T0-0.25 (ppb)	T0-0.5 (ppb)	T0-1.0 (ppb)	T24-0.0625 (ppb)	T24-0.125 (ppb)	T24-0.25 (ppb)	T24-0.5 (ppb)	T24-1.0 (ppb)	T96-0.0625 (ppb)	T96-0.125 (ppb)	T96-0.25 (ppb)
Naphthalene	30.1	42.3	87.3	175	288	<RL	<RL	<RL	6.68	59.8	<RL	<RL	<RL
Methylnaphthalene, 2-	41.2	47.9	111	253	416	<RL	<RL	<RL	<RL	30.8	<RL	<RL	<RL
Methylnaphthalene, 1-	33.1	41.2	88.7	195	310	0.0535	0.184	<RL	<RL	52.6	<RL	<RL	<RL
Dimethylnaphthalene, 2,6-	16.1	20.4	51.2	129	204	<RL	<RL	<RL	7.44	26.2	<RL	<RL	<RL
Trimethylnaphthalene, 2,3,5-	5.05	6.93	21.7	46.4	79.1	<RL	0.0583	0.950	7.56	9.14	<RL	<RL	<RL
Naphthalenes, C1 -	75.8	92.0	206	465	963	0.0637	0.228	<RL	<RL	87.2	<RL	<RL	<RL
Naphthalenes, C2 -	78.4	97.6	227	545	1207	<RL	<RL	<RL	37.0	126	0.131	0.806	<RL
Naphthalenes, C3 -	47.9	60.4	178	459	962	0.171	0.258	2.48	85.1	103	0.160	<RL	<RL
Naphthalenes, C4 -	13.9	21.0	59.6	156	242	0.574	1.31	10.9	31.4	27.5	0.125	<RL	1.93
Biphenyl	5.80	7.03	15.7	35.8	57.0	<RL	<RL	<RL	<RL	9.29	<RL	<RL	<RL
Acenaphthylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Acenaphthene	0.725	<RL	<RL	8.18	9.9	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	2.53	<RL	7.54	17.6	27.8	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluorene, 1-	4.63	6.68	15.8	41.6	63.2	<RL	0.0557	0.951	8.16	7.63	<RL	<RL	<RL
Fluorenes, C1 -	8.07	11.7	27.8	71.1	110	0.0667	0.130	1.12	14.1	14.7	<RL	<RL	<RL
Fluorenes, C2 -	9.76	14.7	38.0	97.7	155	0.662	1.30	7.87	21.3	20.8	0.120	0.604	1.73
Fluorenes, C3 -	8.81	14.5	37.1	88.4	170	1.016	2.32	9.29	21.5	5.59	0.187	<RL	3.19
Dibenzothiophene	4.06	5.72	13.6	32.4	49.6	0.463	0.487	<RL	<RL	7.02	0.899	1.42	1.23
Methyldibenzothiophene, 4-	4.74	6.99	17.8	46.0	69.6	0.439	0.465	0.804	9.27	8.51	0.194	<RL	1.07
Dibenzothiophenes, C1 -	9.41	13.8	35.2	90.5	136	0.791	0.926	1.65	16.6	17.2	0.503	1.01	2.14
Dibenzothiophenes, C2 -	10.8	15.7	42.2	111	170	1.304	2.18	9.57	23.7	20.1	0.160	0.530	2.87
Dibenzothiophenes, C3 -	6.44	11.6	28.5	75.0	114	1.521	2.59	7.81	17.0	15.4	0.331	1.16	4.20
Phenanthrene	4.84	6.50	17.1	40.2	61.4	<RL	<RL	<RL	<RL	8.42	<RL	<RL	<RL
Methylphenanthrene, 1-	3.09	<RL	11.4	29.6	45.8	<RL	<RL	<RL	5.58	5.47	<RL	<RL	<RL
Dimethylphenanthrene, 3,6-	0.96	<RL	<RL	11.0	19.0	0.0896	0.111	0.615	<RL	<RL	<RL	<RL	<RL
Phenanthrene/Anthracene, C1 -	12.5	18.0	47.1	121	186	0.0673	0.103	1.08	21.2	24.3	<RL	<RL	<RL
Phenanthrene/Anthracene, C2 -	16.3	26.2	70.8	181	285	1.27	2.43	12.8	<RL	43.7	0.130	1.21	3.62
Phenanthrene/Anthracene, C3 -	9.05	14.5	38.5	101	154	2.03	3.44	11.4	22.6	21.1	0.307	1.45	5.59
Phenanthrene/Anthracene, C4 -	4.84	7.53	19.8	50.9	79.1	1.34	2.11	5.95	10.4	10.0	0.321	1.07	3.20
Anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluoranthene, 2-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene/Pyrenes, C1 -	1.77	<RL	8.45	20.6	35.1	0.275	0.600	2.10	<RL	<RL	<RL	<RL	0.973
Pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benz[a]anthracene	<RL	<RL	<RL	<RL	7.03	0.0696	0.119	<RL	<RL	<RL	<RL	<RL	<RL
Chrysene	<RL	<RL	<RL	<RL	<RL	<RL	0.0783	<RL	<RL	<RL	<RL	<RL	<RL

Chrysenes, C1 -	0.928	<RL	<RL	10.4	15.3	0.204	0.351	1.14	<RL	<RL	0.0566	<RL	0.594
Chrysenes, C2 -	1.04	<RL	<RL	11.2	18.3	0.269	0.412	1.28	<RL	<RL	0.104	<RL	0.737
Chrysenes, C3 -	0.687	<RL	<RL	7.53	12.8	0.197	0.312	0.893	<RL	<RL	0.078	<RL	0.508
Benzo(b)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(k)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(e)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	0.0591	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(a)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Indeno(1,2,3-c,d)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Dibenz(a,h)anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(g,h,i)perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

PAHs in WAF test #3

WAF Test #3	-T0-0.5 (ppb)	T0-1.0 (ppb)	T0-2.0 (ppb)	T0-4.0 (ppb)	T0-8.0 (ppb)	T24-0.5 (ppb)	T24-1.0 (ppb)	T24-2.0 (ppb)	T24-4.0 (ppb)	T24-8.0 (ppb)	T96-0.5 (ppb)	T96-1.0 (ppb)	T96-2.0 (ppb)
Naphthalene	44.1	42.8	27.4	48.4	9.70	0.0217	0.0209	0.0251	0.0196	0.0517	0.0278	0.0216	0.0113
Methylnaphthalene, 2-	22.1	16.6	11.7	20.6	14.1	0.00946	0.0112	0.0110	0.00874	0.0133	0.0216	0.0184	0.00862
Methylnaphthalene, 1-	18.3	14.6	10.0	17.5	12.6	0.00537	0.00708	0.00618	<RL	<RL	0.0263	0.0215	0.00738
Dimethylnaphthalene, 2,6-	3.80	3.09	3.41	3.27	35.0	<RL	<RL	<RL	<RL	<RL	0.0104	0.00885	<RL
Trimethylnaphthalene, 2,3,5-	0.552	0.495	0.620	0.647	7.62	<RL	<RL	0.00651	0.0132	0.0671	<RL	0.00511	0.00553
Naphthalenes, C1 -	42.1	32.5	22.5	39.6	27.7	0.0138	0.0170	0.0176	0.0115	0.0185	0.0503	0.0419	0.0165
Naphthalenes, C2 -	17.5	15.5	11.0	18.0	18.0	0.0151	0.0199	0.0229	0.0254	0.0436	0.0707	0.0460	0.0267
Naphthalenes, C3 -	4.65	4.34	4.88	4.77	6.39	0.0137	0.0185	0.0340	0.0388	0.121	0.0452	0.0475	0.0479
Naphthalenes, C4 -	0.638	0.686	0.766	0.841	11.3	0.0177	0.0139	0.0320	0.0590	0.121	0.0111	0.0224	0.0189
Biphenyl	2.95	2.22	2.55	2.41	25.4	<RL	<RL	<RL	<RL	0.00662	<RL	<RL	<RL
Acenaphthylene	<RL	0.0668	0.0846	0.0785	0.845	<RL	<RL	<RL	<RL	0.0101	<RL	<RL	<RL
Acenaphthene	<RL	0.178	0.187	0.185	1.93	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluorene	0.783	0.650	0.761	0.684	8.65	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluorene, 1-	0.544	0.522	0.557	0.531	6.89	<RL	<RL	<RL	<RL	0.0606	<RL	<RL	<RL
Fluorenes, C1 -	0.955	0.916	0.974	0.950	12.3	<RL	0.00533	0.00804	0.0107	0.0811	<RL	<RL	0.00542
Fluorenes, C2 -	<RL	0.455	0.465	0.472	6.17	0.0208	0.0182	0.0388	0.0710	0.136	0.0130	0.0129	0.0273
Fluorenes, C3 -	<RL	0.233	0.298	0.276	3.46	0.0140	0.0155	0.0311	0.0477	0.0709	0.0147	0.0168	0.0200
Dibenzothiophene	0.749	0.553	0.647	0.636	8.94	0.167	0.123	0.0426	0.0169	0.0295	0.311	0.217	0.108
Methyldibenzothiophene, 4-	<RL	0.264	0.260	0.274	3.48	0.0379	0.0339	0.0190	0.00840	<RL	0.0243	0.0189	0.0208
Dibenzothiophenes, C1 -	0.692	0.706	0.789	0.778	10.0	0.0455	0.0523	0.0447	0.0497	0.0787	0.0430	0.0467	0.0579
Dibenzothiophenes, C2 -	<RL	0.618	0.663	0.640	8.10	0.0745	0.105	0.147	0.167	0.314	0.0515	0.0718	0.116
Dibenzothiophenes, C3 -	<RL	0.336	0.398	0.377	5.54	0.0453	0.0759	0.116	0.146	0.291	0.0215	0.0373	0.0875
Phenanthrene	0.689	0.424	0.526	0.548	8.59	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylphenanthrene, 1-	<RL	0.162	0.174	0.174	2.29	<RL	0.00626	0.00945	0.00868	0.0204	<RL	<RL	<RL
Dimethylphenanthrene, 3,6-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Phenanthrene/Anthracene, C1 -	0.659	0.666	0.702	0.708	9.36	0.00640	0.0101	0.0177	0.0169	0.0360	0.00576	0.00762	0.0125
Phenanthrene/Anthracene, C2 -	0.541	0.678	0.674	0.599	7.65	0.0196	0.0249	0.0468	0.0738	0.135	0.0117	0.0116	0.0254
Phenanthrene/Anthracene, C3 -	<RL	0.274	0.248	0.199	2.83	0.00719	0.0109	0.0214	0.0331	0.0608	0.00760	0.0090	0.0147
Phenanthrene/Anthracene, C4 -	<RL	0.074	0.0668	0.0672	0.890	<RL	0.0107	0.0144	0.0156	0.0341	<RL	0.0062	0.00981
Anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	0.0092	<RL	<RL	<RL
Fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Methylfluoranthene, 2-	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Fluoranthene/Pyrenes, C1 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	0.00737	0.0108	<RL	<RL	<RL
Pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benz[a]anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysenes, C1 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL

Chrysenes, C2 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Chrysenes, C3 -	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(b)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(k)fluoranthene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(e)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(a)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Indeno(1,2,3-c,d)pyrene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Dibenz(a,h)anthracene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL
Benzo(g,h,i)perylene	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL	<RL