

**ACUTE AND CHRONIC EFFECTS OF CRUDE AND DISPERSED OIL ON CHINOOK  
SALMON SMOLTS (*ONCORHYNCHUS TSHA WYTSCHA*)**

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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. 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 smolts of Chinook salmon (*Onchorhynchus tshawytscha*). A closed, flow-through test system for exposing salmon 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 hydrocarbons by gas chromatography-flame ionization detection. After exposure to various concentrations of oil or dispersed oil for 96 hours, the LC50 of the WAF tests was approximately 20 fold lower than that of the CEWAF tests. The mortality results suggest that application of oil dispersants decreased the toxicity of oil. The surviving fish were then scarified to examine the metabolic impacts of WAF and CEWAF. After removing muscle and liver tissues from the smolts, the tissues were flash frozen. The low molecular weight metabolites were extracted and analyzed using one-dimensional  $^1\text{H}$  and projections of two-dimensional  $^1\text{H}$ ,  $^1\text{H}$  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. The PCA results from the NMR spectra of muscle from animals exposed to WAF showed a distinct dose-response profile. Peaks of specific metabolites were further integrated and semi-quantified. The changes of specific metabolite levels were not always consistently related to dose. The patterns in dose response of metabolites are metabolite and organ dependent. In our experiments, both WAF and CEWAF appear to cause similar metabolic effects (albeit at different concentrations) in muscle. Exposure to WAF and CEWAF resulted in the shunting of the production of fuel molecules (ATP or phosphocreatine) and organic osmolytes (e.g. glycerophosphorylcholine) in favor of increased amino acid synthesis. However, even though a decrease of fuel molecules (i.e. phosphocreatine) and glycerophosphorylcholine were found in liver and muscle after WAF exposure, the decrease of lactate and glucose and increase of formate were only observed in liver. NMR-based metabolomics provide a sensitive whole-organism assessment of metabolic health. We conclude that dispersant treatment decreases the toxicity of crude oil (by possibly reducing its bioavailability), and that NMR-based metabolomics is a rapid, high throughput, sensitive, and more affordable approach to identify metabolic fingerprints as well as developing biomarkers for environmental contaminants.

**Keywords:** metabolomics, Chinook salmon, 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 smolts entering the ocean. In particular, there is concern that oil dispersant application could increase the toxicity of crude oil in 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 *Oncorhynchus 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 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 salmon populations during key periods of their migration, particularly when smolts are entering the ocean from native streams and rivers. Due to sea 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 coastal spill response activities.

This project will compare 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 smolts of chinook salmon (*Onchorhynchus tshawytscha*). The null hypothesis to be tested is: *The toxic impacts of crude oil on chinook salmon smolts are not increased by application of oil dispersants.*

## 2.0 Objectives

The primary objectives of this project were to develop a closed, flow-through test system for exposing salmon smolts to declining concentrations of the WAF or CEWAF of PBCO, and to conduct short-term 96-h declining exposures to assess the relative toxicities of dispersed and non-dispersed PBCO. 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. The project was also supported by matching funds from both the California Department of Fish & Game (CDFG) – Office of Spill Prevention and Response (OSPR), and the UC Davis Oiled Wildlife Care Network (OWCN).

Chinook salmon smolts (~6 cm), obtained from the CDFG Feather River Hatchery, will be exposed for 96 h to various concentrations of either the WAF or CEWAF of PBCO using methods we have also standardized through CROSERF and a declining exposure protocol, also previously developed in our laboratory. The WAF and CEWAF will be fully characterized for hydrocarbons by gas chromatography (GC-FID). Declining exposure concentrations will better simulate actual spill conditions, where both dilution and dispersion occur, and will be monitored using total hydrocarbon content (THC) analysis. Animals will be evaluated for mortality. Experiments will be repeated a minimum of three times for statistical validity. Some surviving smolts will be placed in clean seawater, where they will be routinely weighed and measured for an additional 12 months to



determine any chronic effects. Others will be sacrificed and flash frozen for later  $^1\text{H-NMR}$  metabolomic analysis to identify stressful metabolic changes that could impair natural stress responses. For instance, glycolytic and TCA cycle intermediates, as well as high-energy phosphagens, can be measured, providing an indication of their metabolic status and ability to recover. Metabolic viability of the acutely exposed fish after long-term growth will be measured too.

### 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. MPSL is a flowing seawater and freshwater facility where aquatic toxicology research is conducted cooperatively by the California Department of Fish and Game and UC Davis. Metabolomic analysis of tissues from exposed smolts were conducted at the UC Davis Nuclear Magnetic Resonance (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 Prudhoe Bay crude oil (PBCO) obtained from Resource Technology Corporation (Laramie, WY, USA), and 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 water-accommodated fraction (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 (22L) of 0.45  $\mu\text{m}$ -filtered natural seawater ( $\sim 33\text{‰}$  salinity) in a 23-L polycarbonate carboy (resulting in a standardized 22–23% headspace, by volume). Mixing energy was provided by magnetic stirrers at a rate of  $110 \pm 10$  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 aspirator bottles, stirrers, and volume of seawater. These solutions were designated chemically-enhanced water-accommodated fractions (CEWAFs) because dispersants 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 gas-tight Hamilton syringes (after Singer *et al.*, 1998). Exact masses of oil and dispersant 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

Measurement of total hydrocarbon content (TPH: C<sub>10</sub>–C<sub>36</sub>) was accomplished using a Hewlett-Packard 6890 gas chromatograph fitted with a flame ionization detector (FID). This instrument allowed reliable resolution of compounds from methyl-cyclohexane up to approximately *n*-C<sub>36</sub>. One liter water samples were extracted three times with dichloromethane (DCM) by liquid-liquid extraction. After each extraction, the solvent phase was collected and combined, with the final extract made up to 1-10 ml volume depending on the color in the extract. Extracts were stored in the refrigerator-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 to better represent the number and relative proportions of the various DCM-soluble compounds contained in the oil (Payne, 1994). A stock solution was first prepared by adding a measured mass of oil directly into a volumetric flask containing DCM. This stock was then serially diluted volumetrically with DCM.

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 total hydrocarbon content (THC = BTEX C<sub>6</sub>–C<sub>9</sub> + TPH C<sub>10</sub>–C<sub>36</sub>), and thus was not biased by quantitating a specific set of target analytes (Girling *et al.*, 1994). Concentrations of the volatile hydrocarbons benzene, toluene, ethylbenzene and xylenes (BTEX) were analyzed using a Hewlett-Packard 6890-5973 GC-MS with a Hewlett-Packard 7695 purge & trap concentrator and a Teledyne Tekmar autosampler (US EPA Method 8260).

### 3.3 Toxicity Test Procedures

Spiked-exposure 96-h toxicity tests were completed with salmon smolts (*Oncorhynchus tshawytscha*) using established test procedures (Singer *et al.* 2000), modified to accommodate larger organisms. Chinook salmon smolts (~6 cm), obtained from the CDFG Feather River Hatchery, and acclimated to seawater culture conditions prior to testing. Two cohorts of fish were used in these experiments. The first was transported from the hatchery on May 12, 2005, and these fish were used in all three WAF tests and the first two CEWAF tests. The second cohort was transported from the hatchery on June 1, 2005, and these fish were used in the third CEWAF 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.

Water temperature, DO, and pH were monitored daily during testing. Diluent was natural seawater filtered to ~20 µm at ambient salinity (~33‰). Spiked exposures were conducted in sealed, 18-L polycarbonate flow-through exposure chambers. Tests involved six treatments: five WAF or CEWAF treatments and a seawater 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.

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 seawater at a rate of approximately 200 mL/min (Figure 1).

After test initiation, concentrations in all chambers were monitored hourly for 7 h using total carbon (TC) analysis, accomplished by 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 analyzed immediately. TC data were used to assess acceptability of oil decline rates (Singer *et al.*, 1998).

### **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 (LC50) were estimated using the trimmed Spearman-Kärber procedure (Hamilton *et al.*, 1977). Test acceptability was assessed with both biological and chemical criteria. Biological criteria considered 80% survival in controls to be acceptable. Chemical criteria for test acceptance were those set out in Singer *et al.* (1991); a combination of linear regression (log-transformed TC concentration versus time), ANCOVA, and ANOVA procedures were used to verify that concentration decline rates of each test treatment were not statistically different either within or among any tests.

### **3.5 Animal and Experimental Design for Metabolomics studies**

Each complete experiment consisted of five different concentrations of WAF or CEWAF and a control, each replicated three times (for a total of 18 chambers containing at least 8 fish each). Experiments for metabolomic analyses were repeated 3 times for statistical validity. Additional long-term grow studies (one WAF and one CEWAF tests) were also analyzed. Therefore, total 4 WAF and 4 CEWAF tests were processed metabolic analysis. After 96-h exposure, two surviving fish from each replicate tank were sacrificed. Muscle and liver tissues were immediately dissected, flash frozen in liquid N<sub>2</sub>, and stored at -80°C until extraction.

### **3.6 Metabolite Extraction**

Extraction methods were taken from previous work (Lin *et al.* submitted). Briefly, frozen muscle or liver samples from surviving fish were pulverized together in a liquid N<sub>2</sub>-cooled mortar and lyophilized overnight. The homogenous dry tissue powder was weighed, and then extracted with 30 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 (10,000 g, 10 min, 4°C), 0.46 mL of supernatant was removed and then lyophilized prior to NMR analyses.

### **3.7 1D <sup>1</sup>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<sub>2</sub>O (0.1 M, pH 7.4) containing sodium 3-trimethylsilyl-2,2,3,3-d<sub>4</sub>-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 9- $\mu$ s ( $60^\circ$ ) pulse, 6-kHz spectral width, 2.5-s relaxation delay with presaturation of the residual water resonance, and 100 transients collected into 32k data points, requiring a 9-min total acquisition time. All data sets were zero-filled to 64k points, exponential line-broadenings of 0.5 Hz were applied before Fourier transformation, the spectra were phase and baseline corrected and then calibrated (TMSP, 0.0 ppm) using XWINNMR 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 6 kHz in F2 (chemical shift axis) and 40 Hz in F1 (spin-spin coupling constant axis). A 3.0-s relaxation delay was employed, giving a total acquisition time of 11 min. Datasets were zero-filled to 128 points in F1, and both dimensions multiplied by sine-bell window functions prior to Fourier transformation. Spectra were tilted by  $45^\circ$ , symmetrized about F1, calibrated (TMSP, 0.0 ppm), and the proton-decoupled skyline projections (p-JRES) obtained, all using XWINNMR.

### 3.8 NMR Spectral Pre-Processing and Statistical Analyses

Each spectrum was segmented into 1960 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 1960 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 mean-centered before multivariate analysis.

Principal component analysis (PCA) of 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 identify potential outliers as well as the degree of similarity 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. For each model built, the loading vector for the PC was examined to identify which metabolites contributed to these clusters. Two-way ANOVAs were conducted on the changes of peak areas between doses and tests using SAS. Only the results of doses will be presented.

## 4.0 Results

### 4.1 System Performance

Flush rates in the exposure aquaria were controlled using manual flow meters which calibrated daily. 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. TC decline rates were compared statistically between individual treatments within each test, and between all WAF and CEWAF tests following methods described in Singer *et al.* (1991). No significant differences were found between treatment slopes (ANCOVA F values  $\leq 0.15$  for all tests), so flush rates in all tests were considered similar and therefore acceptable. Once equality of within-test flush rates was established, comparisons between individual tests were performed using analysis of variance (ANOVA), using the rate constants calculated from regressions of log-

transformed concentrations vs. time. Results of the ANOVAs confirmed that flush rates in the three tests with WAF and the three tests with CEWAF were not statistically different ( $p = 0.46$ ).

The flush rate in these experiments was 200 mL/per minute, and hydrocarbons were measured at three times: T0 at test initiation; T8, after 8 h of exposure; and T24, after 24 h of exposure. Analysis of total hydrocarbon content (THC) in the WAF and CEWAF experiments showed THC concentrations in the treatments were comparable to back round concentrations after 8 h (Figure 3).

## 4.2 Acute Toxicity of WAF and CEWAF

THC ( $C_6 - C_{36}$ ) in these experiments is defined as the sum of BTEX ( $C_6-C_9$ ) 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 LC50s. Based on LC50s calculated using measures of THC in WAFs and CEWAFs, Chinook salmon smolts were considerably more sensitive to WAFs of non-dispersed PBCO than to CEWAFs from oil treated with the dispersant Corexit 9500. The 96-h LC<sub>50</sub>s for the three WAF tests were 11.03, 8.04 and 3.31 mg/L THC (mean WAF LC<sub>50</sub> = 7.46 mg/L THC). The 96-h LC<sub>50</sub>s for the three CEWAF tests were 159.85, 165.52 and 142.42 mg/L THC (mean CEWAF LC<sub>50</sub> = 155.93 mg/L THC; Figure 4; Table 1).

## 4.3 NMR Spectroscopy of Metabolites from Muscle Tissues

Representative <sup>1</sup>H spectrum showing the metabolic fingerprints of muscle extracts are presented in Figure 5. The p-JRES NMR spectra which possess flatter baselines and lower peak densities than corresponding 1D <sup>1</sup>H NMR spectra were also used for peak identification. Major metabolites in both <sup>1</sup>H and p-JRES NMR spectra were assigned by comparison to tabulated chemical shift and the peak multiplicity in <sup>1</sup>H spectra (Fan, 1996; Figure 5 and Table 2). Some metabolites were further confirmed by <sup>1</sup>H-<sup>13</sup>C heteronuclear single quantum coherence (HSQC). Phosphocreatine and several amino acids (i.e. alanine and glycine) were dominant in the spectra. Other observed metabolite classes included carbohydrates (i.e. glucose), nucleotides (i.e. ATP and ADP), glycolytic products (i.e. lactate), and citric acid cycle intermediates (i.e. succinate).

## 4.4 NMR Spectroscopy of Metabolites from Liver Tissues

The <sup>1</sup>H NMR spectra of liver tissue contained congested areas, probably due to significant amounts of macromolecules in the extracts. Such congestion can bury weak peaks and interfere with the accuracy of peak assignment and integration. p-JRES spectra (Figure 6) provide a much flatter baseline with clearer peaks as opposed to <sup>1</sup>H NMR. Tabulated chemical shifts and data from previous studies (Fan, 1996) were used for peak assignment in both <sup>1</sup>H and p-JRES spectra (Figure 6; Table 3). The assignment between <sup>1</sup>H and p-JRES spectra were comparable. Taurine, lactate, and alanine were abundant metabolites in the liver tissues. Other major metabolites identified in liver tissue included amino acids (isoleucine, valine, alanine, arginine/phosphoarginine, glutamate, glutamine, glycine, aspartate), carbohydrates (i.e. glucose), nucleotides (i.e. ATP and ADP), and glycerophosphorylcholine.

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

Although the <sup>1</sup>H spectra of the muscle extracts exhibited some degree of peak overlap, especially between chemical shifts 3 to 4 ppm, the metabolic fingerprints provided valuable information for differentiating metabolic effects. The <sup>1</sup>H spectra of 3 WAF tests and 2 CEWAF tests which used the same cohort of fish were

analyzed by an unsupervised method of PCA. PCA summarizes the similarity of NMR spectra and yields a simple graphical output where one spot denotes one sample. Scores plots show separation among 3 WAF tests along PC1 scores (38.72%; Figure 7). Further, the PC2 scores of WAF (19.43 %) demonstrated a trend of dose-dependent metabolic impacts. As the dose which was characterized as total hydrocarbon content (THC) increased, the PC2 scores decreased. Each test (including control and several doses) was then analyzed individually to avoid variation due to experimental condition and animal physiological status between each test. Figure 8 provides examples of scores plots showing metabolic dose-response information from individual WAF test. In the WAF1 test, there is separation of metabolic profiles from animals subjected to the higher dose to those subjected to lower doses along PC1 (40.27%). Metabolic profiles of animals treated with highest dose were separated out from the lower doses in WAF2 and WAF3, along PC1 (38.14% and 29.86% respectively). In WAF2 and WAF3, the animals treated with highest dose of WAF tend to cluster together with negative PC1 scores, while the control and animals exposed to low doses have positive PC1 scores.

The <sup>1</sup>H NMR spectra of 3 CEWAF tests were analyzed using PCA. PCA scores plot illustrates the similarity of samples. Samples from three different tests can be separated along PC1 and PC2 (Figure 9). Each test was then analyzed by PCA individually in order to avoid the variation from each experiment as well as the physical condition and aging of fishes. Results from the analyses show no significant dose-response in the metabolic profiles of first two CEWAF tests (Figure 10a and 10b). However, a trend of dose-response along PC2 in the third CEWAF test was seen in Figure 10c. While the dose, which characterized as THC, increased, the PC2 scores decreased.

#### **4.6 Dose-Response of Metabolic Profiles in Livers of Smolts Exposed to WAF or CEWAF**

Since the p-JRES spectra of liver extracts provided better-resolved peaks, the metabolic profiles from these spectra were analyzed using an unsupervised PCA method, which summarizes the overall metabolic variation between samples. Major identified peaks in p-JRES spectra were integrated and compared between each WAF dose (3 tests) and each CEWAF dose (4 tests). The liver samples from the first WAF test were not analyzed using JRES NMR. The PCA scores plots separated metabolic profiles from animals treated with different doses of WAF or CEWAF. However, there was no clear trend of dose-response in scores plot for each or the combination of WAF/CEWAF exposure.

#### **4.7 Specific Metabolic Changes in Muscles after Exposures**

Examples of PCA scores plots from analysis of individual of WAF or CEAF exposure showed metabolic dose-response (Figure 11a and b). PCA loadings plots (Figure 12a and b) demonstrated peaks within the spectra contributing to the variation in corresponding principle components. Peaks with positive loadings (i.e. glycerophosphorylcholine in Figure 12b) correspond to metabolites that have higher levels in the control. Therefore, the plots showing intensity changes after WAF or CEWAF exposure provide information for potential biomarker development. Several peaks that show the greatest change in the loadings plots in response to exposure have been identified. Only well resolved peaks were selected for further integration and comparison between doses among 4 tests of WAF or CEWAF.

Results of two-way ANOVA are presented in Tables 4 and 5 and these summarize the changes and *p* values of identified metabolites in muscles according to doses in 4 tests of WAF and CEWAF, respectively. The changes in specific metabolites were calculated by subtracting the average peak area by that of the corresponding control from each test. Therefore, a negative number means decrease of metabolite in those dosed animals. For

example, succinate decreased after WAF exposure, but increased after CEWAF. The decrease of succinate was significant ( $p < 0.05$ ) only at the intermediate WAF dose. A significant increase in succinate was observed at the lowest CEWAF dose ( $p < 0.05$ ). The results illustrate that metabolic changes are dose and treatment dependent; however, it is not clear why succinate concentrations in muscles differed between WAF and CEWAF exposures.

A summary of significant changes in muscle tissues due to increasing WAF and CEWAF doses is provided in Figure 13. Amino acids including valine, alanine, arginine/phosphoarginine, glutamate, and glutamine increased after WAF or CEWAF exposure. The pattern of metabolite increases among doses is metabolite dependent. Statistically-significant increases were observed at low doses of WAF (i.e. glutamine;  $p < 0.05$ ), intermediate doses of WAF (i.e. alanine;  $p < 0.01$ ), and high doses of WAF (i.e. arginine/phosphoarginine;  $p < 0.05$ ). Significant metabolite increases were also observed at both low and high doses of CEWAF (i.e. valine;  $p < 0.05$ ) but not intermediate doses. The increase of taurine is significant at a high dose of WAF, and at the lowest dose of CEWAF ( $p < 0.05$ ). Glycerophosphorylcholine decreased in all doses of WAF or CEWAF ( $p < 0.05$ ).

#### 4.8 Specific Metabolite Changes in Livers after Exposures

Two-way ANOVA was used to analyze the specific metabolite changes in smolt tissues at different doses among 3 tests of WAF and 4 tests of CEWAF, using major peaks identified from p-JRES. This analysis included peaks at 7.093, 7.865, 8.458, and 8.593, identified from the  $^1\text{H}$  NMR spectra, since these peak intensities were better resolved. The changes in specific metabolites were derived from subtraction of the average of specific peak areas in the control from those of the treatments. Tables 6 and 7 summarize the changes and  $p$  value of metabolites among different doses of WAF or CEWAF, respectively. Figure 14 highlights the metabolites which were significantly lower or higher among doses in both the WAF and CEWAF treatments.

Valine concentrations in liver were significantly higher ( $p < 0.01$ ) in fish exposed to high concentrations of WAF and CEWAF. The amino acids glutamine and glycine also were significantly increased at intermediate ( $p < 0.01$ ) and high ( $p < 0.05$ ) concentrations of CEWAF, respectively. Phosphocreatine and glycerophosphorylcholine both decreased significantly at intermediate doses of WAF ( $p < 0.05$ ). Both  $\alpha$  and  $\beta$  glucose showed increases and decreases after WAF or CEWAF treatment. Formate increased significantly ( $p < 0.01$ ) after exposure to middle and high concentrations of WAF. There was also a decrease of AMP after exposure to high doses of WAF and CEWAF ( $p < 0.05$ ).

#### 4.9 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 smolts. Surviving 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. Unfortunately, the maintenance crew accidentally released anoxic seawater contaminated with hydrogen sulfide into the supply lines leading into the long-term culture tanks – most of the fish died within minutes. They were weighed, but there was insufficient growth to show differences from controls (data not shown).

To generate additional fish for long-term growth studies, one additional WAF test was conducted on September 8, 2005, and one additional CEWAF test was conducted on October 10, 2005. Some of these fish were used for

additional metabolomic analysis and the remaining smolts were cultured for 3 mo. Smolt survival was poor in these post-exposure cultures, and there was an insufficient number of fish to allow statistical comparisons between control and treatment fish. It is not clear why survival was so poor, but most of the mortality occurred in the 3 mo after the initial WAF and CEWAF exposures (Table 8). The fish were from a second cohort provided by the CDFG American River Hatchery. We observed a higher rate of mortality in this cohort relative to the first one obtained from the same hatchery. Our culture logs show that a total 678 smolts died in the 5 culture tanks used to hold the cohort 2 fish prior to their use in the oil experiments, while only 34 smolts died from cohort 1 during culture. Fish from cohort 1 were used in the 3 WAF tests and the first two CEWAF tests. Fish from the second cohort were used for the final CEWAF test, and the WAF and CEWAF tests that were repeated to provide additional fish for long-term growth and metabolomic studies. The high mortality in the second cohort of fish suggests these fish were somehow weaker, although no obvious signs of disease were observed, and both cohorts were treated the same in culture. Additional WAF and CEWAF tests are planned for summer 2007, to provide fish for long-term growth and metabolomic analyses. These tests will include TPH and BTEX analyses of WAF and CEWAF solutions.

## 5.0 Discussion

### 5.1 Relative Toxicity of Dispersed and Non-Dispersed Oil

Our results show that based on THC, the mean LC<sub>50</sub> of the WAF tests (LC<sub>50</sub> = 7.46 mg/L THC) was approximately 20 fold lower than that of the CEWAF tests (LC<sub>50</sub> = 155.93 mg/L THC). This suggests that although there were much higher concentrations of total hydrocarbons present in the CEWAF solutions, hydrocarbon bioavailability to salmon smolts was lower under dispersed conditions. Reduced hydrocarbon bioavailability in CEWAFs might be attributed to several factors. Addition of dispersant to oil in seawater results in a multiphase system consisting of dissolved hydrocarbons, dissolved surfactants, micelles, and particulate bulk-oil (Singer *et al.*, 1998). Consequently, the salmon smolts were potentially exposed to many toxicants having differing modes of action (NRC 2005). The majority of constituents in dispersed oil are less bioavailable than the lighter hydrocarbon fractions present in WAFs, particularly when compared to the unweathered PBCO WAF used in the present study. Oil chemistry in this study was characterized using measures of total petroleum hydrocarbons, and the most volatile fractions were characterized as BTEX. We did not measure individual PAHs in these experiments, so it is not possible to quantify relative concentrations of lighter hydrocarbon fractions in the WAF and CEWAF tests. Examination of the Time 0 BTEX concentrations show that at the loadings corresponding to the LC<sub>50</sub>s, there was a greater mean concentration of total BTEX in the WAF tests than in the CEWAF tests. The mean total BTEX in the three WAF tests was 5,867 ± 5072 µg/L at the loading LC<sub>50</sub>. The mean total BTEX in the three CEWAF tests was 4,042 ± 976 µg/L at the loading LC<sub>50</sub>. However, the greater relative concentration of BTEX in the WAF tests (1.5 fold) does not seem to be sufficient to account for the 20-fold difference in LC<sub>50</sub>s observed in these experiments. Because PAHs were not measured in this study, the degree to which variable PAH concentrations may have influenced the acute toxicity of WAF and CEWAF cannot be determined. Analysis of these constituents would have been useful to help interpret the results, particularly in terms of determining relative effects of PAHs on short-term non-polar narcosis (NRC 2005). Future studies should include this component of hydrocarbon analysis.

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. Chromatograms of WAF and CEWAF solutions in the current study were visually compared to investigate qualitative differences in relative concentrations of lighter oil fractions in dispersed and non-dispersed oil solutions. Three chromatograms from the WAF oil loading of 1.56 g/L PBCO; Figure 15) were compared to three chromatograms from CEWAF loadings of 0.25 g/L PBCO; Figure 16). The chromatograms showed that WAF solutions contained a much greater proportion of lighter fractions and fewer heavier fractions relative to the CEWAF solutions. 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 provide a sensitive whole-organism assessment of metabolic health. In our study, significant metabolic changes were characterized at concentrations of WAF and CEWAF well below the lethal thresholds identified by the bioassays. This is particularly true in the CEWAF experiments, where the dose causing metabolic change was 6.5 times less than the LC50 (155.93 mg/L THC). This approach provides a much more sensitive indicator of environmental stress, and may be used as early diagnostic indicator of the health of salmon smolts exposed to oil spills.

Our research on the metabolic responses of salmon smolts to oil provides information on the potential mechanisms of oil toxicity to salmon early life stages. In our experiments, both WAF and CEWAF appear to cause similar metabolic effects (albeit at different concentrations) in muscle. Exposure to WAF and CEWAF resulted in the shunting of the production of fuel molecules (ATP or phosphocreatine) and organic osmolytes (glycerophosphorylcholine) towards increased amino acid synthesis. Increased amino acid production is necessary for protein synthesis required for cellular repair. The imbalance of amino acid supply and usage to generate energy may lead to a delay in development, reproduction, or ability to adjust to additional stress. Elevated amino acids in muscle may also have resulted from protein degradation and potential cell injury. Our studies also demonstrate that metabolic effects are organ specific. Even though the decrease of fuel molecules (i.e. phosphocreatine) and glycerophosphorylcholine were found in liver and muscle after WAF exposure, decrease of lactate and glucose, and increase of formate were only observed in liver.

## 5.3 Dose-Dependent Metabolic Changes

PCA, which may be characterized an unsupervised analytical method, shows the direction of most variance. In some cases of our studies, the result is not according to the experimental design, but just background noises. These experiments were done at different time due to the limitation of space and equipments. It is very likely that the physical condition of fish changed during the period of tests; therefore, the background noises and metabolic responses of fish were different. Factors that have been shown to affect a metabolic fingerprint include strain, gender, diurnal variation (Plumb *et al.*, 2003), age (Plumb *et al.*, 2005) and intestinal microflora (Williams *et al.*, 2002). Some researchers have also demonstrated that metabolic variation can also be due to animal cages (Robertson *et al.*, 2002). We had also tried to avoid the variances from diet and exposure condition. However, it is possible that slight experimental condition, such as during WAF or CEWAF preparation, contribute to large metabolic effects.

PCA scores plots provided information on the similarity of metabolic profiles among samples. The PCA scores plot of muscle from animals exposed to WAF showed a distinct dose-response profile, which showed lower

PC2 scores relative to control, and demonstrated that higher concentrations of CEWAF increased metabolic impact. Metabolic disruption may be due to the same biochemical pathways leading to toxicity (Viant *et al.*, 2005a). However, after integrating specific metabolites identified by loadings plots, their changes were not consistently in relation to dose. This was because significant changes sometimes occurred only from an intermediate dose, or only at low or high doses. PCA effectively resolved changes in metabolite profiles among doses, but is not sufficient to suggest the trend of dose-response for each metabolite.

The PCA results from liver extracts do not indicate a trend of metabolite dose-response. After analyzing individual metabolites, several metabolites stood out. Some changes (both increase and decrease) were dose-dependent, but some were not. This illustrated the complexity of metabolic networks and regulation, and suggests that metabolomic studies for biomarker development need to further examine specific metabolites with consideration to hydrocarbon doses.

#### **5.4 Role of Endogenous Metabolites**

Concentrations of numerous amino acids (i.e. valine, alanine, arginine) decreased significantly at high doses of WAF and CEWAF. Malmendal *et al.* (2006) also reported a decrease of alanine in fruit flies subjected to heat stress. In protein catabolism, proteins are broken down by protease enzymes into their constituent amino acids. An increase in amino acids may therefore result from protein degradation in addition to cell repair. The imbalance of amino acid supply and usage may lead to a delay in development, reproduction, or ability to adjust to stress. Phosphocreatine decreased significantly in muscle tissues of smolts exposed to high doses of CEWAF. It also decreased in smolt liver tissues exposed to WAF. Phosphocreatine's primary role is as a spatial and temporal buffer of ATP. It donates its phosphate group to ADP ( $ATP + Creatine \leftrightarrow ADP + Phosphocreatine$ ), which is regulated by creatine kinase. Once phosphocreatine decreases, the energy supply to the smolt development or movement may potentially be affected. Decrease in phosphocreatine was also observed in medaka embryos exposed to trichloroethylene (Viant *et al.*, 2005a), and hypoxic challenge (Pincetich *et al.*, 2005). Phosphocreatine decreased in eyed eggs of Chinook salmon exposed to pesticides (Viant *et al.*, 2006), and in juvenile steelhead trout subjected to heat stress (Viant *et al.*, 2003a). These earlier metabolomic studies also suggested that the decrease of phosphocreatine co-occurred with the decrease of ATP. In our study, there was a decrease in ATP, but it was not statistically significant.

Glycerophosphorylcholine, an organic osmolyte in cells, decreased significantly in smolt muscle exposed to all concentrations of CEWAF. It also decreased in smolt liver after exposure to all concentrations of WAF. Decrease in glycerophosphorylcholine leads to disruption of total intracellular osmolyte concentrations, which may impact the ability of salmon smolts to adjust osmotic stress during seaward migration. Succinate, which decreased in smolt muscles after exposure to WAF, is a key component of the citric acid cycle. Succinate reduces ubiquinone by donating electrons to the electron transfer chain. However, it increased in muscles when smolts were exposed to CEWAF. The reason for this differential response in WAF and CEWAF is unclear. An increase in succinate was found in earthworms exposed to 3-trifluoromethyl-aniline (Warne *et al.*, 2000) and under starvation and cooling stress (Lenz *et al.*, 2005).

#### **5.5 Potential Biomarkers of WAF or CEWAF Exposures**

Several small endogenous metabolites show consistent changes at different doses, suggesting these metabolites are more suitable as endpoints of WAF or CEWAF toxicity and exposure. For example, formate increased significantly in liver after WAF exposure, while glycerophosphorylcholine decreased significantly in muscle

after CEWAF exposure. Thus, both chemicals may potentially serve as biomarkers for toxicity or exposure in 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 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 20-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 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. Unfortunately, we were unable to determine whether short-term oil exposures resulted in long-term growth effects in this study. We were also unable to investigate whether there were any long-term metabolic impacts after acute exposures due to problems with long-term cultures in this study. These experiments will be repeated in summer 2007 as part of on-going research supported by the California Department of Fish and Game – Office of Spill Prevention & Response.

## 6.0 Technology Transfer

Currently, there is limited information available on the effects of oil or chemically-dispersed oil on the 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 Publication

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

Lin CY, Anderson B, Phillips BM, Peng AC, Hsieh F, Viant MR, and Tjeerdema RS. Metabolic Impacts on Chinook Salmon Smolts Exposed to Crude Oil or Dispersed Oil Using Nuclear Magnetic Resonance Spectroscopy. (in prep)

### 7.2 Presentation

Lin CY, Anderson B, Phillips B, Peng A, Hsieh F, Viant M, and Tjeerdema R. “Toxic Effects of Dispersed and Non-dispersed Oil on Chinook Salmon Smolts (*Oncorhynchus tshawytscha*) Using Metabolomics” Coastal Response Research Center Workshop “Polycyclic Aromatic Hydrocarbon Toxicity Summit”, Seattle, WA (2006)

Lin CY, Anderson B, Phillips B, Peng A, Hsieh F, Viant M, and Tjeerdema R. “Metabolic Impacts on Chinook Salmon Smolts Exposed to Crude Oil or Dispersed Oil Using One- and Two-dimensional Nuclear Magnetic Resonance Spectroscopy”. The Second Scientific Meeting of the Metabolomics Society, Boston, MA. (2006)

Lin CY, Wu H, Tjeerdema RS, and Viant MR. “Evaluation of metabolite extraction strategies from tissue samples using NMR metabolomics”. The Second Scientific Meeting of the Metabolomics Society, Boston, MA. (2006)

Lin CY, Viant M, Anderson B, and Tjeerdema R. “Metabolomic Analyses of Chinook Salmon Smolts Exposed to Crude Oil or Dispersed Oil”. Society of Toxicology 45<sup>th</sup> annual meeting, San Diego, CA. (2006)

Lin CY, Viant M, Anderson B, and Tjeerdema R. “Acute Effects of Crude Oil and Dispersed Oil on Chinook Salmon Smolts Using NMR-based Metabolomics”. SETAC North America 26<sup>th</sup> annual meeting, Baltimore, MD. (2005)

Lin CY, Viant M, Anderson B, and Tjeerdema R. “Evaluation of Tissue Extraction Methods in NMR Based Metabolomics”. The First International Conference of the Metabolomics Society, Tsuruoka City, Japan. (2005)

Lin CY, Anderson B, Viant M, and Tjeerdema R. “Acute and Chronic Effects of Crude Oil and Dispersed Oil on Chinook Salmon Smolts (*Oncorhynchus tshawytscha*)” Coastal Response Research Center meeting, Silver Spring, MD. (2005)

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Figure 1. 20-L polycarbonate carboys used for WAF and CEWAF preparation (above) and 18-L polycarbonate aquaria used for smolt exposures (below).





Figure 2. Avance DRX-500 NMR spectrometer.

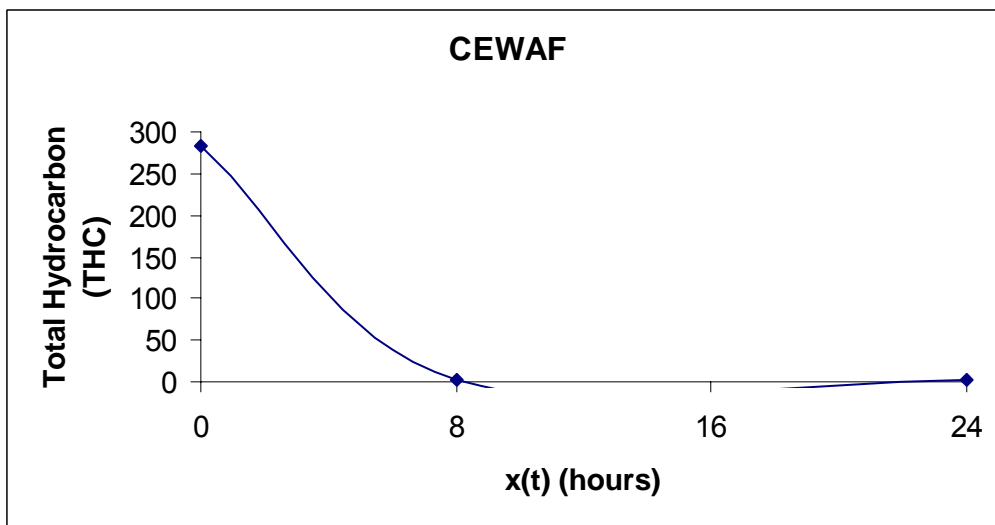
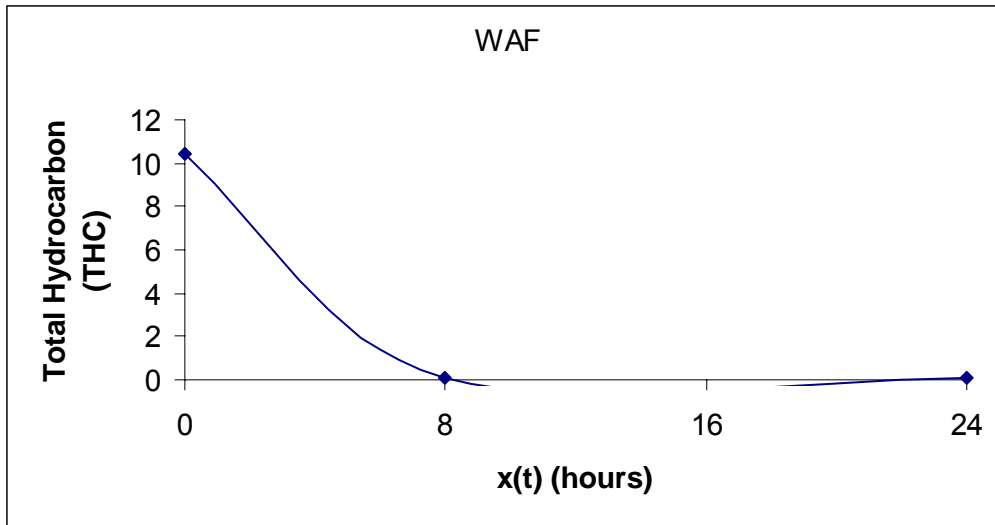
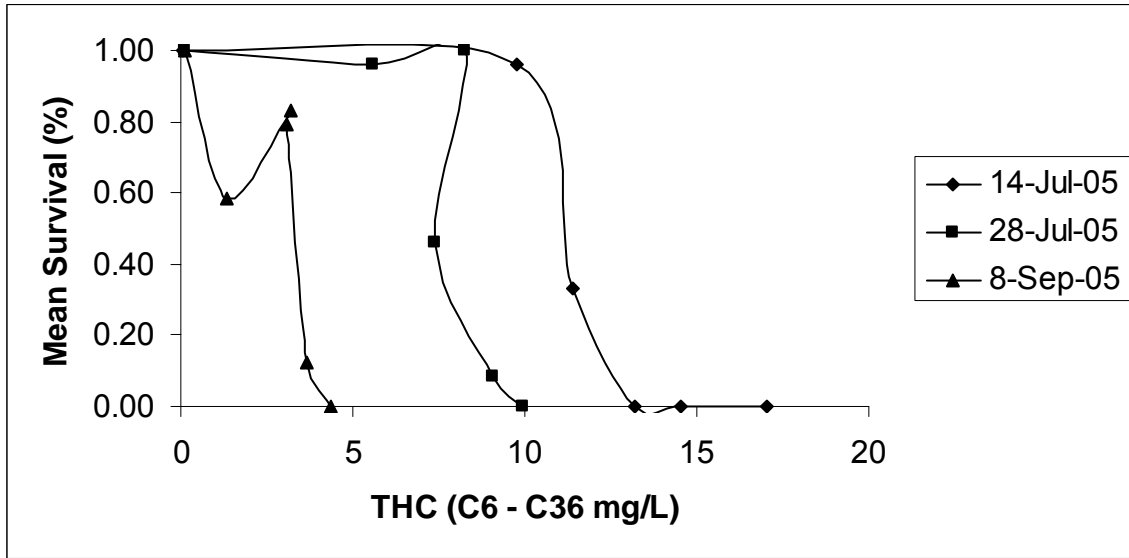


Figure 3. Average WAF and CEWAF decline rates. Amount of WAF or CEWAF were characterized by total hydrocarbon content (THC) (mg/L).

WAF tests



CEWAF tests

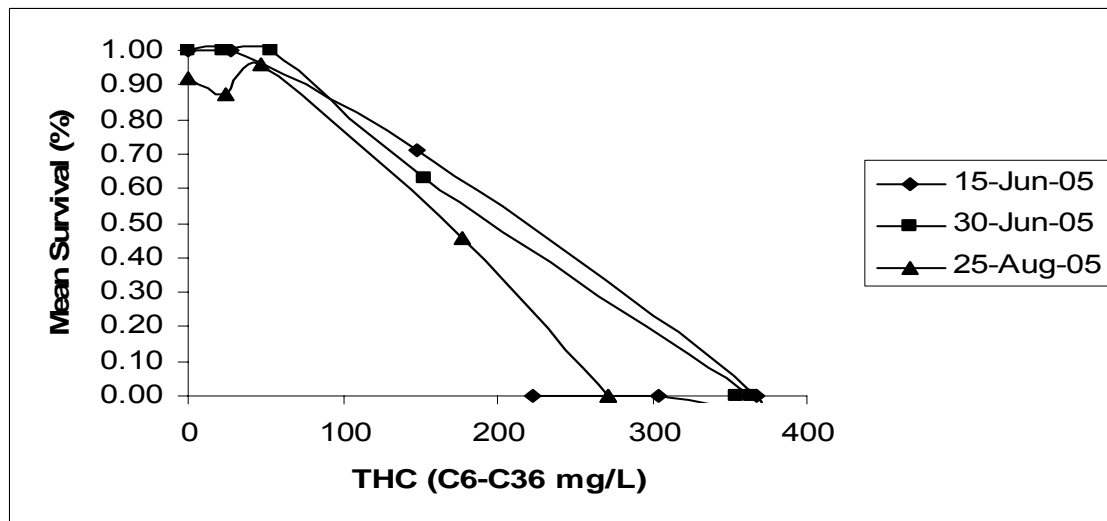


Figure 4. Salmon smolt survival in three WAF (upper) and CEWAF (lower) tests after 96-h. THC = total hydrocarbon content.

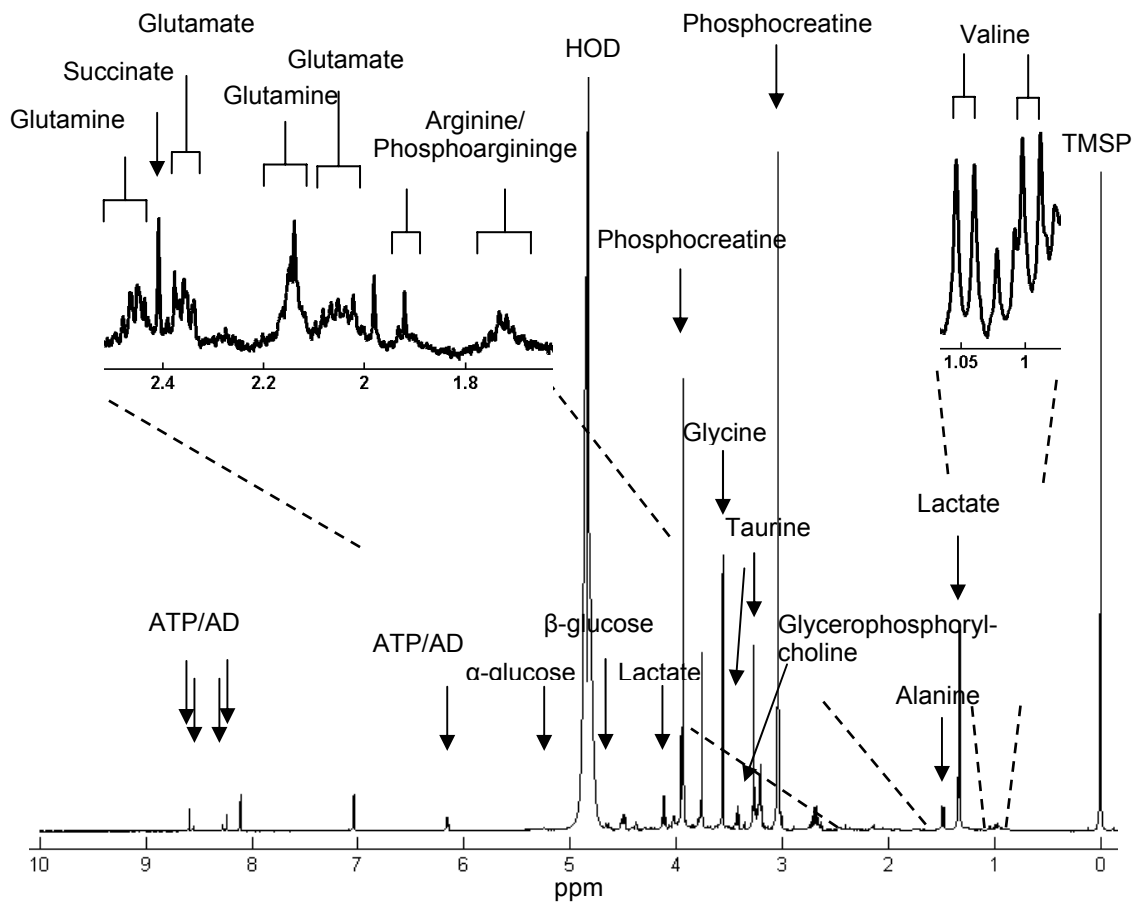


Figure 5. Representative 1D <sup>1</sup>H NMR spectrum of the smolt muscle extracts.

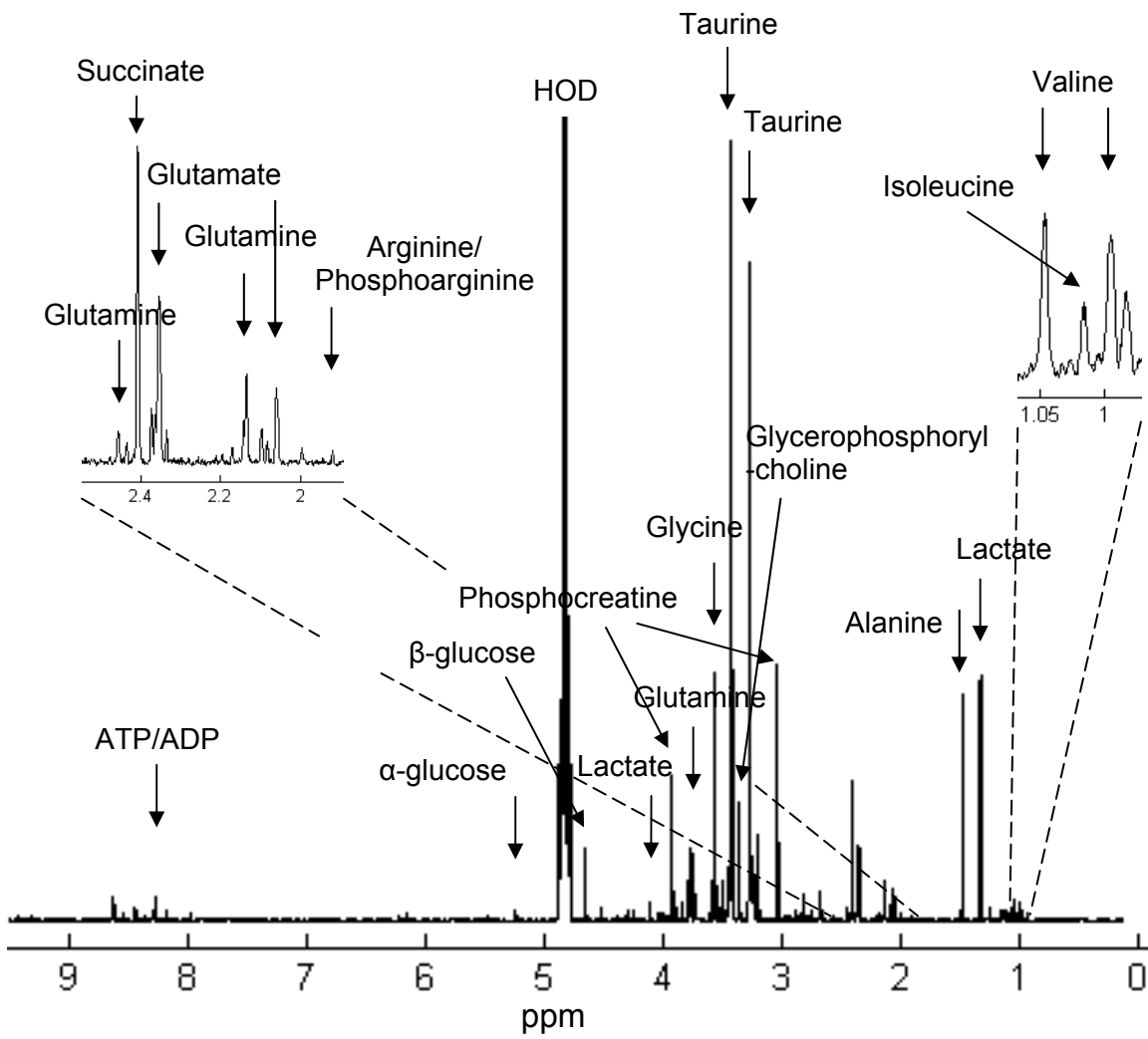


Figure 6. Representative 2D J-resolved NMR spectrum of smolt liver extracts.

Scores Plot for WAF 1/2/3 Muscle

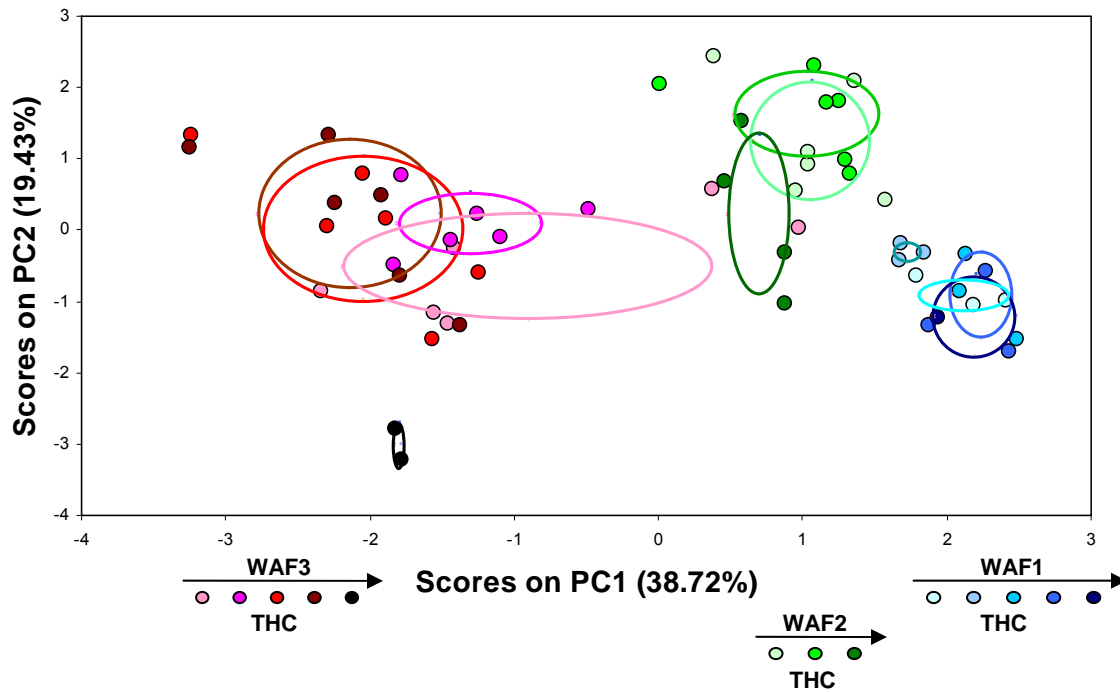
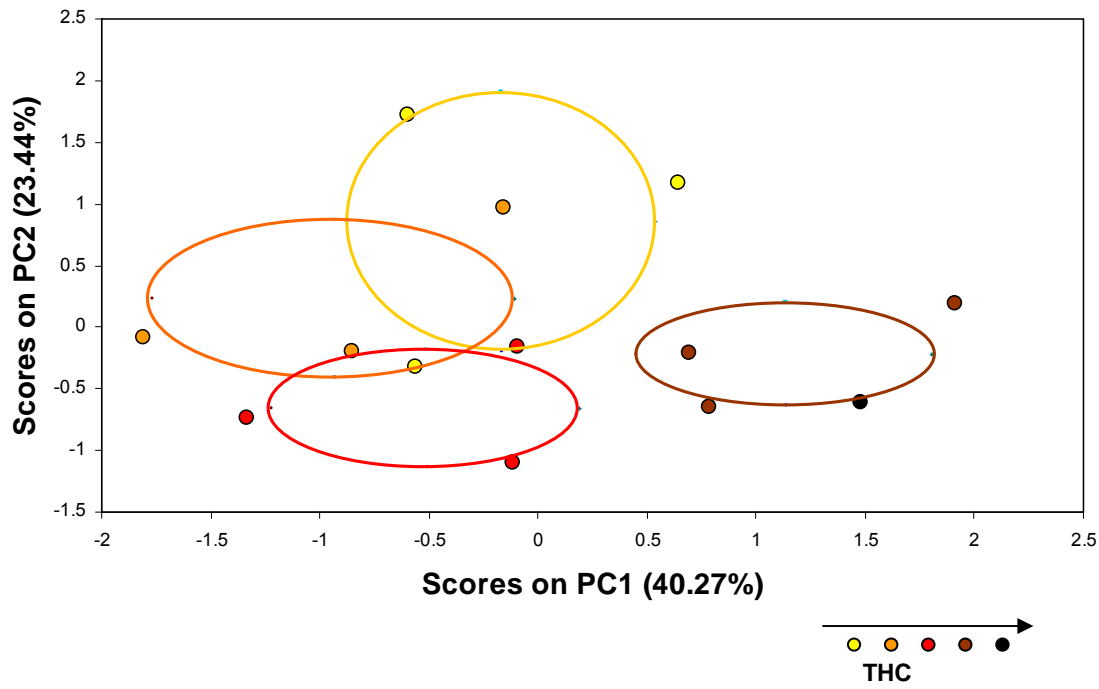


Figure 7. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts from 3 tests of WAF studies.

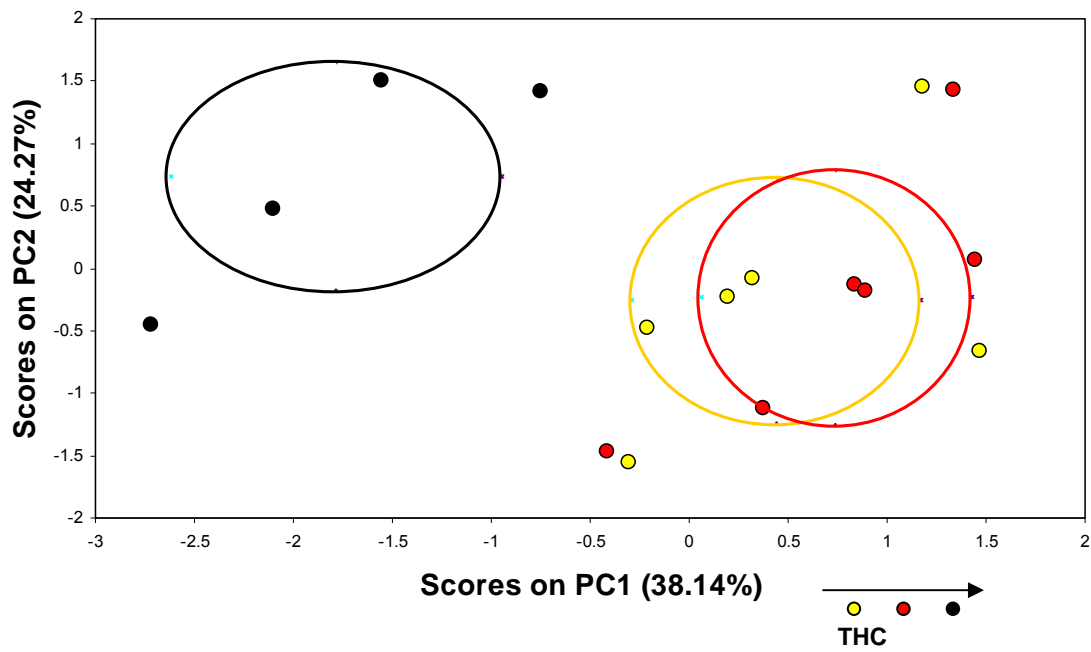
a.

Scores Plot for WAF1 Muscle



b.

Scores Plot for WAF2 Muscle



c.

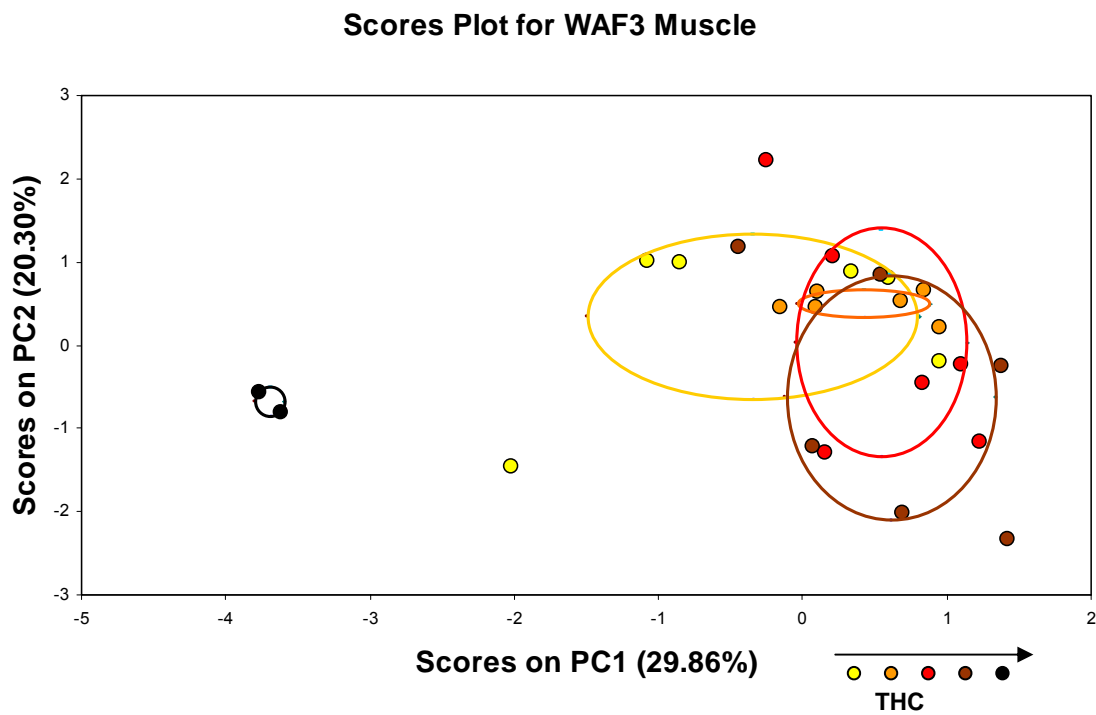


Figure 8. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts from 3 individual WAF tests (a, b, c).



Scores Plot for CEWAF 1/2/3 Muscle

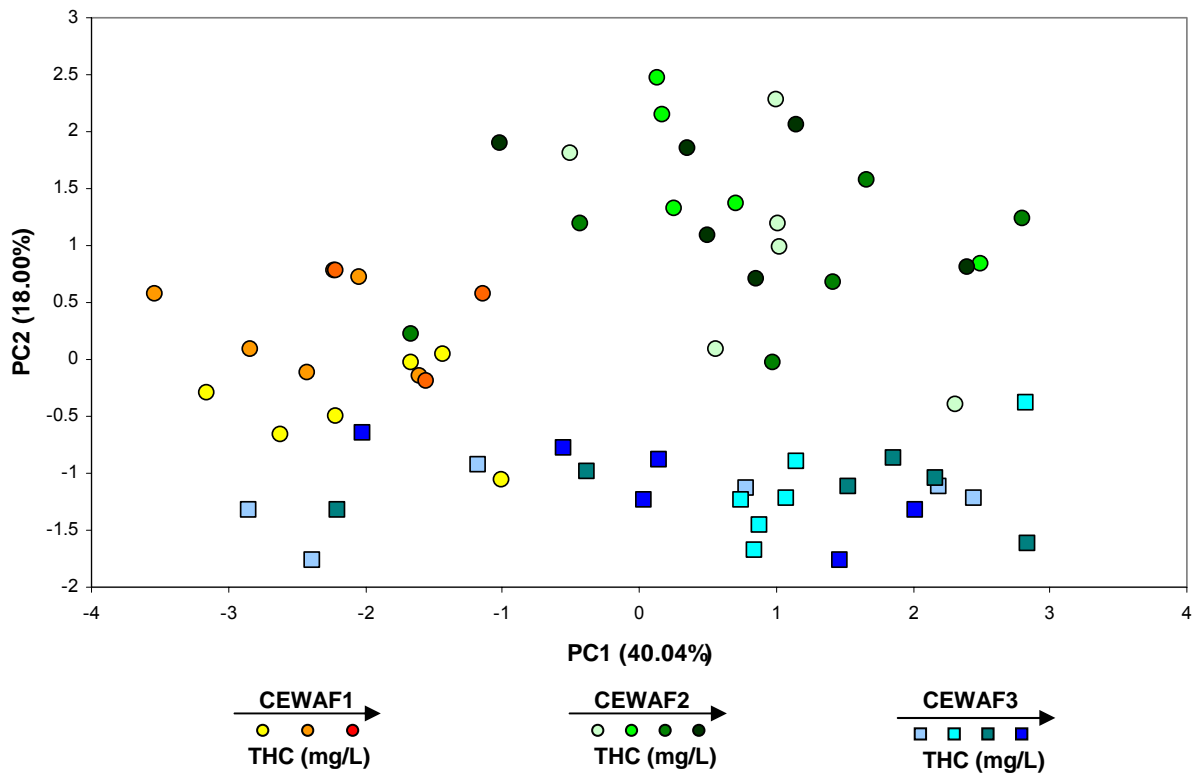
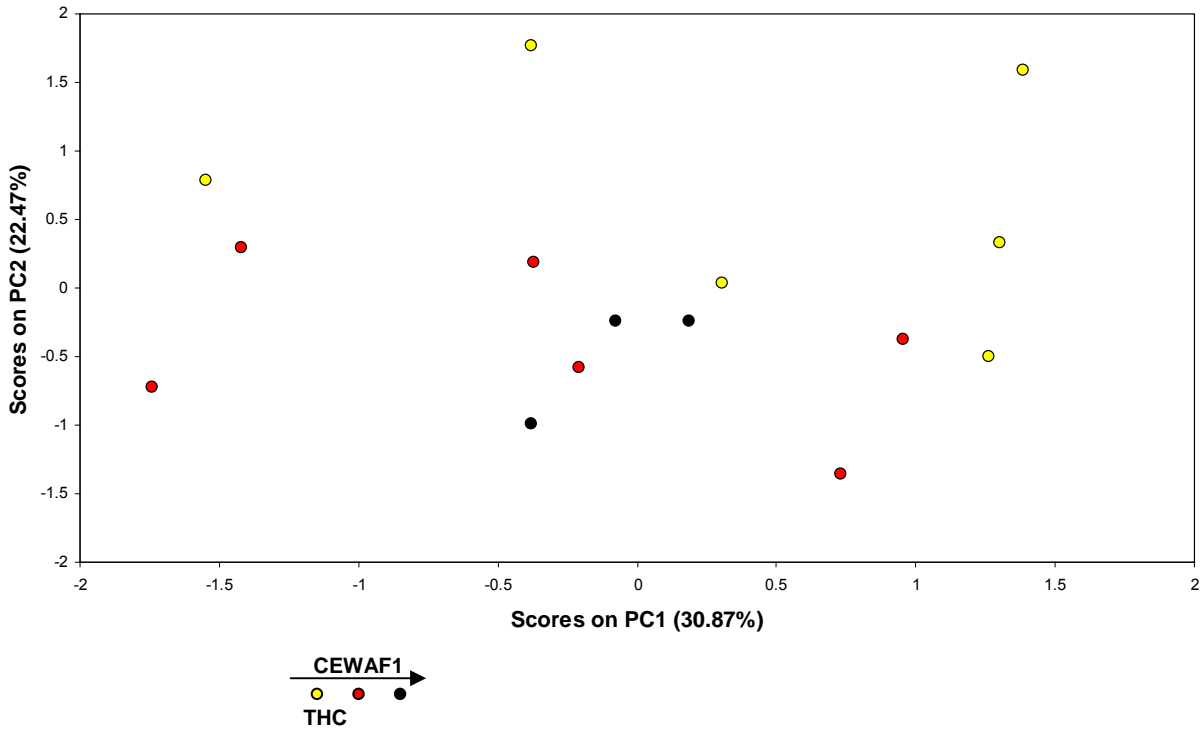


Figure 9. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts from 3 tests of CEWAF studies.

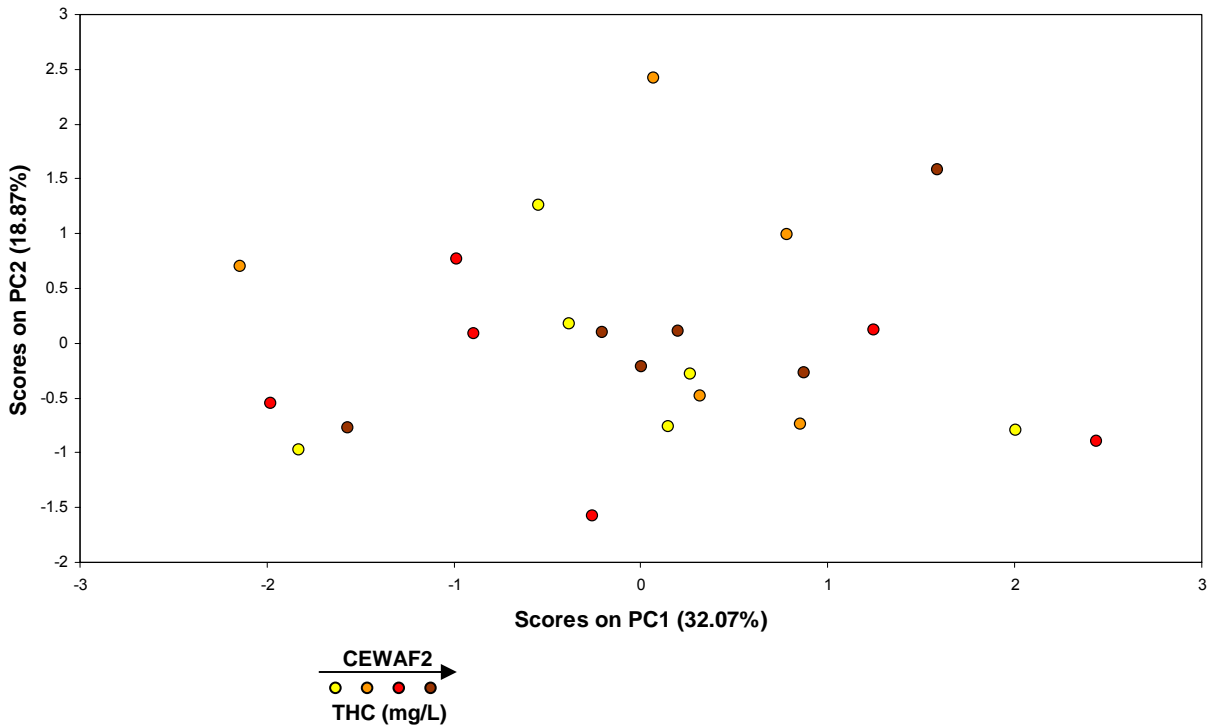
a.

Scores Plot for CEWAF1 Muscle



b.

Scores Plot for CEWAF2 Muscle



C.

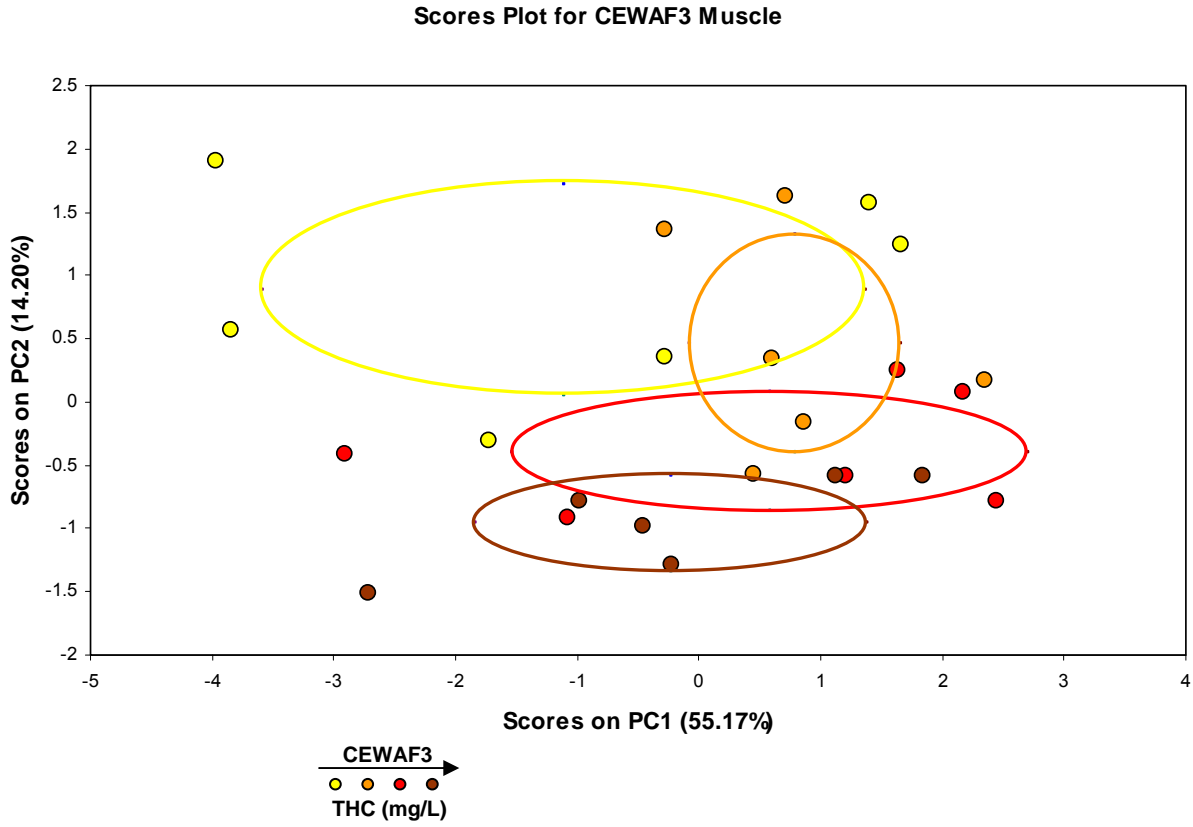


Figure 10. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts from 3 individual CEWAF tests (a, b, c).

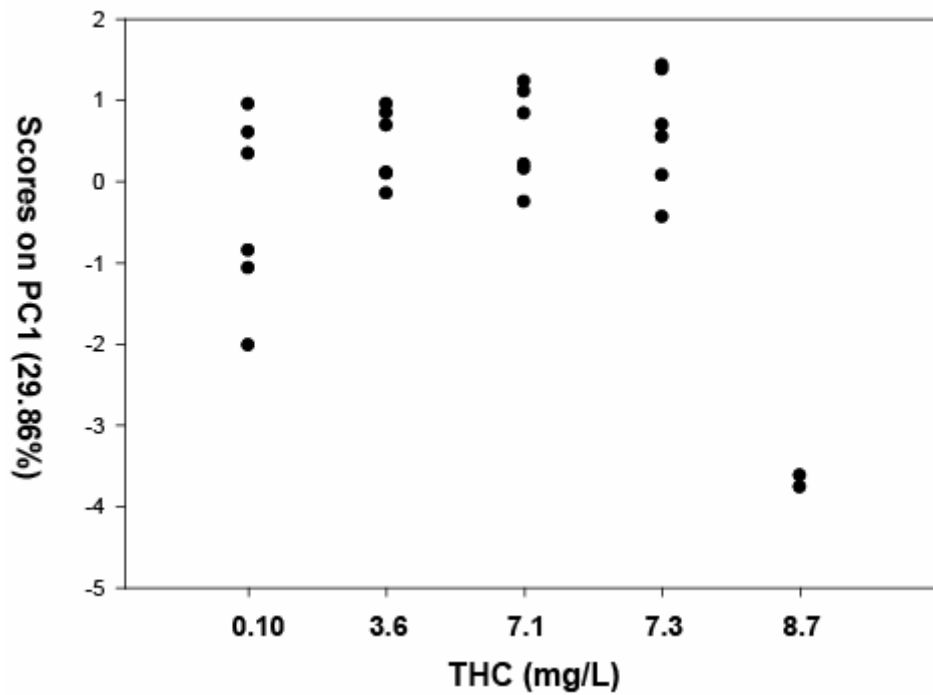


Figure 11a. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts exposed to variable doses of WAF (n=6, except n=2 for the highest dose).

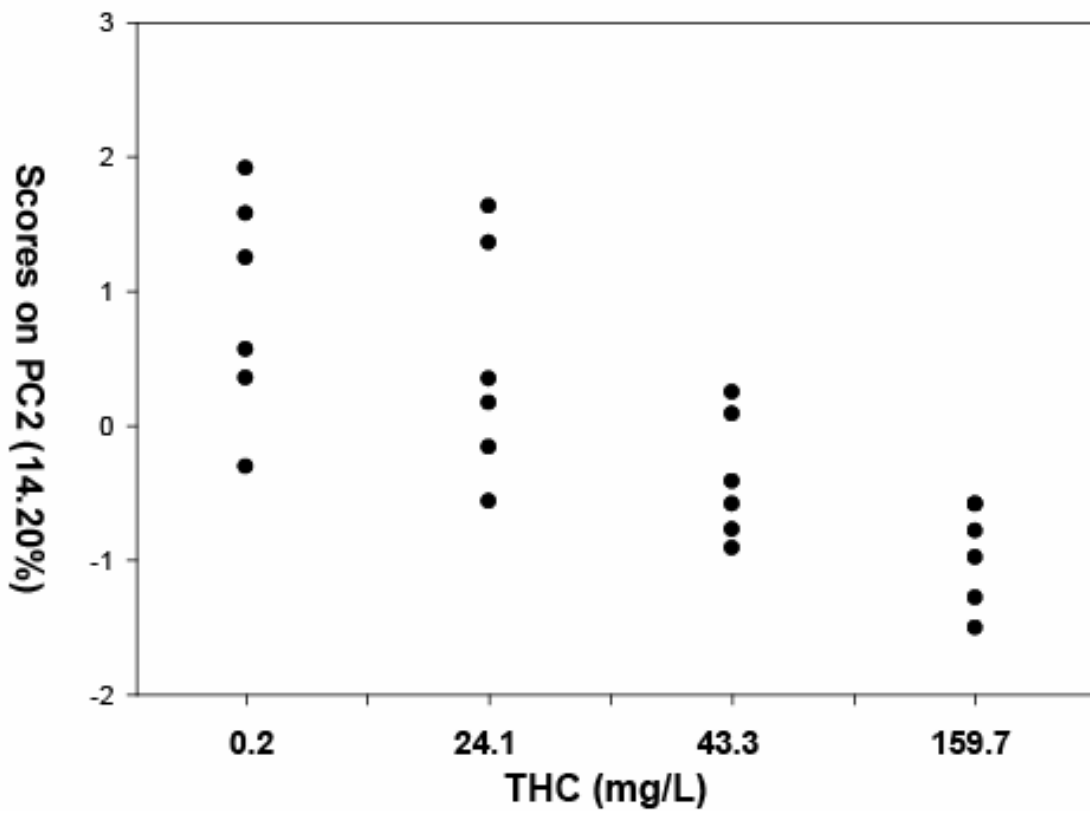


Figure 11b. PCA scores plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts exposed to variable doses of CEWAF (n=6).

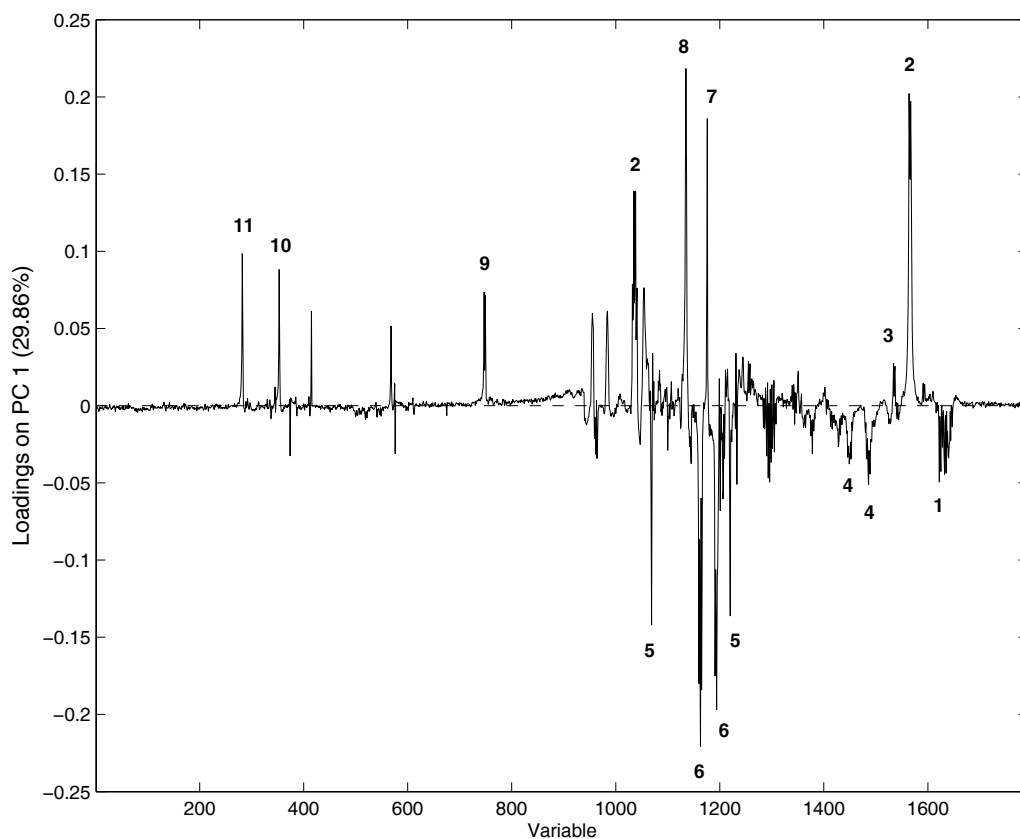


Figure 12a. PC1 loadings plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts exposed to WAF. Metabolite assignments: 1. valine, 2. lactate, 3. alanine, 4. arginine/phosphoarginine, 5. phosphocreatine, 6. taurine, 7. glycerophosphoryl choline, 8. glycine, 9. ATP/ADP, 10. IMP, 11. AMP.

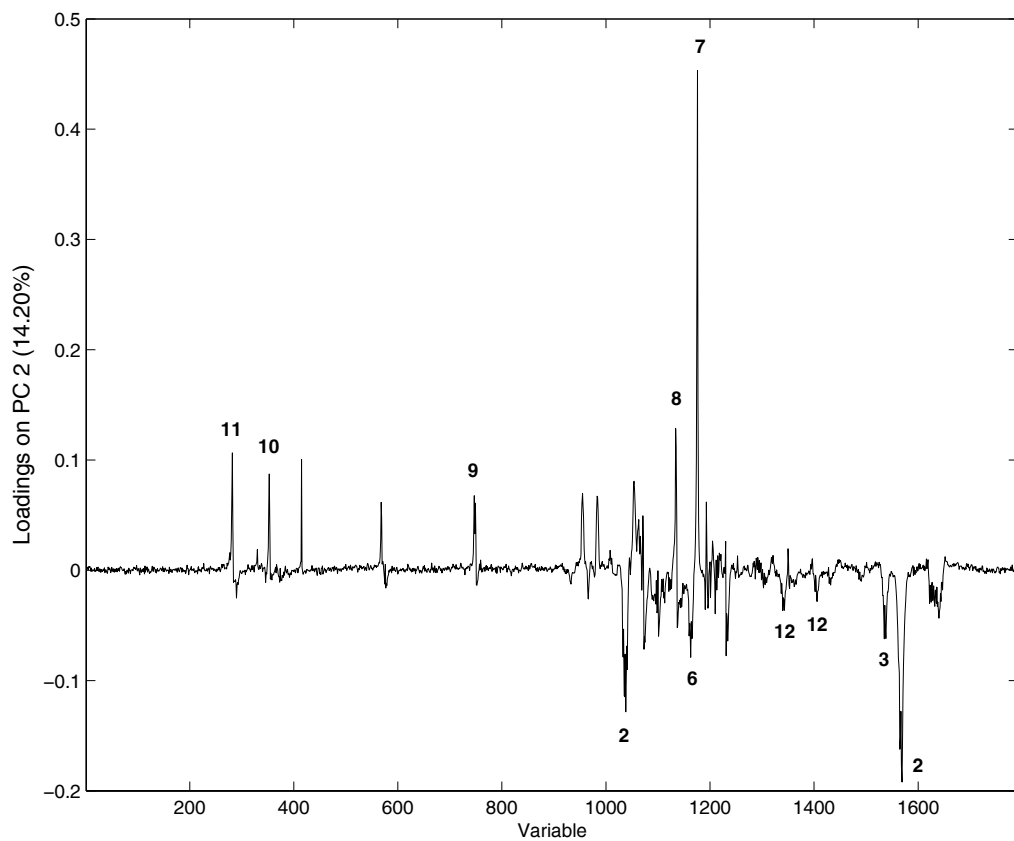


Figure 12b. PC2 loadings plot from the analysis of the 1D  $^1\text{H}$  NMR spectra of the muscle of smolts exposed to CEWAF. Metabolite assignments: 2. lactate, 3. alanine, 6. taurine, 7. glycerophosphorylcholine, 8. glycine, 9. ATP/ADP, 10. IMP, 11. AMP, 12. glutamine.

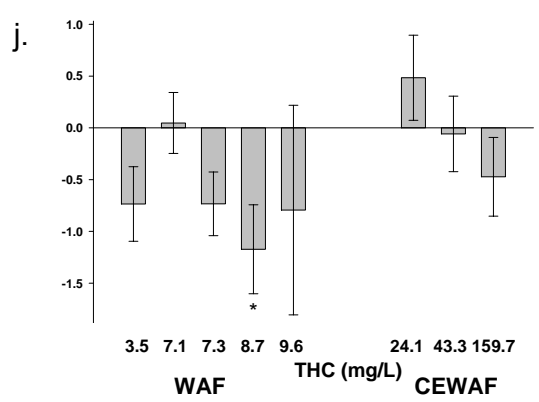
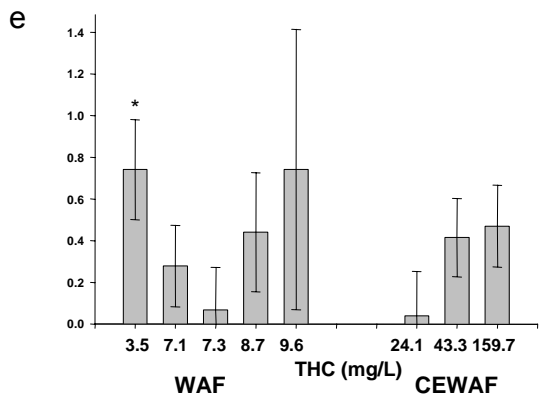
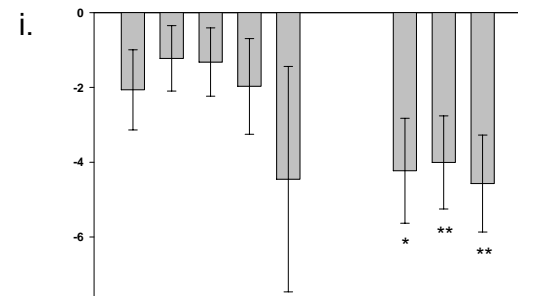
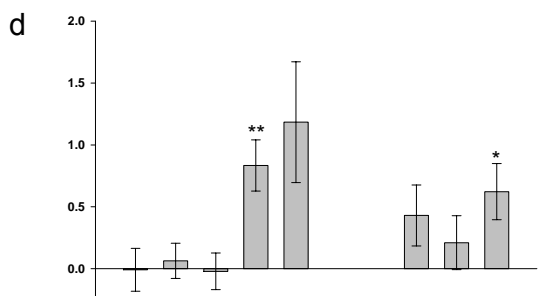
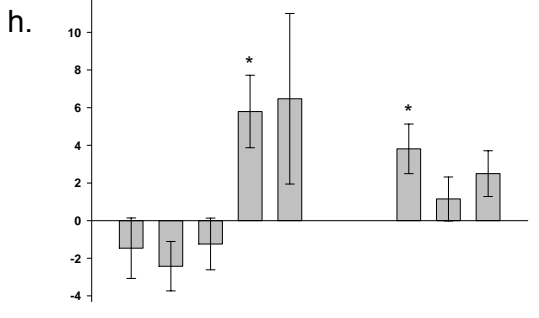
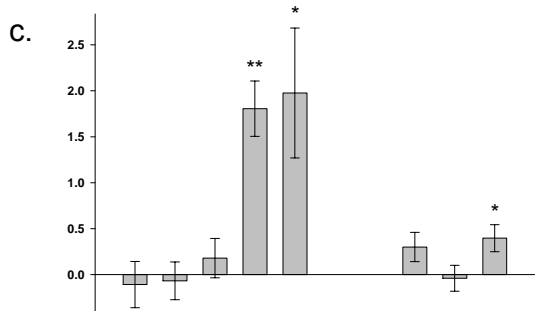
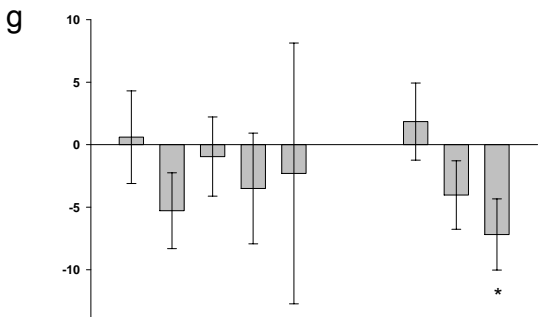
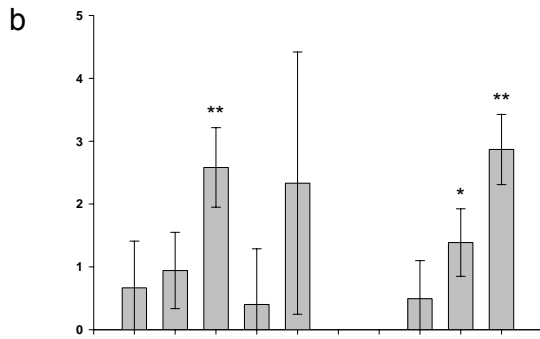
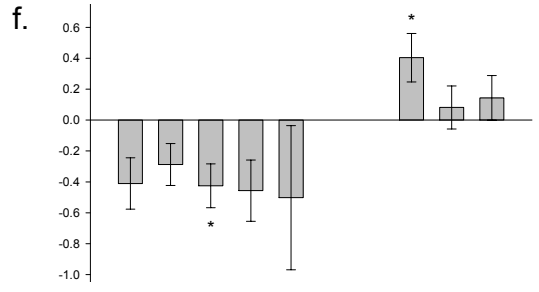
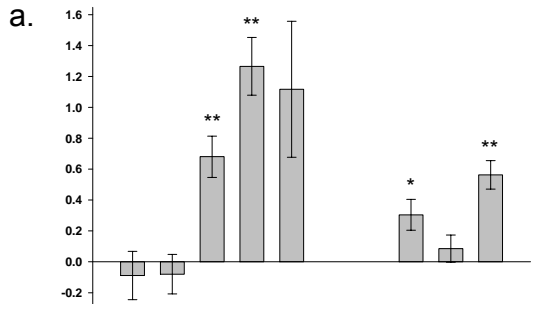




Figure 13. Change in amounts of specific metabolites including valine (a), alanine (b), arginine/phosphoarginine (c), glutamate (d), glutamine (e), succinate (f), phosphocreatine (g), taurine (h), glycerophosphorylcholine (i), ATP/ADP (j) in the muscle of smolts exposed to variable doses of WAF or CEWAF. The changes were calculated by subtracting the average peak area by that in the control of each test. Four tests were conducted in each WAF or CEWAF exposure. \* Denotes  $p < 0.05/5$  and \*\* denotes  $p < 0.01/5$  for WAF exposures. \* Denotes  $p < 0.05/3$  and \*\* denotes  $p < 0.01/3$  for CEWAF exposures.

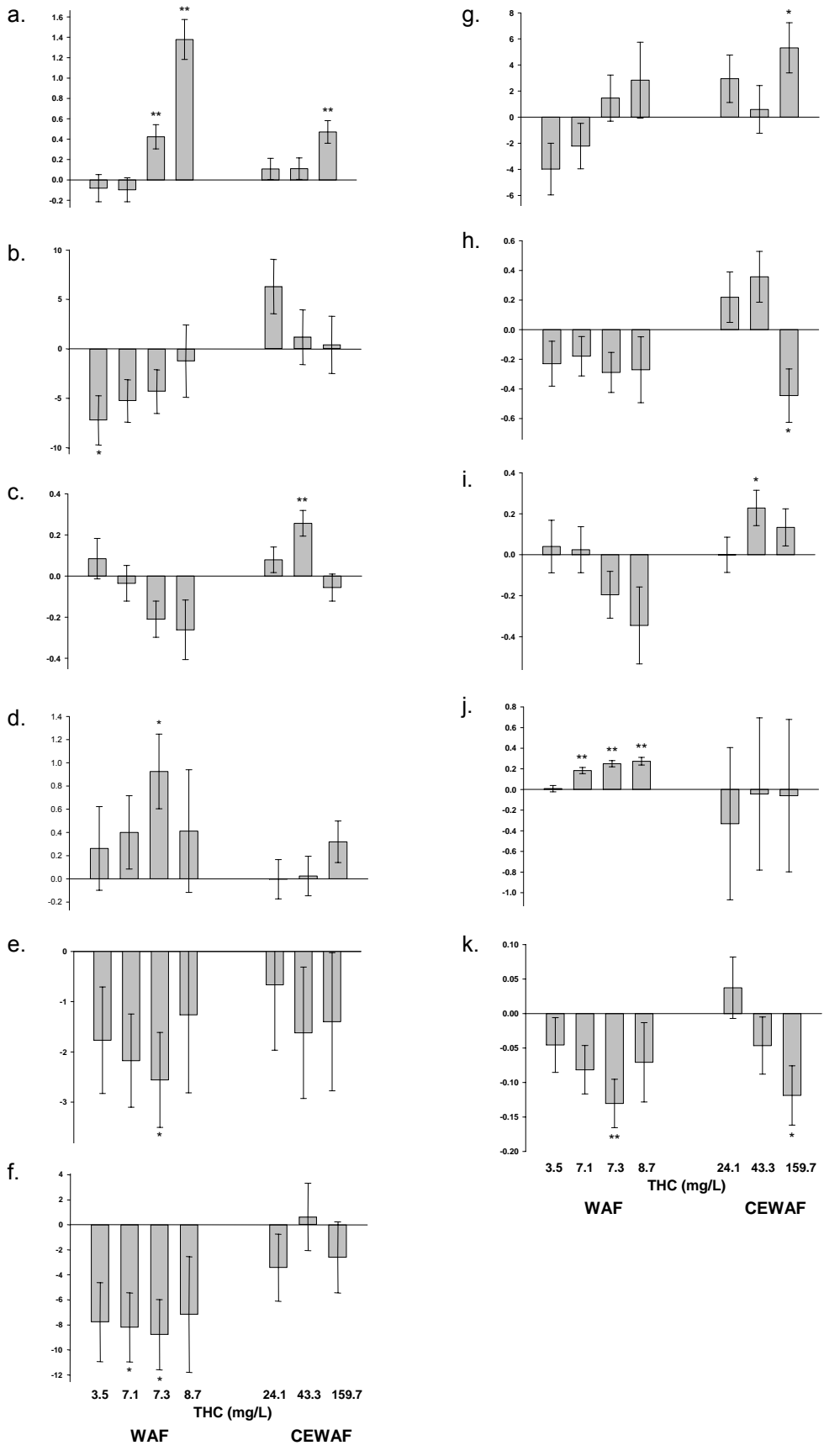


Figure 14. Change in amounts of specific metabolites including valine (a), lactate (b), glutamine (c), aspartate (d), phosphocreatine (e), glycerophosphorylcholine (f), glycine (g),  $\alpha$ -glucose (h), ATP/ADP (i), formate (j), AMP (k) in the liver of smolts exposed to variable doses of WAF or CEWAF. The changes were calculated by subtracting the average peak area by that in the control of each test. Four tests were conducted in each WAF or CEWAF exposure. \* Denotes  $p < 0.05/5$  and \*\* denotes  $p < 0.01/5$  for WAF exposures. \* Denotes  $p < 0.05/3$  and \*\* denotes  $p < 0.01/3$  for CEWAF exposures.

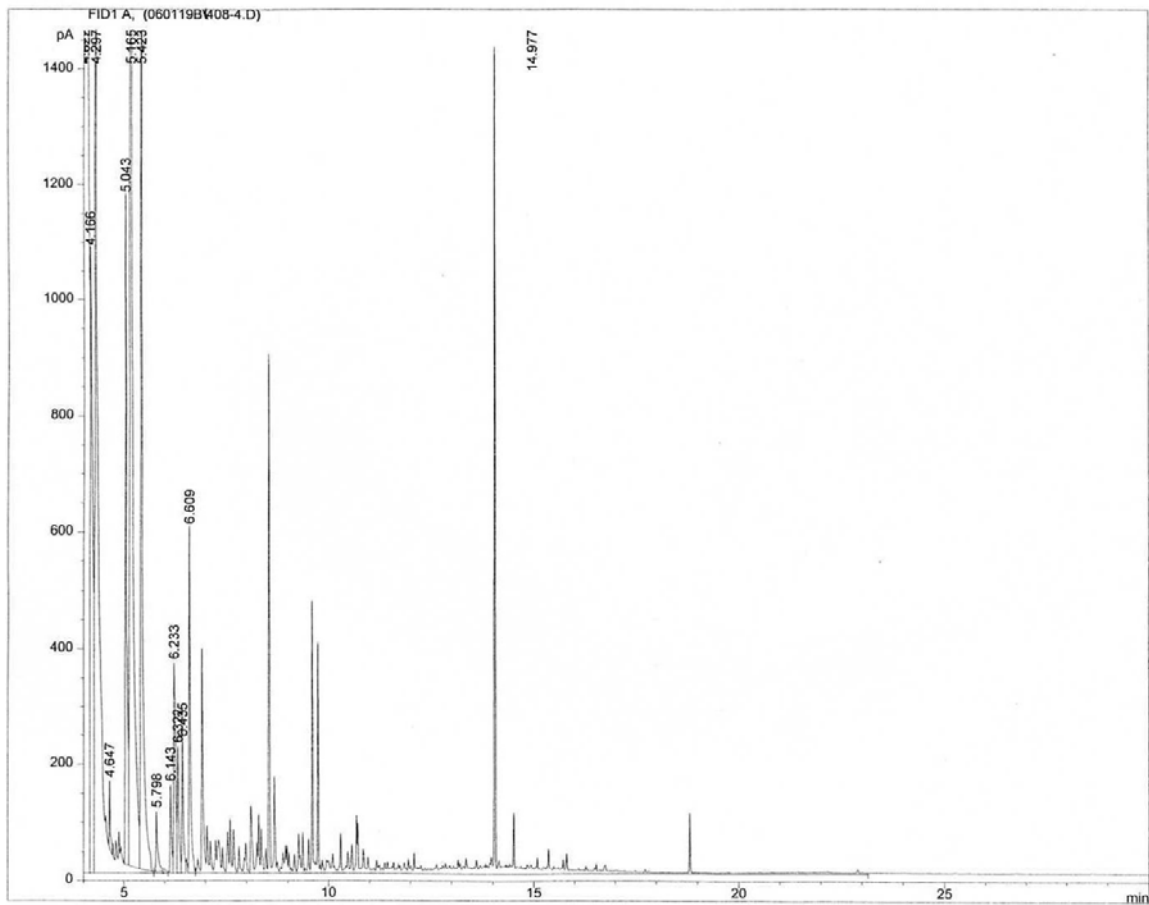


Figure 15. Representative chromatogram from WAF solution at nominal loading of 1.56 g/L of PBCO.

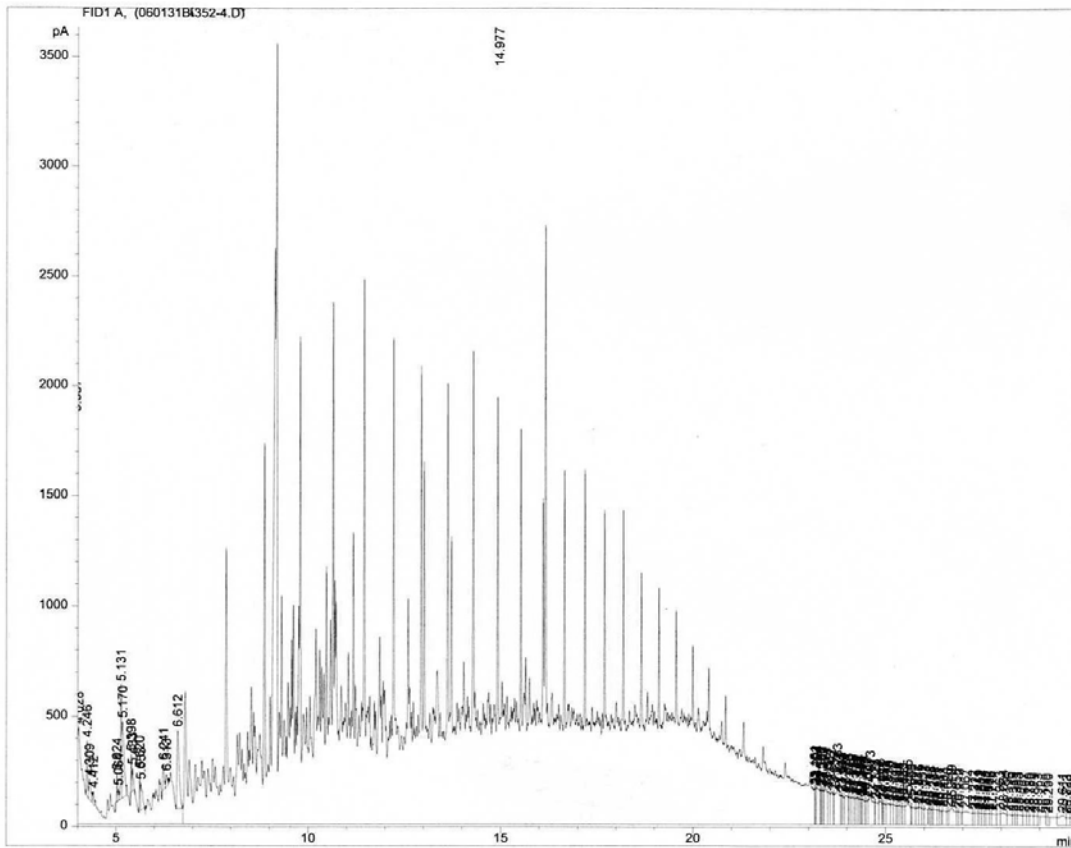


Figure 16. Representative chromatogram from CEWAF solution at nominal loading of 0.25 g/L of PBCO.

Table 1: Daily and final 96-h mean salmon smolt survival in three CEWAF and WAF tests.

**CEWAF Test 1: 6/15/05**

Oil Loading Concentration	THC conc.@ T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.1846	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.125	27.841	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.25	147.758	1	8	7	7	7	7	0.71	0.19
		2	8	4	4	4	4		
		3	8	6	6	6	6		
0.5	366.8245	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
1	303.772	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
2	223.49	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		

**CEWAF Test 2: 6/30/05**

Oil Loading Concentration	THC conc. at T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.3226	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.0625	23.4122	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.125	54.335	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.25	153.44	1	8	7	7	7	7	0.63	0.33
		2	8	6	6	6	6		
		3	8	2	2	2	2		
0.5	364.581	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
1	354.348	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		

**CEWAF Test 3: 8/25/05**

oil Loading Concentration	THC conc. at T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.12062	1	8	8	8	8	6	0.92	0.14
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.0625	24.699	1	8	8	8	8	7	0.88	0.13
		2	8	8	8	6	6		
		3	8	8	8	8	8		
0.125	47.77	1	8	8	8	8	7	0.96	0.07
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.25	177.93	1	8	3	3	3	3	0.46	0.14
		2	8	4	4	4	3		
		3	8	5	5	5	5		
0.5	272.04	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
1	272.03	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		

**WAF Test 1: 7/14/05**

Oil Loading Concentration	THC conc. at T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.0776	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.78	9.778	1	8	8	8	7	7	0.96	0.07
		2	8	8	8	8	8		
		3	8	8	8	8	8		
1.56	11.383	1	8	5	5	5	5	0.33	0.31
		2	8	3	3	3	3		
		3	8	0	0	0	0		
3.125	13.204	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
6.25	14.55	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		
12.5	17.02	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		

**WAF Test 2: 7/28/05**

Oil Loading Concentration	THC conc. at T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.1206	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.39	5.5675	1	8	8	8	8	8	0.58	0.36
		2	8	8	5	3	3		
		3	8	8	3	3	3		
0.78	8.2305	1	8	8	4	4	4	0.83	0.29
		2	8	8	8	8	8		
		3	8	8	8	8	8		
1.56	7.377	1	8	8	8	7	6	0.79	0.07
		2	8	7	7	7	7		
		3	8	6	6	6	6		
3.125	9.07	1	8	2	2	2	2	0.13	0.13
		2	8	1	1	1	0		
		3	8	3	3	3	1		
6.25	9.936	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		

**WAF Test 3: 9/08/05**

Oil Loading Concentration	THC conc. at T0	Rep	Day 0	Day 1	Day 2	Day 3	Day 4	Mean Survival	SD
0	0.1096	1	8	8	8	8	8	1.00	0.00
		2	8	8	8	8	8		
		3	8	8	8	8	8		
0.39	1.332	1	8	8	8	8	8	0.58	0.36
		2	8	8	5	3	3		
		3	8	8	3	3	3		
0.78	3.17	1	8	8	4	4	4	0.83	0.29
		2	8	8	8	8	8		
		3	8	8	8	8	8		
1.56	3.093	1	8	8	8	7	6	0.79	0.07
		2	8	7	7	7	7		
		3	8	6	6	6	6		
3.125	3.687	1	8	2	2	2	2	0.13	0.13
		2	8	1	1	1	0		
		3	8	3	3	3	1		
6.25	4.386	1	8	0	0	0	0	0.00	0.00
		2	8	0	0	0	0		
		3	8	0	0	0	0		



Table 2. Metabolites identified from muscle tissues of salmon smolts by NMR<sup>a</sup>.

Metabolites	<sup>1</sup> H NMR assignment (ppm) <sup>c</sup>
Valine	0.995 (d), 1.047* (d)
Lactate <sup>b</sup>	1.328* (d), 4.115* (q)
Alanine <sup>b</sup>	1.488* (d), 3.785 (m)
Arginine/Phosphoarginine	1.725 (m), 1.918* (m)
Glutamate	2.070 (m), 2.355 (t)
Glutamine <sup>b</sup>	2.138 (m), 2.455* (m)
Succinate	2.410* (s)
Phosphocreatine <sup>b</sup>	3.039* (s), 3.934 (s)
Taurine <sup>b</sup>	3.268* (t), 3.423* (t)
Glycerophosphoryl- choline	3.358* (s)
Glycine <sup>b</sup>	3.563* (s)
β-glucose <sup>b</sup>	4.650 (d)
α-glucose	5.238* (d)
ATP/ADP <sup>b</sup>	6.158 (d), 8.273* (s), 8.546* (s)
IMP	8.238* (s)
AMP	8.593* (s)

\* Peaks are taken to quantify and compared between treatments.

<sup>a</sup> Representative metabolites have been selected to illustrate the wide range of metabolite classes detectable with NMR spectroscopy. All peaks are confirmed by p-JRES spectra.

<sup>b</sup> Metabolite assignment is confirmed by 2D NMR (HSQC).

<sup>c</sup> Peaks observed as a singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m).

Table 3. Metabolites identified from liver tissues of salmon smolts by NMR<sup>a</sup>.

Metabolites	<sup>1</sup> H NMR assignment (ppm) <sup>c</sup>	p-JRES assignment (ppm)
Isoleucine		0.943, 1.015
Valine	1.047 (d)	0.995*, 1.047*
Lactate <sup>b</sup>	1.328 (d), 4.115 (q)	1.328*, 4.115*
Alanine <sup>b</sup>	1.488 (d), 3.785 (m)	1.488*, 3.785
Arginine/Phosphoarginine	1.725 (m), 1.918 (m), 3.833 (t)	1.918*
Glutamate <sup>b</sup>	2.070 (m), 2.355 (t)	2.355*
Glutamine <sup>b</sup>	2.138 (m), 2.455 (m), 3.763 (m)	2.138*, 2.455, 3.763
Succinate	2.410 (s)	2.410
Hypotaurine	2.658 (t)	2.658*
Aspartate	2.817 (dd), 3.900 (dd)	2.817*, 3.900
Phosphocreatine	3.039 (s), 3.934 (s)	3.039*, 3.934*
Taurine <sup>b</sup>	3.268 (t), 3.423 (t)	3.268*, 3.423*
Glycerophosphoryl-choline	3.358 (s)	3.358*
Glycine <sup>b</sup>	3.563 (s)	3.563*
β-glucose <sup>b</sup>	4.650 (d)	4.650*
α-glucose	5.238 (d)	5.238*
NAD <sup>+</sup> / NADP <sup>+</sup>	6.042 (d), 6.093 (d), 8.174 (s), 8.833* (d), 9.147* (d), 9.342* (s)	8.174
ATP/ADP <sup>b</sup>	6.158 (d), 8.273 (s), 8.546 (s)	6.158, 8.273*
Formate	8.458* (s)	
AMP	8.593* (s)	8.593

\* Peaks are taken to quantify and compared between treatments.

<sup>a</sup> Representative metabolites have been selected to illustrate the wide range of metabolite classes detectable with NMR spectroscopy. All spectra were normalized by total spectral area.

<sup>b</sup> metabolite assignment is confirmed by 2D NMR (HSQC).

<sup>c</sup> Peaks observed as a singlet (s), doublet (d), triplet (t), quartet (q), or multiplet (m).

Table 4. Metabolic changes in the muscle of smolts exposed to WAF<sup>a, b</sup>

Metabolites	chemical shift (ppm)	3.5 mg/L – Ctrl (p)	7.1 mg/L – Ctrl (p)	7.3 mg/L – Ctrl (p)	8.7 mg/L – Ctrl (p)	9.6 mg/L – Ctrl (p)
<b>Valine</b>	1.047 (d)	-0.09 (0.5707)	-0.08 (0.5707)	0.68 ( $<0.0001$ ) ↑**	1.26 ( $<0.0001$ ) ↑**	1.12 (0.0133)
<b>Lactate</b>	1.328 (d)	10.07 (0.0990)	7.73 (0.1212)	10.46 (0.0454)	-15.02 (0.0404)	-16.06 (0.3465)
	4.115 (q)	2.52 (0.0620)	1.97 (0.0731)	2.18 (0.0579)	-3.21 (0.0467)	-3.58 (0.3414)
<b>Alanine</b>	1.488 (d)	0.67 (0.3718)	0.94 (0.1248)	2.58 (0.0001) ↑**	0.40 (0.6518)	2.33 (0.2674)
<b>Arginine/ Phosphoarginine</b>	1.918 (m)	-0.11 (0.6679)	-0.07 (0.7451)	0.18 (0.4047)	1.81 ( $<0.0001$ ) ↑**	1.98 (0.0067) ↑*
<b>Glutamine</b>	2.455 (m)	0.74 (0.0028) ↑*	0.28 (0.1591)	0.07 (0.7375)	0.44 (0.1268)	0.74 (0.2739)
<b>Succinate</b>	2.410 (s)	-0.41 (0.0158)	-0.29 (0.0373)	-0.43 (0.0037) ↓*	-0.46 (0.0241)	-0.50 (0.2851)
<b>Phosphocreatine</b>	3.039 (s)	0.60 (0.8725)	-5.28 (0.0855)	-0.95 (0.7645)	-3.50 (0.4313)	-2.30 (0.8256)
<b>Taurine</b>	3.268 (t)	-4.74 (0.1860)	-4.80 (0.1027)	-3.74 (0.2202)	10.14 (0.0195)	9.39 (0.3503)
	3.423 (t)	-1.46 (0.3660)	-2.42 (0.0700)	-1.24 (0.3690)	5.79 (0.0036) ↑*	6.47 (0.1571)
<b>Glycerophosphoryl- Choline</b>	3.358 (s)	-2.07 (0.0579)	-1.22 (0.1669)	-1.32 (0.1524)	-1.97 (0.1277)	-4.45 (0.1437)
<b>Glycine</b>	3.563 (s)	-3.38 (0.3552)	1.41 (0.6374)	0.44 (0.8886)	-6.88 (0.1172)	5.31 (0.6047)
<b>α-glucose</b>	5.238 (d)	0.19 (0.2482)	0.14 (0.2313)	-0.21 (0.0770)	-0.21 (0.3347)	-0.32 (0.3424)
<b>ATP/ADP</b>	8.273 (s)	0.43 (0.0470)	-0.01 (0.9438)	-0.08 (0.6674)	-0.32 (0.2172)	-0.49 (0.4156)
	8.546 (s)	0.71 (0.0389)	-0.01 (0.9602)	-0.09 (0.7677)	-0.48 (0.2387)	-0.70 (0.4629)
<b>IMP</b>	8.238 (s)	-0.97 (0.0304)	0.09 (0.8061)	-0.38 (0.3099)	-0.48 (0.3596)	-0.09 (0.9405)
<b>AMP</b>	8.593 (s)	-1.48 (0.0212)	0.10 (0.8440)	-0.53 (0.3247)	-0.65 (0.3889)	-0.07 (0.9689)

<sup>a</sup> All spectra were normalized by total spectral area. The changes in metabolites were derived from subtraction of the average of that peak area in the control from that of the treatment. WAF was characterized by total hydrocarbon content (mg/L).

<sup>b</sup> \* $p < 0.05/5$ , \*\*  $p < 0.01/5$

Table 5. Metabolic changes in the muscle of smolts exposed to CEWAF<sup>a, b</sup>

Metabolites	Chemical shift (ppm)	24.1 mg/L – Ctrl (p)	43.3 mg/L – Ctrl (p)	159.7 mg/L – Ctrl (p)
<b>Valine</b>	1.047 (d)	0.30 (0.0031) ↑*	0.09 (0.3373)	0.56 (<.0001) ↑**
<b>Lactate</b>	1.328 (d)	-10.12 (0.1164)	11.18 (0.0515)	3.18 (0.5904)
	4.115 (q)	-1.94 (0.1691)	2.54 (0.0437)	1.09 (0.3981)
<b>Alanine</b>	1.488 (d)	0.49 (0.4174)	1.39 (0.0116) ↑*	2.87 (<.0001) ↑**
<b>Arginine/ Phosphoarginine</b>	1.918 (m)	0.30 (0.0625)	-0.04 (0.7821)	0.40 (0.0084) ↑*
<b>Glutamine</b>	2.455 (m)	0.04 (0.8509)	0.42 (0.0303)	0.47 (0.0190)
<b>Succinate</b>	2.410 (s)	0.40 (0.0119) ↑*	0.08 (0.5583)	0.14 (0.3249)
<b>Phosphocreatine</b>	3.039 (s)	1.84 (0.5514)	-4.03 (0.1442)	-7.18 (0.0135) ↓*
<b>Taurine</b>	3.268 (t)	7.23 (0.0048) ↑*	1.98 (0.3721)	4.09 (0.0792)
	3.423 (t)	3.81 (0.0048) ↑*	1.15 (0.3264)	2.50 (0.0429)
<b>Glycerophosphoryl- choline</b>	3.358 (s)	-4.23 (0.0035) ↓*	-4.01 (0.0019) ↓**	-4.57 (0.0007) ↓**
<b>Glycine</b>	3.563 (s)	3.99 (0.2049)	-0.68 (0.8066)	2.73 (0.3473)
<b>α-glucose</b>	5.238 (d)	-0.01 (0.9295)	-0.08 (0.4080)	0.15 (0.1517)
<b>ATP/ADP</b>	8.273 (s)	-0.19 (0.3655)	-0.11 (0.5272)	-0.16 (0.3913)
	8.546 (s)	-0.39 (0.2217)	-0.20 (0.4782)	-0.25 (0.3826)
<b>IMP</b>	8.238 (s)	0.61 (0.2232)	0.11 (0.8009)	-0.14 (0.7601)
<b>AMP</b>	8.593 (s)	0.89 (0.2106)	0.17 (0.7882)	-0.22 (0.7367)

<sup>a</sup> All spectra were normalized by total spectral area. The changes in metabolites were derived from subtraction of the average of that peak area in the control from that of the treatment. CEWAF was characterized by total hydrocarbon content (mg/L).

<sup>b</sup> \*p<0.05/3, \*\* p<0.01/3

Table 6. Metabolic changes in the liver of smolts exposed to WAF<sup>a, b</sup>

Metabolites	chemical shift (ppm)	3.5 mg/L – Ctrl ( <i>p</i> )	7.1 mg/L – Ctrl ( <i>p</i> )	7.3 mg/L – Ctrl ( <i>p</i> )	8.7 mg/L – Ctrl ( <i>p</i> )
<b>Valine</b>	0.995	-0.11 (0.4630)	-0.12 (0.3586)	0.54 (0.0002) ↑**	1.61 (<0.0001) ↑**
	1.047	-0.08 (0.5492)	-0.10 (0.4116)	0.42 (0.0008) ↑**	1.38 (<0.0001) ↑**
<b>Lactate</b>	1.328	-7.22 (0.0051) ↓*	-5.27 (0.0171)	-4.32 (0.0562)	-1.24 (0.7359)
	4.115	-0.57 (0.0217)	-0.42 (0.0523)	-0.28 (0.2037)	-0.17 (0.6386)
<b>Alanine</b>	1.488	-10.44 (0.0254)	-1.80 (0.6543)	-2.40 (0.5574)	-7.49 (0.2666)
<b>Arginine/ Phosphoarginine</b>	1.918	-0.11 (0.6059)	-0.21 (0.3356)	0.39 (0.0836)	0.21 (0.4411)
<b>Glutamine</b>	2.138	0.41 (0.3102)	0.01 (0.9714)	0.03 (0.9254)	0.41 (0.4006)
<b>Glutamate</b>	2.355	0.76 (0.3774)	0.73 (0.3306)	1.37 (0.0763)	2.05 (0.1061)
<b>Phosphocreatine</b>	3.039	-3.26 (0.1039)	-4.15 (0.0196)	-4.50 (0.0132)	-1.87 (0.5210)
	3.934	-1.77 (0.1002)	-2.17 (0.0225)	-2.56 (0.0089) ↓*	-1.26 (0.4194)
<b>Hypotaurine</b>	2.658	-0.18 (0.7434)	0.02 (0.9653)	0.88 (0.1163)	1.74 (0.0316)
<b>Aspartate</b>	2.817	0.26 (0.4709)	0.40 (0.2100)	0.93 (0.0055) ↑*	0.41 (0.4392)
<b>Phosphocreatine</b>	3.039	-3.26 (0.1039)	-4.15 (0.0196)	-4.50 (0.0132)	-1.87 (0.5210)
	3.934	-1.77 (0.1002)	-2.17 (0.0225)	-2.56 (0.0089) ↓*	-1.26 (0.4194)
<b>Taurine</b>	3.268	-7.95 (0.0289)	-4.65 (0.1405)	-4.61 (0.1514)	-5.18 (0.3240)
	3.423	-8.93 0.0358	-5.44 (0.1409)	-4.45 (0.2357)	-5.27 (0.3913)
<b>Glycerophosphoryl- choline</b>	3.358	-7.78 (0.0163)	-8.19 (0.0043) ↓*	-8.78 (0.0027) ↓*	-7.17 (0.1255)
<b>Glycine</b>	3.563	-3.98 (0.0493)	-2.22 (0.2073)	1.46 (0.4115)	2.84 (0.3333)
<b>β-glucose</b>	4.650	-1.74 (0.0539)	-1.79 (0.0249)	-1.75 (0.0303)	-1.40 (0.2859)
<b>α-glucose</b>	5.238	-0.23 (0.1349)	-0.18 (0.1821)	-0.29 (0.0370)	-0.27 (0.2287)
<b>Unknown</b>	7.093 (s)	0.04 (0.4968)	0.11 (0.0352)	0.16 (0.0026)	0.11 (0.1243)

				↑*	
<b>Unknown</b>	7.865 (s)	0.05 (0.3692)	0.09 (0.0692)	0.13 (0.0070)	0.09 (0.1597)
<b>ATP/ADP</b>	8.273	0.04 (0.7551)	0.02 (0.8281)	-0.20 (0.0924)	-0.35 (0.0711)
<b>Formate<sup>c</sup></b>	8.458 (s)	0.01 (0.7852)	0.18 ( $<0.0001$ )	0.25 ( $<0.0001$ )	0.27 ( $<0.0001$ )
<b>AMP<sup>c</sup></b>	8.593 (s)	-0.05 (0.2601)	-0.08 (0.0279)	-0.13 (0.0009)	-0.07 (0.2296)
<b>NAD/NADP<sup>+</sup><sup>c</sup></b>	8.833 (d)	-0.03 (0.5086)	0.04 (0.3118)	0.05 (0.1787)	0.10 (0.0512)
	9.147 (d)	-0.03 (0.5926)	0.04 (0.3068)	0.08 (0.0717)	0.09 (0.0970)
	9.342 (s)	-0.01 (0.7628)	0.04 (0.2904)	0.09 (0.0261)	0.08 (0.1397)

<sup>a</sup> All spectra were normalized by total spectral area. The changes in metabolites were derived from subtraction of the average of that peak area in the control from that of the treatment. WAF was characterized by total hydrocarbon content (mg/L).

<sup>b</sup> \*  $p < 0.05/4$ , \*\*  $p < 0.01/4$  (based on 4 sets of p-JRES spectra), except <sup>c</sup> \*  $p < 0.05/5$ , \*\*  $p < 0.01/5$  (based on 5 sets of <sup>1</sup>H NMR spectra).

Table 7. Metabolic changes in the liver of smolts exposed to CEWAF<sup>a, b</sup>

Metabolites	chemical shift (ppm)	24.1 mg/L – Ctrl ( <i>p</i> )	43.3 mg/L – Ctrl ( <i>p</i> )	159.7 mg/L – Ctrl ( <i>p</i> )
Valine	0.995	0.14 (0.1938)	0.10 (0.3620)	0.43 (0.0002) ↑**
	1.047	0.11 (0.3067)	0.11 (0.2980)	0.47 (<0.0001) ↑**
Lactate	1.328	6.32 (0.0248)	1.19 (0.6702)	0.42 (0.8865)
	4.115	0.71 (0.0340)	0.11 (0.7432)	-0.04 (0.9169)
Alanine	1.488	6.09 (0.1134)	5.75 (0.1347)	1.44 (0.7215)
Arginine/ Phosphoarginine	1.918	0.16 (0.6581)	-0.69 (0.1359)	N.A.
Glutamine	2.138	0.12 (0.6357)	0.83 (0.0013) ↑**	0.32 (0.2211)
Glutamate	2.355	0.16 (0.7932)	1.44 (0.0196)	1.00 (0.1189)
Hypotaurine	2.658	-1.29 (0.2136)	-1.30 (0.1756)	-0.01 (0.9926)
Aspartate	2.817	-0.00 (0.9838)	0.02 (0.8884)	0.32 (0.0786)
Phosphocreatine	3.039	-1.12 (0.6466)	-2.83 (0.2518)	-2.51 (0.3341)
	3.934	-0.67 (0.6100)	-1.62 (0.2196)	-1.40 (0.3120)
Taurine	3.268	2.35 (0.4447)	-0.35 (0.9091)	5.14 (0.1156)
	3.423	4.07 (0.2485)	1.23 (0.7271)	7.73 (0.0402)
Glycerophosphoryl- choline	3.358	-3.43 (0.2034)	0.63 (0.8154)	-2.61 (0.3598)
Glycine	3.563	2.94 (0.1099)	0.59 (0.7483)	5.32 (0.0072) ↑*
β-glucose	4.650	1.80 (0.0899)	2.49 (0.0206)	-2.29 (0.0416)
α-glucose	5.238	0.22 (0.2030)	0.36 (0.0408)	-0.45 (0.0158) ↓*
Unknown	7.093 (s)	0.04 (0.4465)	-0.00 (0.9918)	-0.01 (0.8143)
Unknown	7.865 (s)	0.04 (0.4470)	-0.00 (0.9975)	-0.02 (0.7103)
ATP/ADP	8.273	-0.00 (0.9993)	0.23 (0.0101) ↑*	0.13 (0.1461)
Formate	8.458 (s)	-0.33 (0.6579)	-0.04 (0.9537)	-0.06 (0.9360)
AMP	8.593 (s)	0.04 (0.4038)	-0.05 (0.2650)	-0.12 (0.0071) ↓*
NAD/NADP+	8.833 (d)	0.07	0.01	0.04

		(0.1068)	(0.7489)	(0.3467)
	9.147 (d)	0.06 (0.1202)	0.01 (0.8845)	0.03 (0.5116)
	9.342 (s)	0.04 (0.3022)	0.01 (0.8236)	0.01 (0.7790)

<sup>a</sup> All spectra were normalized by total spectral area. The changes in metabolites were derived from subtraction of the average of that peak area in the control from that of the treatment. CEWAF was characterized by total hydrocarbon content (mg/L).

<sup>b</sup> \* $p < 0.05/3$ , \*\*  $p < 0.01/3$



Table 8. Smolt survival and growth after exposure to PBCO WAF and CEWAF.

Test	WAF 4					CEWAF5				
	9/12/05	12/12/05	1/9/06	2/9/06	3/16/06	9/26/05	12/15/05	1/9/06	2/9/06	3/16/06
Dates weighed										
Loading (g/L)	<b>0.00</b>					<b>0.00</b>				
# of Fish	18.00	4.00	4.00	4.00	3.00	14.00	2.00	1.00	0.00	0.00
Mean	19.33	27.23	27.10	37.80	37.87	19.55	24.55	27.60		
SD	4.25	6.58	11.05	12.88	16.40	5.31	5.87			
Loading (g/L)	<b>0.39</b>					<b>0.0625</b>				
# of Fish	8.00	3.00	2.00	2.00	2.00	3.00	2.00	1.00	1.00	1.00
Mean	17.14	17.57	21.45	24.60	39.40	27.00	42.20	52.30	60.70	67.50
SD	2.83	3.00	7.99	9.33	12.73	4.60	8.49			
Loading (g/L)	<b>0.78</b>					<b>0.1250</b>				
# of Fish	14.00	6.00	6.00	6.00	6.00	9.00	4.00	4.00	2.00	2.00
Mean	18.81	29.92	34.27	35.98	55.62	20.07	26.60	25.15	31.25	51.70
SD	4.42	8.64	10.35	15.40	17.03	3.18	3.98	7.15	12.23	7.35
Loading (g/L)	<b>1.56</b>					<b>0.250</b>				
# of Fish	13.00	4.00	4.00	3.00	1.00	2.00	1.00	1.00	1.00	1.00
Mean	21.18	29.93	35.73	38.27	99.30	20.65	35.50	33.60	33.50	46.40
SD	5.04	12.38	17.10	25.70		1.34				

