RELATIVE TOXICITY OF WEATHERED PRUDHOE BAY CRUDE OIL AND DISPERSED OIL ON SPAWNING TOPSMELT *(ATHERINOPS AFFINIS*)

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Abstract

Crude oil contamination from tanker spills remains a potentially serious problem along coastal Californi,a particularly within the San Francisco Estuary where oil spills present a serious threat to one of the most important ecosystems on the West Coast. Use of oil dispersants to mitigate spill impacts in this and similar systems requires information on the relative toxicity of chemically dispersed oil on coastal habitats. This includes data on the relative toxicity of chemically- and physically-dispersed oil on near-shore fish species. This study examined the relative toxicities of physically- and chemically-dispersed weathered Prudhoe Bay Crude Oil (PBCO) on topsmelt (*Atherinops affinis*). This species was selected because it is a dominant fish species in California estuaries and bays, and based on its ecological relevance, it is an appropriate indicator for risk of oil spills to fish populations in the San Francisco Estuary. The study assessed toxicity of the water-accommodated fraction of physically-dispersed oil (WAF) and chemically-dispersed oil (CEWAF) on spawning adult topsmelt, as well as on the developing embryos of this species. The study incorporated metabolomic analyses of tissues from adult and embryonic life stages to provide biomarker information on sublethal effects of hydrocarbons on topsmelt.

Results of experiments comparing the relative toxicity of WAF and CEWAF demonstrated that chemical dispersion of weathered PBCO resulted in greater hydrocarbons in seawater relative to physical dispersion. Higher total hydrocarbon content (THC) and higher concentrations of polynuclear aromatic hydrocarbons (PAHs) resulted in greater toxicity of CEWAFs to both adult and embryonic topsmelt. Physical dispersion of weathered PBCO (WAF) did not result in toxic concentrations of hydrocarbons in seawater to either adult or embryonic topsmelt. Therefore no LC50s could be calculated for adult or embryonic topsmelt after exposure to WAF. While the responses of embryonic tospmelt to CEWAF suggest inhibited development was due to specific hydrocarbon constituents, it is not clear whether mortality of adults exposed to CEWAF was due to a toxic response of topsmelt to increased hydrocarbon bioavailability, or to physical reactions of adult fish to the complex mixtures of the larger hydrophilic complex consisting of dissolved hydrocarbons, dissolved surfactants and particulate bulk-oil in CEWAF.

Adult topsmelt exposed to sublethal concentrations of WAF and CEWAF survived and grew after the initial 96h exposure. There were no differences in growth between control fish and those exposed to WAF or CEWAF after 78 days in culture. A dose-dependent reduction in the number of embryos produced was observed in both WAF and CEWAF-exposed adult fish. There was no apparent relationship between embryo viability and *in vivo* exposure to WAF or CEWAF. Corexit 9500 by itself was completely toxic to adult fish at 100 mg/L.

Following 96 hour exposures to sublethal concentrations of chemically and physically dispersed oil, one surviving fish from each replicate exposure tank was sacrificed for metabolomic analysis. Low molecular weight metabolites were extracted from muscle tissue and analyzed using one-dimensional ^{1}H nuclear magnetic resonance (NMR) spectroscopy. Following spectral processing, metabolites were investigated using principal components analysis (PCA) and metabolic changes were identified for each concentration of WAF and CEWAF. Metabolomic analysis results for the WAF and CEWAF tests demonstrated an increase in amino acids and an increase in ATP. This suggests the breakdown of proteins possibly for cellular repair, but the decreased use of energy suggests the use of amino acid skeletons for energy production.

Physical dispersion of weathered PBCO at loadings as high as 25 g/L did not result in sufficient hydrocarbons in seawater to inhibit development and hatching success of embryos exposed directly to WAF. Chemical dispersion of PBCO resulted in much higher concentrations of THC and tri-cyclic PAHs, and these inhibited embryo larval development at the lowest oil loading tested. CEWAF solutions created from dispersion of weathered PBCO caused cardiovascular abnormalities and resulted in high rates of embryo mortality. Furthermore, metabolomic analyses for embryos resulted in variations of increasing and decreasing metabolic changes. Phosphocreatine was identified only in embryo extracts of those exposed to WAF and CEWAF directly; suggesting the use of energy to repair damage resulting from the exposure.

Since topsmelt are among the most numerous of estuarine species in California, they are an important indicator species. Toxicity results with this species provides information to resource managers regarding the relative risk to coastal and estuarine fish of treating oil spills with Corexit 9500 dispersant. Experiments using environmentally realistic declining exposures of

weathered crude oil suggest that dispersion of oil could affect adult survival and reproduction, and could also inhibit development in situations where embryos were subjected to elevated hydrocarbons during critical stages of development.

Keywords: metabolomics, Topsmelt, toxicity, oil spill, dispersed oil, Prudhoe Bay Crude Oil, Corexit 9500

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1.0 Introduction

Topsmelt (*Atherinops affinis*) are an ecologically-important dominant fish species found in the coastal bays, estuaries and the near-shore coastal waters of California. They reproduce in San Francisco Estuary, and their larvae migrate throughout this system for further development. Due to its refining capacity, the San Francisco Bay is at risk of a tanker spill similar or larger to the COSCO Busan spill of November 2007. There is serious concern that oil spills within the bay and delta waterways may impact the development and survival of spawning and developing fish in this system. Because of its distribution, life history characteristics, and ecological relevance, the topsmelt is an appropriate indicator species for risk of oil spills to fish populations in the San Francisco Estuary.

Chemical dispersants are used to minimize damage that may occur from an oil spill threatening coastal shorelines. Use of Corexit 9500 dispersant (Nalco/Exxon Energy Chemicals) is intended to penetrate high viscosity oils and emulsions (Singer *et. al*, 1996) but its effectiveness may be limited by the various hydrocarbons within the oil, viscosity and density of the crude oil and salinity of the water (Blondina *et. al*, 1999). It is generally thought that dissolving crude oil results in small molecular-weight polycyclic aromatic hydrocarbons (PAHs) becoming more bioavailable to many aquatic organisms. However, dispersants are designed to aggregate hydrocarbons into micelles, potentially reducing their bioavailability by formation of a larger hydrophilic complex consisting of dissolved hydrocarbons, dissolved surfactants and particulate bulk-oil (Singer *et el.*, 1998).

Highly lipophilic oil components are potentially toxic to aquatic organisms and may target their tissues (i.e. skeletal muscle and embryo membranes) and organs (i.e. liver) containing a large amount of lipids (DiGiulio, 2008). Of those targets, the liver and skeletal muscles play important metabolic roles and are subject to experiencing adverse effects of xenobiotics. Crude and dispersed oil may also have an effect on fish development. Critical stages in development occur during embryogenesis and larval growth, due to their large lipid content, making these life stages particularly susceptible to xenobiotic damage. Oil components thus may interfere with reproduction, development, growth and survival of aquatic organisms.

This project is designed to provide information regarding the relative toxicities of treated and untreated oiled seawater to spawning adult topsmelt and their embryos. This work is a continuation of OSPR-OWCN studies designed to examine the effects of dispersed and un-dispersed oil on the survival and development of coastal fish species. In the event of a coastal oil spill, resource managers need information on the risk associated with treating spills with dispersants, particularly when breeding populations of coastal fish are present. The project combines toxicological information from acute exposures examining lethal effects with biomarker endpoints using NMR-based metabolomics, and chronic studies of longer-term growth effects.

2.0 Objectives

This 2-year project was designed to address multiple objectives. The primary objective was to assess the relative toxicities of dispersed and un-dispersed weathered PBCO to adult and embryonic topsmelt following short-term declining exposures.

Specific objectives are as follows:

Objective 1: Compare the toxicity of physically dispersed oil (water accommodated fraction; WAF) and chemically dispersed oil (chemically enhanced water accommodated fraction; CEWAF) on gravid adult topsmelt; conduct short-term 96-h exposures to assess the relative toxicities of both WAF and CEWAF.

Objective 2: Examine the residual effects of WAF and CEWAF sublethal exposures on the ability of topsmelt to spawn.

Objective 3: Examine the residual effects of WAF and CEWAF on the viability of topsmelt embryos spawned by the exposed gravid adults.

Objective 4: Compare the effects of WAF and CEWAF on embryos developed from ova exposed *in vivo* (Objective 3) to those on developing topsmelt embryos exposed after fertilization.

Objective 5: Assess long term survival and growth of post-spawned adult topsmelt after short-term 96-h exposures to WAF and CEWAF.

Objective 6: Examine the effects of WAF and CEWAF exposure on the long term growth of topsmelt larvae through their juvenile development.

In addition to these objectives, additional experiments were conducted to assess the relative toxicity of Corexit 9500 to topsmelt adults and embryos. Results of these experiments provide additional information on the relative risk of using these dispersant in coastal waters.

3.0 Methods

This project was conducted at three laboratories. Oil exposures were conducted at the Marine Pollution Studies Laboratory (MPSL) at Granite Canyon (Monterey, CA). Metabolomic analysis of tissues from exposed topsmelt adults and embryos was 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 (WPCL), 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 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 (mass to mass). Untreated oil testing was performed using the WAF of weathered PBCO. Weathering consisted of heating the oil to 70 °C

until the oil mass was reduced by 20% (Marty et al. 1997). All PBCO was weathered in separate lots, then composited for use in the WAF and CEWAF experiments.

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 laboratory seawater 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 seawater. 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 masses 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 were 21 h, followed by 3 h of settling time to allow the largest oil droplets to resurface (Singer *et al.,* 2000). The 21-h mix: 3-h CEWAF settling regimen was used to match the 24-h total preparation time used for WAFs.

3.2 Toxicity Test Procedures

Adult Topsmelt Experiments

Topsmelt were exposed for 96 h to various concentrations of the WAF and CEWAF of weathered PBCO using methods standardized through Chemical Response to Oil Spills Environmental Research Forum (CROSERF) and a declining exposure protocol. Range-finder tests were conducted to determine optimal definitive test concentrations. All definitive test solutions were fully characterized for total hydrocarbon content (THC) by

gas chromatography (GC-FID), and samples were also characterized for polynuclear aromatic hydrocarbons constituents using GC-Mass Spectroscopy.

Acute 96-h toxicity tests with WAF were completed with gravid topsmelt (*Atherinops affinis*) using established test procedures (Singer *et al.* 2000). Gravid topsmelt, were obtained from Elkhorn Slough, Monterey County, California, and were held at the MPSL in 4-ft dia. flow-through systems, under ambient temperature and spring lighting conditions; consisting of overhead fluorescent lights and light timers.

Spiked exposures were conducted in sealed, 18-L polycarbonate flow-through exposure chambers. Tests involved six treatments: five WAF or CEWAF concentrations and a seawater control, with each treatment having three replicates. Water temperature, dissolved oxygen (DO), and pH were monitored daily during testing.

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, fifteen adult topsmelt were added to each chamber by unsealing the chamber lid, introducing the animals by hand, and resealing the chamber. Tests were initiated by immediate commencement of flushing of all chambers with clean, seawater at a rate of approximately 40 mL/min. Flush rates were monitored before and during the exposures by direct measures of seawater flow to each aquarium.

Prior to conducting definitive experiments with topsmelt, preliminary experiments were conducted with PBCO WAF to determine the optimal flushing rate to approximate hydrocarbon declines predicted by oil spill models designed for San Francisco Bay (Pond et al., 2000). The results showed that a flushing rate of 40 mL per minute resulted in an approximately 88% decline in hydrocarbons after 24 h, which is comparable to the model

predictions. This flush rate is 20% of that used in our previous experiments with salmon smolts (i.e., 200 mL/min; Tjeerdema et al. 2006). Fish survival was acceptable in experiments using the lower flush rate. This flush rate was used in all experiments using topsmelt. When the definitive WAF test was conducted with mature adult topsmelt, DO concentrations began to decline to unacceptably low levels in the test aquaria within the first 6 hours of the exposure. Aeration was provided to each aquarium to increase the DO. It is likely the lower DO levels in the definitive test were due to the use of larger topsmelt. Aeration was used in exposures of adult topsmelt in all subsequent experiments. Aeration was not used in experiments with topsmelt embryos.

At the termination of each test, one surviving fish from each of replicate tank was dissected for metabolomic analyses (described below), and the remaining survivors were transferred to culture tanks for long-term spawning and growth studies.

Surviving fish were induced to spawn following exposures by manipulating culture temperatures and lighting conditions using established methods (Middaugh *et al.* 1993). Spawning success was quantified as the number and size of successive spawns over a two-week period. Selected cohorts of spawned embryos were isolated and cultured through embryonic development and larval hatching, and developmental abnormalities and hatching success were monitored following methods of Anderson *et al*. (1991).

In addition to WAF and CEWAF exposures, topsmelt adults were exposed to Corexit 9500 alone to establish baseline toxicity information for the dispersant. Three adult fish were exposed in the same 18L aquaria used in the WAF and CEWAF experiments. Each concentration was replicated three times and experiments were conducted at 20 ºC for 96 h, using static aerated exposures.

Median-lethal concentrations (LC_{50}) were estimated using the trimmed Spearman-Karber procedure (Hamilton *et al.,* 1977). Rangefinder and definitive tests were considered acceptable if control survival was greater than 80% (adults) and 70% (embryos).

Topsmelt Embryo Experiments

Topsmelt embryos were provided by Aquatic Bio Systems (Fort Collins, CO). Early blastula stage embryos were shipped using overnight courier in chilled seawater and on ice packs and the embryos arrived at MPSL at approximately 13 ºC. Some of the experiments were conducted with late gastrula stage embryos, but the majority of exposures were conducted with embryos that had been held for an additional 24h at 13 ºC to ensure consistent development sufficient to meet the control hatching success criterion (≥70 %). These embryos were at stage 16 (after Lagler et al. 1962) at Time 0. At this stage the fish had developed optic lobes without optic vessicles, but there was no development of the heart and cardiovascular system.

Direct exposures of topsmelt embryos to WAF and CEWAF solutions were conducted in multiwell polycarbonate tissue culture plates (Fisher Scientific) submerged in 1L polycarbonate plastic food trays (Rubbermaid Corp.). Each tissue culture plate contained 24 wells, and the volume of each well was 5 mL. For these experiments, WAF and CEWAF solutions were prepared using the methods described above. The WAF and CEWAF solutions were distributed to the 1L food trays, and one tissue culture container was then submerged in each food tray. Topsmelt embryos were then pipetted into the individual wells to initiate exposure to the oiled seawater. For range-finding experiments, one embryo was placed in each tissue culture well, and for definitive experiments, two embryos were placed in each well. After the embryos were distributed to the tissue culture plates, the food trays were flushed with clean seawater at a rate of 2 ml per minute using digital positive-pressure peristaltic pumps. This flushing rate was proportionate to that used in the adult topsmelt exposures. Chemistry samples for THC and PAH analyses were collected at the initiation of the embryo exposures (T0) and after the 24h declining exposure (T24).

The embryos were exposed to WAF and CEWAF solutions for 24 hours under declining exposure conditions at 15 ºC. At 24 hours, the embryos were transferred to 20 mL scintillation vials containing 10 mL of the test solution collected from the exposure chambers. Transferring the embryos to vials allowed for observations of the embryos as

they continued development in the diluted WAF and CEWAF solutions. The embryos were allowed to develop at 20 ºC for the remaining exposure period. All embryos were observed using an inverted microscope on day 5. At this stage the embryonic heart was fully developed and circulation was evident throughout the body. Observations of developmental abnormalities were based on the scoring system developed by Weis and Weis (1982) and included craniofacial, cardiovascular and skeletal abnormalities. A subset of 10 to 15 live embryos was preserved for metabolomic analyses by freezing the embryos in liquid nitrogen and holding them in a – 80 ºC freezer. The remaining embryos were allowed to continue development until hatching or death.

In addition to observations of developmental abnormalities, larval hatching success was recorded. The length and width of the yolk sac of hatched larvae was measured and the yolk sac area was normalized to total larval length to assess the degree of yolk sac edema (Anderson et al. 2009). The occurrence of periocardial edema in hatched larvae was also noted.

3.3 Gas Chromatography

Chromatographic measurement of total hydrocarbon content (TPH – C10–C36), were accomplished using a Hewlett-Packard 6890 gas chromatograph fitted with a flame ionization detector (FID). This instrument set-up typically allows 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 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 included 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 are not directly measured by this method, their inclusion in the mass of oil used to prepare standards allows them to be accounted for in response factor calculations (Payne, 1994). This technique does not allow for direct quantitation of individual hydrocarbons; however, it produces concentrations based on the total response of the 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 is 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 also analyzed using HP GC/MS 6890-5973 with a HP 7695 purge & trap concentrator and a Teledyne Tekman autosampler (method US EPA 8260).

Extraction methods for analyses of polynuclear aromatic hydrocarbons (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 blowdown. Internal standard was added to the extract before analysis by gas chromatography-mass spectrometry using selected ion monitoring. The trace level substituted PAH analysis method that was employed was developed and validated by the WPCL and is based on EPA Method 8270B.

3.4 Animal and Experimental Design for Metabolomics studies

Adult Exposures

Following the 96-h exposures, one surviving fish from each replicate tank (a total of 18 tanks, five WAF and CEWAF concentrations and a control, replicated three times) was sacrificed. Muscle tissues were immediately dissected, flash frozen in liquid N_2, and stored at -80^{o}C until further tissue preparation and metabolite extraction was conducted. Surviving adult fish were transferred to 4' diameter tanks for long-term culture, as described above.

As these fish spawned, embryos from select spawns were isolated to evaluate embryo viability, and a subset of these embryos was selected for metabolomic analysis. These embryos were preserved on day 5 of development, after they had developed a complete circulatory system. Approximately 5 to 15 embryos were transferred to cryovials then flash frozen in liquid N_2, and stored at -80^{o}C until further tissue preparation and metabolite extraction was conducted.

Embryo Direct Exposures

A subset of embryos exposed directly to WAF and CEWAF solutions were also preserved on Day 5 of the experiments. As above, living embryos that had progressed to the point where a complete circulatory system had developed were flash frozen in liquid N_2, and stored at -80^{o}C until metabolomic analysis.

3.5 Metabolite Extraction

Extraction methods were taken from previous work (Lin *et al*., 2009; Van Scoy *et al*., 2010). Briefly, frozen muscle samples were individually 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 a methanol/water (2/1) solution. 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

lyophilized prior to NMR analyses. Methods for metabolomic analysis of topsmelt embryos were the same as those described for adult tissues except that rather than assessing tissues from individual fish, the individual embryos were removed from the exposure vials on Day 5 and composited into groups of 10 to 15 embryos per replicate to provide sufficient tissue for analysis.

3.6 1D ^{1}H NMR Spectroscopy

Metabolomic analyses were performed as previously described, with slight modifications (Lin *et al*., 2009; Van Scoy *et al*., 2010). Extracts were re-suspended in a sodium phosphate buffer in D_2O (0.1 M, pH 7.4) containing sodium 3-trimethylsilyl-2,2,3,3-d_4-propionate (TMSP), as an internal chemical shift standard. One-dimensional (1D) ^{1}H NMR spectra of the adult muscle tissue extracts were measured at 500 MHz using an Avance DRX-500 spectrometer (Bruker, Fremont, CA) with the following acquisition parameters: a 7.0-μs (60˚) pulse, a 7-kHz spectral width, a 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. 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 Topspin software (Version 1.3; Bruker). Extracts obtained from embryos, were measured at 600 MHz using an Avance DRX-600 spectrometer (Bruker, Fremont, CA), following the same acquisition parameters, except for the collection of 150 transients requiring a 12-min total acquisition time.

3.7 NMR Spectral Pre-Processing and Statistical Analyses

Spectra were converted to a format for multivariate analysis using custom-written *ProMetab* software (Viant, 2003) in MATLAB (version 7.8, The MathWorks, Natick, MA, USA). Each spectrum was segmented into chemical shift bins between 0.2 and 10.0 ppm, corresponding to a bin width of 0.005 ppm (2.5 Hz). The spectral area within each bin was integrated to yield a vector containing intensity-based descriptors of the original spectrum and bins representing the residual water peak (from 4.60 to 5.20 ppm) were removed. The total spectral area (TSA) of the remaining bins was normalized to one in order to facilitate

comparison between the spectra. Binned data was log transformed; forming a matrix (i.e. one sample per row and one bin per column) and the columns were mean-centered before multivariate analysis.

Principal component analysis (PCA) of the pre-processed NMR data was conducted using the PLS_Toolbox (Version 5.8; Eigenvector Research, Manson, WA) within MATLAB. Datasets was examined to identify differences between the samples (control and treatments) as well as potential outliers between the metabolic fingerprints, in an unsupervised manner. This pattern recognition technique, employs an algorithm to calculate the highest amount of correlated variation along the first principal component (PC1), with subsequent principal components (PC2, PC3 etc.) containing smaller amounts of variance. Also, PCA loading vectors were examined to identify the metabolites contributing to these PC axes. One-way analysis of variance (ANOVA) tests were conducted on the metabolite peak areas to test for differences between doses (SAS Institute Inc., version 9.1; Cary, NC, USA).

4.0 Results

4.1 System Performance

Flush rates in the exposure aquaria were controlled using manual flow meters. Flow measurements from the individual topsmelt WAF experiment are shown in Appendix I. This data demonstrates the minimal variation between replicate flush rates in the WAF adult test.

Flush rates for the WAF and CEWAF embryo tests were not confirmed. The test containers in the embryo tests were flushed at 2 mL/minute using a digital positive pressure peristaltic pump. Flow rates for this pump were calibrated on 3 occasions within 21d prior to its use in the embryo test and the flow rates were shown to not vary by more than 20% from nominal.

Flush rates in the adult and embryo exposures were designed to flush WAF and CEWAF to approximately 5% of the initial (T0) concentration after 24 hours (T24) following the

NOAA model for the San Francisco Estuary (Pond et al., 2000). Chemistry for hydrocarbon analyses were collected at T0 and after 8 hours (T8) in the adult WAF and CEWAF. Post-flush samples were collected at T8 rather than T24 because previous research showed that THC in WAF was not-detectable after 24h when flushed at 200 ml/min (Tjeerdema et al. 2006). This was due to low T0 hydrocarbon concentrations in WAF. Samples were therefore collected at T8 to allow comparison in flush rates between WAF and CEWAF tests. The THC concentrations at T8 in the adult WAF test ranged from 17% to 49% of the T0 concentration, and the mean T8 THC concentration was 30% of the T0 concentration in this test. The THC concentration at T8 in the adult CEWAF test ranged from 0% to 6% of the T0 concentration, and the mean T8 THC concentration was 3% of the T0 concentration in this test. These results suggest that flush rates differed between the WAF and CEWAF tests, and that the flush rates in the CEWAF tests surpassed those presented in the NOAA model for the SF Estuary.

Chemistry samples for hydrocarbon analyses were collected at T0 and T24 in the embryo WAF and CEWAF tests. Chemistry samples were collected at T24 instead of T8 because the entire contents of the exposure chambers needed to be collected for chemical analysis. The THC concentrations at T24 in the WAF embryo test ranged from 9% to 422% of the T0 concentration, and the mean T24 THC concentration was 162% of the T0 concentration in this test. While WAF THC concentrations were low in all of the loadings in this test, some of the exposure chambers had higher THC at T24 than at T0. T24 THC in three of the loadings (3.125, 12.5, and 25 g/L PBCO) were lowered to 19%, 9%, and 17%, respectively, of the T0 concentrations after 24 hours. T24 THC in the two remaining loading concentrations (1.563 and 6.25 g/L PBCO) were higher, 422% and 344% of the T0 concentration. This resulted in a mean increase in THC after 24 hours. It is not clear why THC concentrations were higher at T24 in the 1.56 and 6.25 g/L WAF treatments. It is possible some stratification of oil remained in replicate aquaria after 24 hours of flushing. The concentration of hydrocarbons in all of the WAF solutions were relatively low, so minor differences in THC between T0 and T24 resulted in large apparent differences in THC, based on a percentage basis.

The THC concentration at T24 in the CEWAF embryo test ranged from 11% to 26% of the T0 concentration, and the mean T24 THC concentration was 18% of the T0 concentration in this test. When compared to the NOAA model (5% dispersed oil at 24h; citation), these results suggest that the flushing rates used in the embryo experiments under-represented those that would be expected to occur when a spill is treated in the SF Estuary.

4.2 Acute Toxicity of WAF and CEWAF to Adult Topsmelt

Prior to definitive experiments, no mortality was observed in the two range-finding tests conducted with weathered WAF concentration as high as 25g/L and 50 g/L loading, respectively (data not shown). The definitive WAF test was conducted oil loadings ranging from 0 to 25g/L to approximate the highest concentration possible in a coastal oil spill (McAuliffe, 1987). No significant mortality was observed in this test (Table 1, Figure 1), and therefore no LC50 could be calculated for adult exposure to WAF. Because of the lack of mortality in all tests, the LC50 is greater than the highest concentration (LC50 > 3.385 mg/L THC).

Two CEWAF range-finding tests were conducted, and these produced variable results. High mortality was observed at all loadings in test one (loading range = 0.63 – 10.0 g/L) and minimal mortality was observed at all loadings in test two (loading range = 0.03 – 0.5 g/L)). A third range-finding test was conducted using loadings of 0.16, 0.31, 0.63, 1.25 and 2.5 g/L. Complete mortality was observed at 1.25 and 2.5 g/L, and this experiment produced a weathered PBCO LC50 of 0.82 g/L oil, based in nominal oil loadings.

The definitive CEWAF adult test was conducted using loadings from 0.06 to 1 g/L (Table 2). Survival (Figure 2) was greater than 90% in THC $\leq$ 26.1 mg/L. Survival was 31% and 7% at the highest two THC concentrations, 58.3 and 270 mg/L, respectively. Based on total hydrocarbon content (THC), the LC50 in this test was 56.4 mg/L. Based on loading, the LC50 was 0.44 g/L PBCO.

A Corexit 9500 dispersant-only test was conducted with a range of dispersant concentrations (Table 3). No mortality was observed at concentrations < 50 mg/L

dispersant, but complete mortality was observed > 50 mg/L. Based on mortality data, a LC50 for Corexit 9500 was calculated to be 70 mg/L.

Table 1. Mean percent survival of adult topsmelt in WAF definitive test. LC50 based on total hydrocarbon content (THC) could not be calculated but would be > 3.385 mg/L. T0 THC concentrations are shown. LC50 based on oil loading could not be calculated but would be > 25 g/L.

WAF Loading	THC	Mean	SD
0.00	0.380	82	20
1.56	0.685	78	10
3.13	0.935	80	12
6.25	1.21	82	8
12.5	1.01	89	10
25.0	3.39	73	12

Table 2. Mean percent survival of adult topsmelt in CEWAF definitive test. LC50 based on total hydrocarbon content (THC) 56.4 mg/L. LC50 based on oil loading = 0.44 g/L.

CEWAF Loading	THC	Mean	SD
0.00	0.380	91	10
0.063	9.63	96	4
0.125	20.1	96	8
0.25	26.1	91	15
0.5	58.3	31	20
1.00	270.0	7	7

Table 3. Mean percent survival of adult topsmelt in Corexit 9500 dispersant-only test. LC50 = 70 mg/L Corexit 9500.

Concentration (mg/L)	PBCO Loading Equivalent (g/L)	Percent Survival	SD
0.00	0.00	100%	0
12.5	0.125	100%	0
25.0	0.250	100%	0
50.0	0.50	100%	0
100.0	1.00	0%	0
200.0	2.00	0%	0

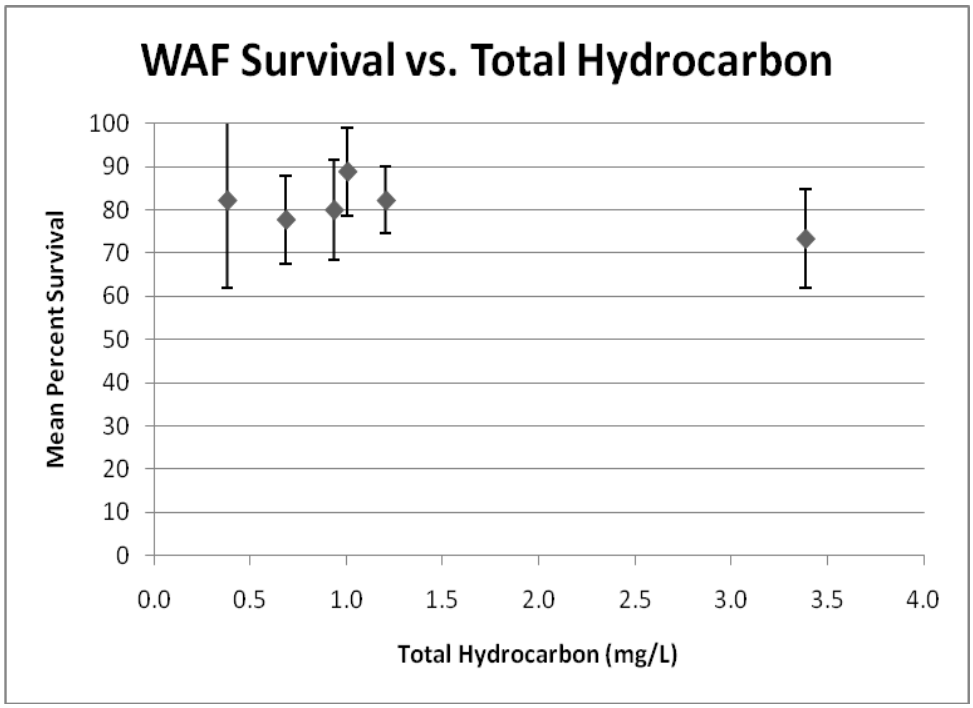


Figure 1. Adult topsmelt survival after 96-h exposure to WAF.

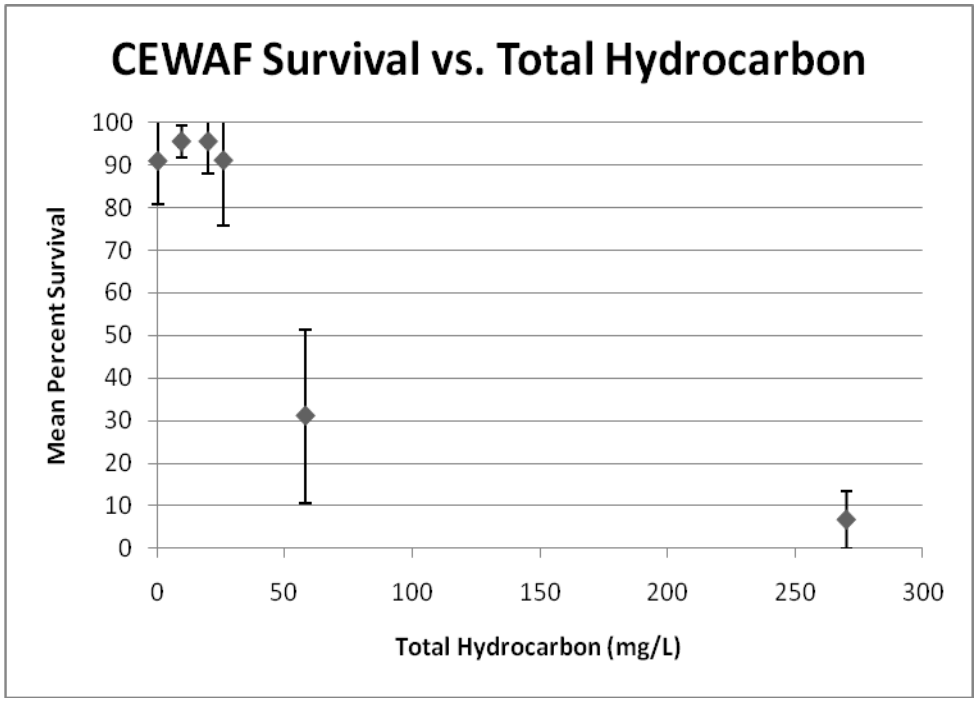


Figure 2. Adult topsmelt survival after 96-h exposure to CEWAF.

4.3 Differences in Hydrocarbon Chemistry Between WAF and CEWAF in Adult Topsmelt Exposures

THC ranged as high as 3.39 mg/L at 25 g/L loading in the WAF test and as high as 270.18 mg/L at 1 g/L loading in the CEWAF test. Comparing THC at similar loadings, 1 g/L in CEWAF vs. 1.56 g/L in WAF, THC in CEWAF was at least 390x that of WAF (Tables 1 and 2).

Relative concentrations of PAH parent compounds also varied considerably between WAF and CEWAF solutions. The CEWAFs contained lower concentrations of naphthalenes than WAFs, except at the highest CEWAF loading. The CEWAF contained higher concentrations of 3 ring PAHs. There were higher concentrations of fluorine in CEWAF, and there were much higher concentrations of phenanthrene and dibenzothiophene. There were also detectable concentrations of 4 ring PAHs at the highest CEWAF loading, but these compounds were not detected in the WAF (Figures 3-5).

The relative toxic contributions from total hydrocarbon loads vs. concentrations from individual PAHs cannot be determined from these results. Napthalenes are considered relatively non toxic to adult fish, while 3 ring PAHs such as phenanthrene are considered to be more potent aryl hydrocarbon receptor (AhR) agonists (Baron et al. 2004). In addition, the experimental design does not allow for differentiating between toxicity to adult topsmelt caused by increased hydrocarbon bioavailability, versus physical reactions of adult fish to the complex mixtures of the larger hydrophilic complex consisting of dissolved hydrocarbons, dissolved surfactants and particulate bulk-oil in CEWAF. Research with rainbow trout suggests oil dispersants increase hydrocarbon (PAH) bioavailability. Ramachandran et al. (2004) found greater CYP1A induction in livers of fish exposed to CEWAF vs WAF, and that the greatest induction occurred in higher viscocity crude oils. Three ring PAHs have been shown to be the principal toxic components to fish embryos in weathered crude oil (e.g., Incardona et al., 2005), and topsmelt embryos exposed to CEWAFdemonstrated symptoms of inhibited cardiovascular development similar to other species exposed to these compounds (see below) .

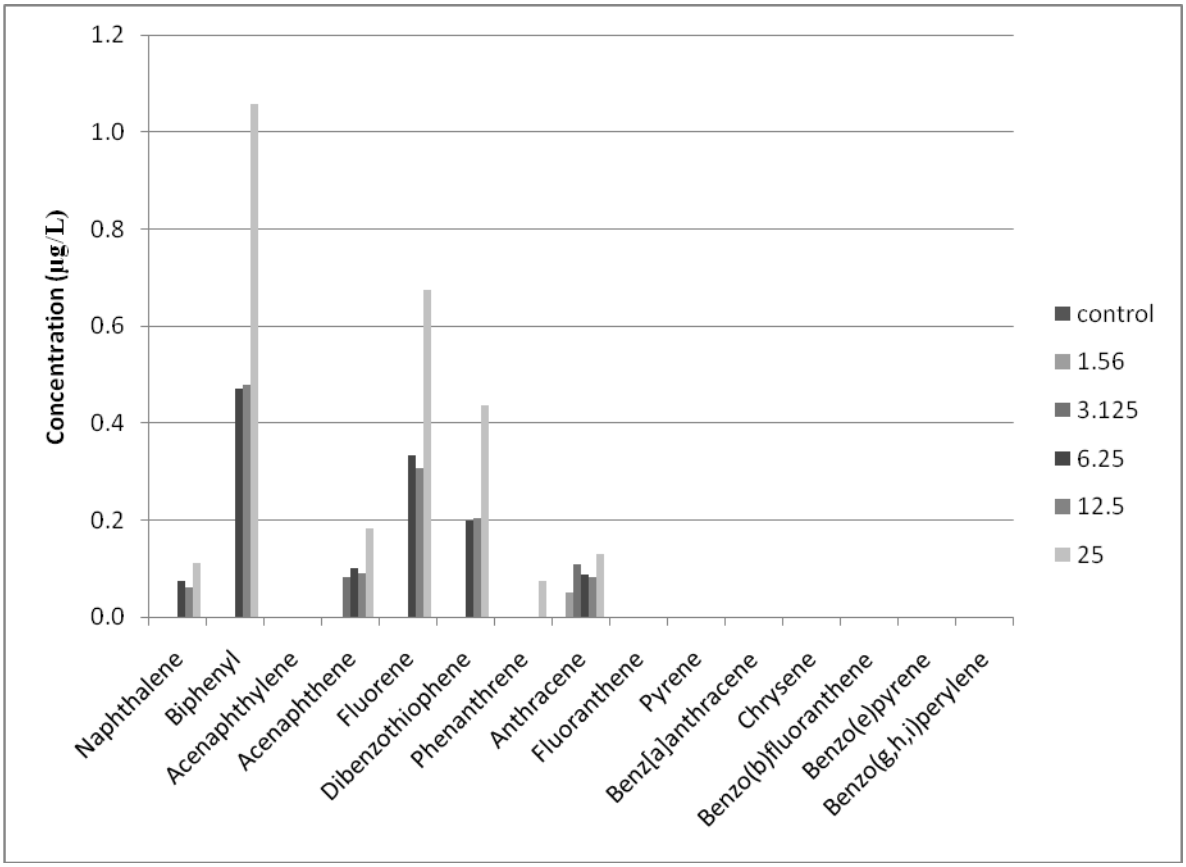


Figure 3. Concentrations of PAH parent compounds (Time 0 concentrations in µg/L) in WAF definitive test using adult topsmelt.

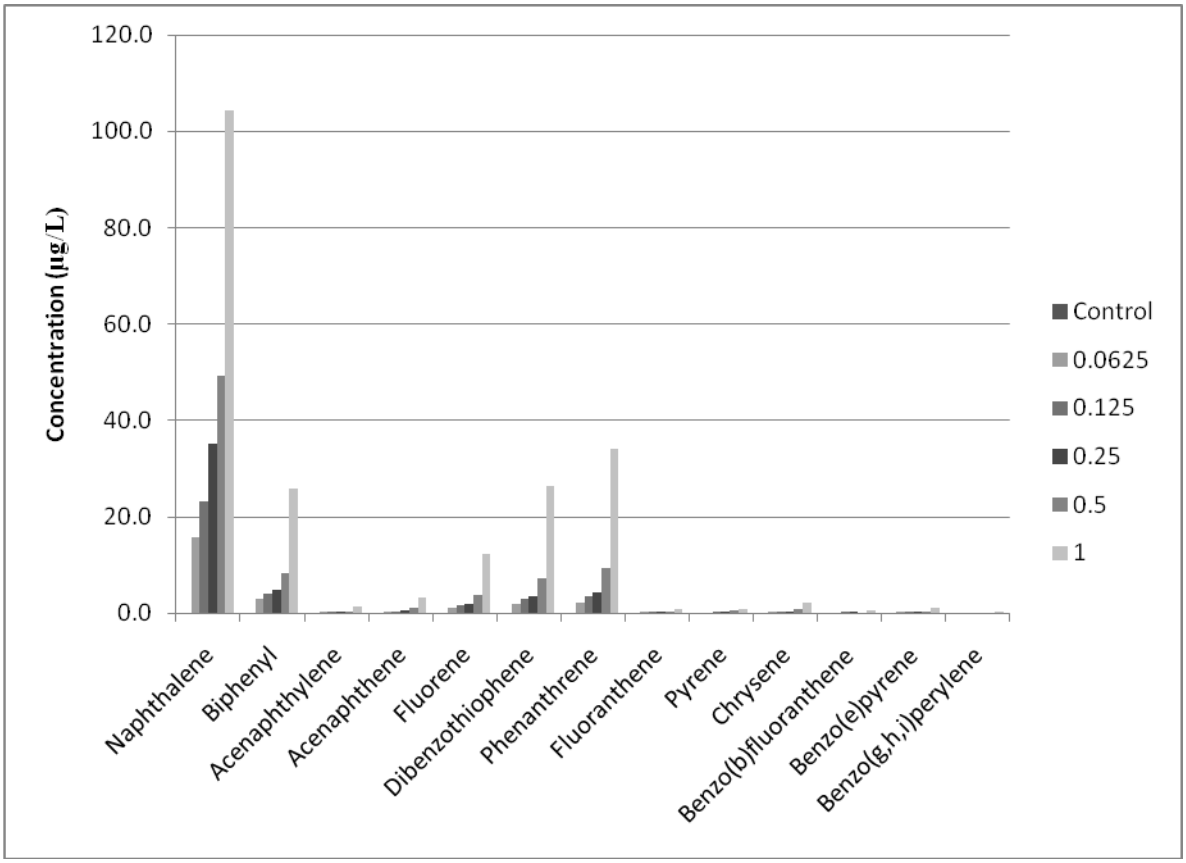


Figure 4. Concentrations of PAH parent compounds (Time 0 concentrations in µg/L) in CEWAF definitive test using adult topsmelt.

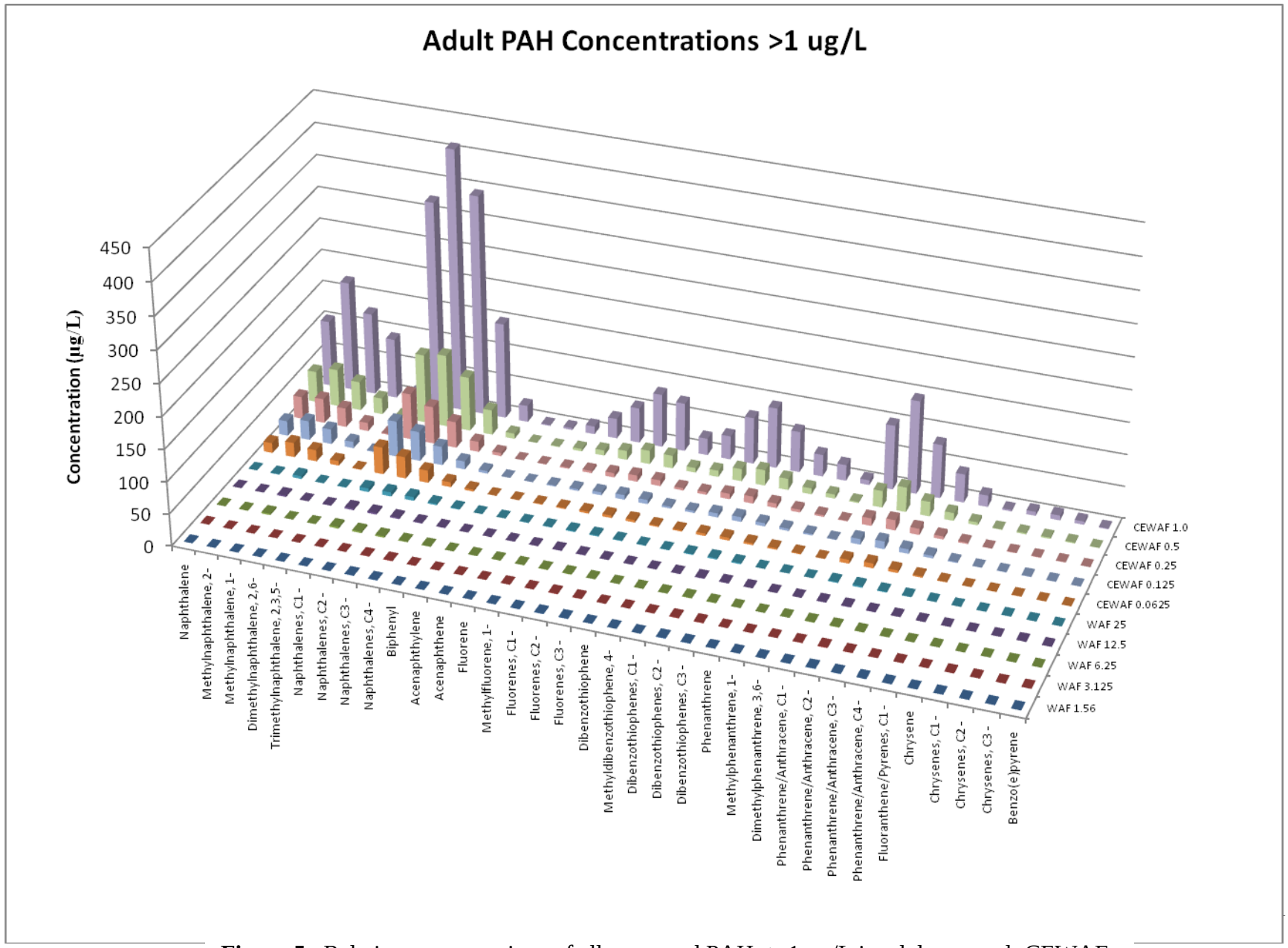


Figure 5. Relative concentrations of all measured PAHs > 1 µg/L in adult topsmelt CEWAF and WAF exposures (Time 0 concentrations in µg/L).

4.4 NMR Spectroscopy of Metabolites from Adult Fish Muscle Tissues and Embryos

A representative 1D ^{1}H NMR spectrum of adult topsmelt muscle extracts is presented in Figure 6. Major metabolites in each sample were assigned by comparison to tabulated chemical shifts and peak multiplicities (Fan, 1996; Table 4). Nucleotides (e.g. ATP) and amino acids (e.g. alanine, histidine and valine) were dominant in the muscle spectra, whereas other metabolites included products of glycolysis (e.g. lactate) and tricarboxylic acid cycle intermediates (e.g. succinate). Both the extracts from the control and treated fish contained similar metabolites which varied in abundance. Also, embryo extracts contained different metabolites that were not observed within the muscle extracts, such as organic osmolyte glycerophosphocholine. The NMR spectra were individually analyzed to avoid variation from changes in experimental conditions and well-resolved peaks were identified and integrated for comparison.

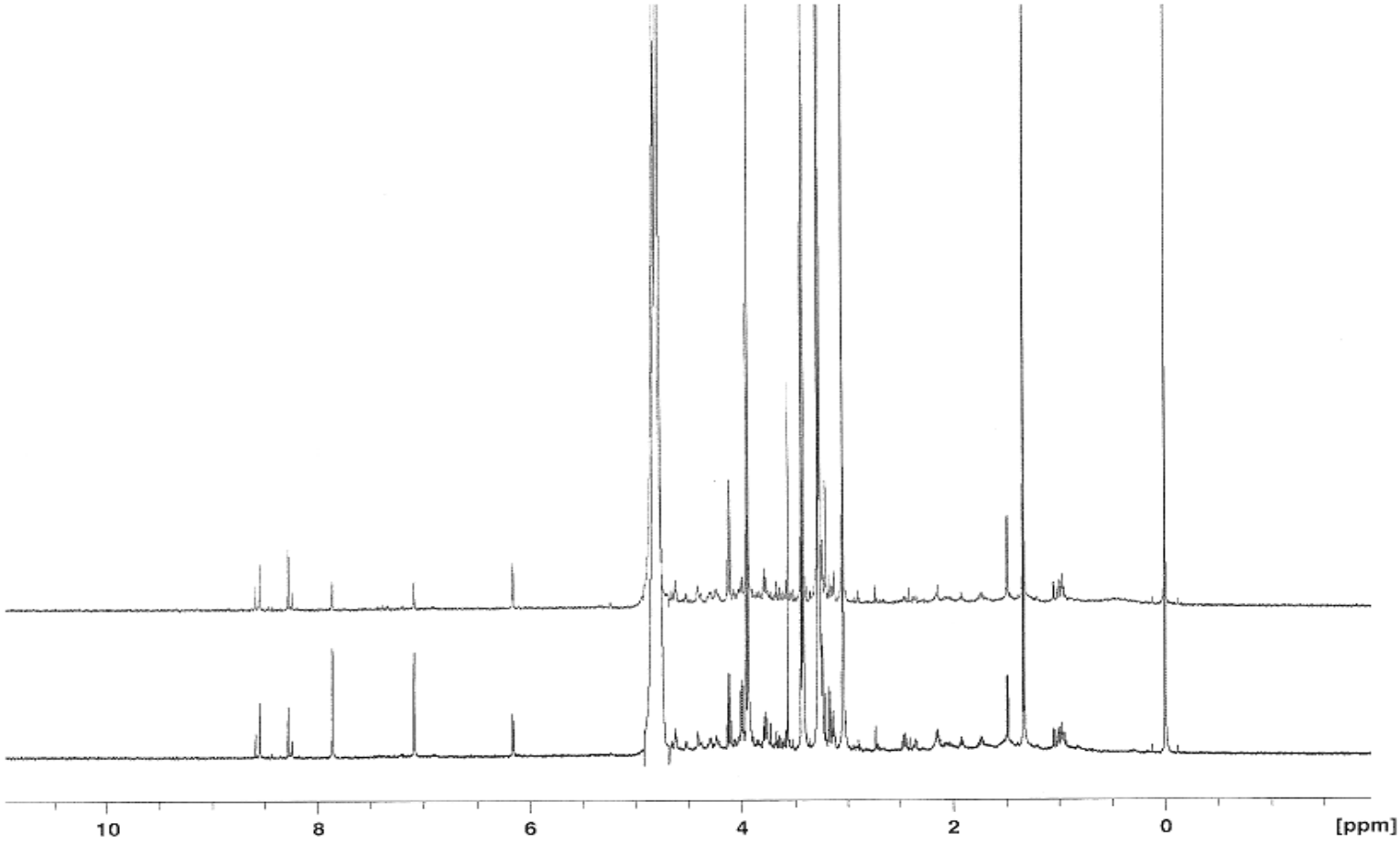


Figure 6. Representative 1D ^{1}H NMR spectrum of topsmelt muscle extracts from a control fish (0.0 g/L) and a WAF exposed fish (6.25 g/L).

Table 4. Metabolites identified by NMR of adult topsmelt muscle [(a)].

Metabolites	^{1}H NMR peak assignment (ppm) [(a, b)]
Valine	1.00 (d), 1.05* (d)
Lactate	1.33* (d), 4.12 (q)
Alanine	1.49* (d)
Arginine/ Phosphoarginine	1.73 (m), 1.92* (m)
Glutamine	2.14 (m), 2.46* (m)
Succinate	2.41* (s)
Phosphocreatine	3.04* (s), 3.94 (s)
Taurine	3.27 (t), 3.43* (t)
Glycine	3.57* (s)
AMP	4.50 (m), 6.16 (d), 8.24 (s), 8.59* (s)
Histidine	7.09 (s), 7.87* (s)
ATP	6.16 (d), 8.28 (s), 8.55* (s)

* Peaks that are quantified 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)

Table 5. Metabolites identified by NMR of embryos spawned from WAF exposed topsmelt [(a)].

Metabolites	^{1}H NMR peak assignment (ppm) [(a, b)]
Valine	1.00 (d), 1.05* (d)
Lactate	1.33* (d), 4.12 (q)
Alanine	1.485* (d)
Glutamate	2.07 (m), 2.36* (t)
Glutamine	2.14 (m), 2.46* (m)
Succinate	2.41* (s)
Taurine	3.27 (t), 3.43* (t)
Glycerophosphocholine	3.36* (s)
Glycine	3.57* (s)

* Peaks that are quantified 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)

Table 6. Metabolites identified by NMR of embryos exposed to WAF or CEWAF [(a)].

Metabolites	^{1}H NMR peak assignment (ppm) [(a, b)]
Valine	1.00 (d), 1.05* (d)
Lactate	1.33* (d), 4.12 (q)
Alanine	1.485* (d)
Glutamate	2.07 (m), 2.36* (t)
Glutamine	2.14 (m), 2.46* (m)
Succinate	2.41* (s)
Phosphocreatine	3.04* (s), 3.94 (s)
Taurine	3.27 (t), 3.425* (t)
Glycerophosphocholine	3.36* (s)
Glycine	3.57* (s)

* Peaks that are quantified 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 Adult Topsmelt Exposed to WAF, CEWAF and Corexit 9500 Dispersant

Each metabolic fingerprint provides information for determining acute metabolic effects resulting from WAF and CEWAF exposure. The ^{1}H NMR spectra were analyzed by PCA; providing a graphical output, comprising of individual samples, summarizing the similarity between the NMR spectra. Fish from the control tanks and from each of the doses were analyzed individually to avoid variation from experimental conditions and possible physiological conditions. The WAF and CEWAF PCA score plots, represents the resultant metabolic dose-response for both the WAF and CEWAF tests. Clustering of the individual samples implies that similar metabolic responses are resulting from different concentrations of WAF and CEWAF; clustering which includes the control samples indicates similar metabolic responses as the controls. Although, the PCA scores plots lack separation between the controls and doses, it is clear that the fish are experiencing similar metabolic perturbations.

The WAF PCA scores plot, shows minimal clustering between individual doses, thus the separation between the control fish and the dosed fish along the PC1 axis (containing 27.41% of variance; Figure 7a), is also minimal. Although there is overlap among the metabolomes, there is a slight indication of a dose –response relationship between the WAF concentrations and a few of the individual samples. This indicates that variable WAF doses are eliciting slight variations in metabolic responses. The CEWAF PCA scores plot lacks separation between the control fish and the dosed fish along PC1 and PC2 (accounting for 30.29% and 24.93% of variance, Figure 7b); a metabolic dose-response relationship is minimal. Replicate fish and similarly muscles from the same fish are expected to result in similar metabolomes and therefore cluster together, however the lack of clustering observed (Figures 5a and 5b), indicates a physiological change may be occurring during the exposure.

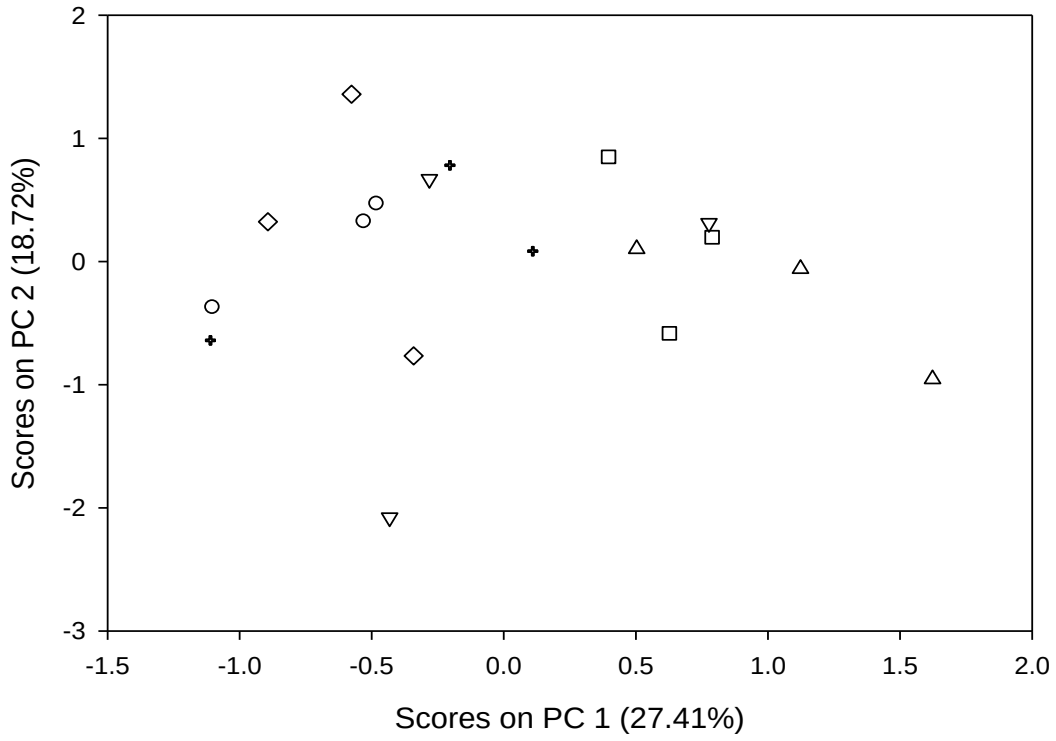
The 78 day grow-out-study resulted in similar metabolome overlap and separation, as the acute study, illustrated in the PCA scores plots (Figure 8a and 8b). The WAF PCA scores plot shows overlap along PC2 (explaining 19.75% of variance; Figure 8a) and clustering of dose 3 and dose 5 exposed fish are separated from the control fish and the remaining doses, therefore resultant metabolic responses are dependent on individual WAF doses. The CEWAF PCA scores plot shows overlap along PC1 and PC2 axes (explaining 47.50% and 20.84% of variance; Figure 8b). Overlap is observed among the control and dose 3 fish whereas dose 1 and dose 2 fish overlap, hence resultant metabolic responses are again dependent on individual CEWAF doses. Overall, the PCA scores plots display minimal separation between doses, but definitive metabolic responses could not be determined strictly from these plots, therefore the change in identified metabolites for each dose and treatment were determined.

Individual doses and controls are expected to result in similar metabolomes with minimal variation; however those of the control fish, between replicates, appear to change, resulting in a change of their PC1 scores. Furthermore, the acute WAF and CEWAF toxicity tests, do not display a metabolic dose-response relationship between concentrations, indicating that the variation in metabolomes may be induced by similar concentrations of

hydrocarbons within the water and not specifically the dose concentrations themselves; similarly determined for salmon pre-smolts (Van Scoy et al, 2010). Overall, the 96-h PCA scores plots display minimal separation between doses, whereas the grow-out fish display a more defined dose-response relationship between individual doses.

The 96-h Corexit 9500 dispersant PCA scores plot (Figure 9) lacks separation between individual doses, thus the control fish are clearly separated from the dosed fish along the PC1 and PC2 axes (representing 41.93% and 16.62% of variance; Figure 1a). Although overlap among the dosed fish metabolomes is observed, there is indication of a dose – response relationship between sample replicates; in particular dose two and dose three exposed fish. This indicates that variable dispersant concentrations are resulting in varied metabolic responses among dosed fish. When compared to both the WAF and CEWAF scores plots, the control and dosed fish are not experiencing similar effects. As previously concluded for WAF and CEWAF, lack of sample separation may be a result of physiological changes during the exposure or similar dissolved hydrocarbon concentrations. However, this exposure resulted in clear separation of the control fish from the dosed fish, further concluding that physiological changes may not be the only determining factor. Furthermore, it is possible that the factor separating the control fish from the dosed fish is the dissolved dispersant components; dissolved components can be increasing with an increase in concentration due to the experiment being conducted in aerated static tanks, ultimately leading to the separation between dose two and three fish.

(a)


Scores on PC 2 (18.72%)
Scores on PC 1 (27.41%)
2
1
0
-1
-2
-3
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0

(b)

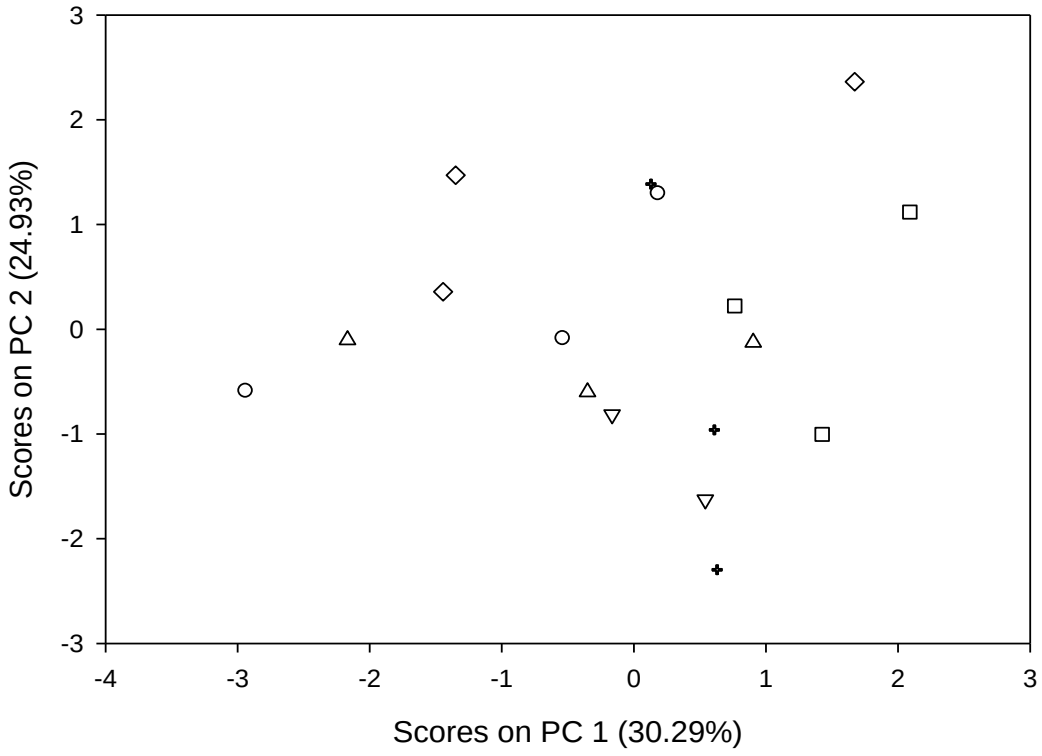
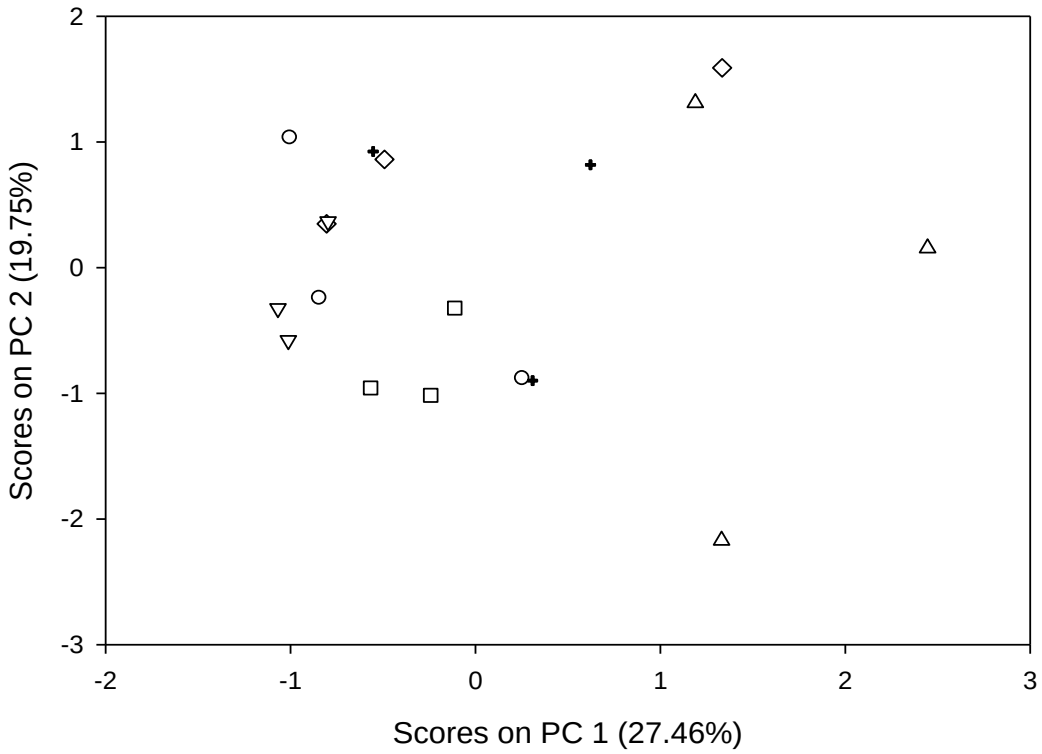


Figure 7. PCA scores plot of the ^{1}H NMR spectra of skeletal muscle extracts from adult topsmelt exposed for 96 h to the (a) WAF and (b) CEWAF of PBCO. All analyses include an unexposed control. Controls and the five concentrations of WAF are characterized as control (◊), 0.685 (○), 0.935 (△), 1.21 (□), 1.01 (+) and 3.39 (▽) mg/L THC. Controls and the five concentrations of CEWAF are characterized as control (◊), 9.63 (○), 20.1 (△), 26.1 (□), 58.3 (+) and 270 (▽) mg/L THC. The symbol shape represents a single dose and each data point represents one biological tissue sample.

(a)



(b)

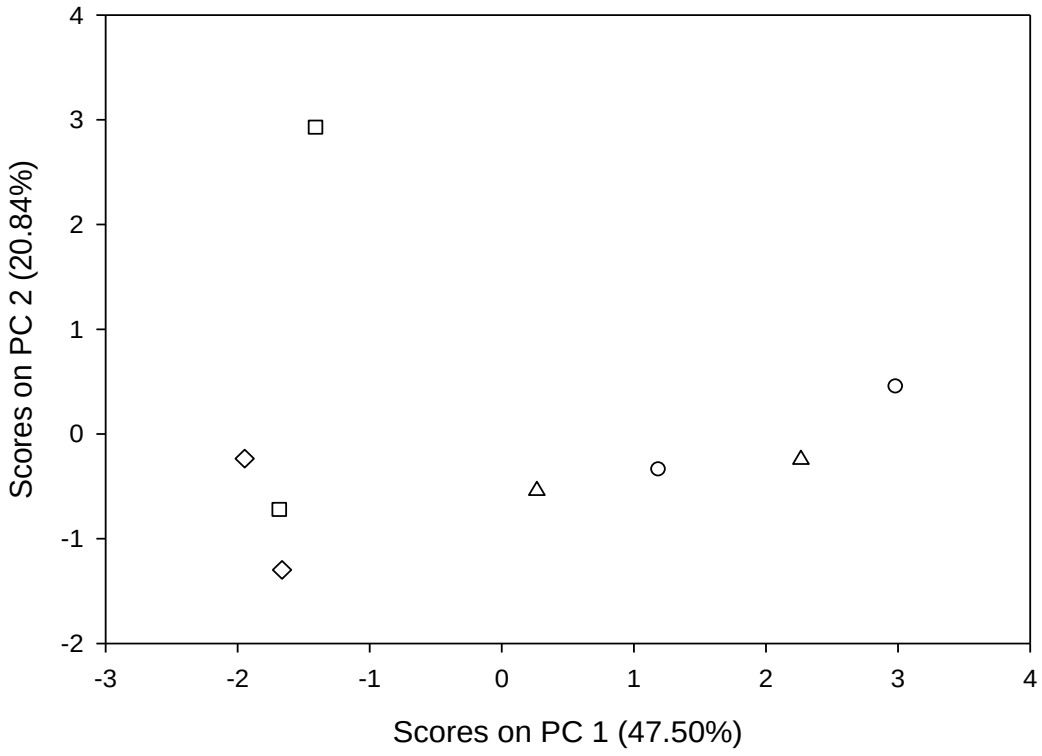


Figure 8. PCA scores plot of the ^{1}H NMR spectra of skeletal muscle extracts from adult topsmelt after 78 days following exposure to the (a) WAF and (b) CEWAF of PBCO. All analyses include an unexposed control. Controls and the five concentrations of WAF are characterized as control (◊), 0.685 (○), 0.935 (△), 1.21 (□), 1.01 (+) and 3.39 (▽) mg/L THC. Controls and the five concentrations of CEWAF are characterized as control (◊), 9.63 (○), 20.1 (△), 26.1 (□), 58.3 (+) and 270 (▽) mg/L THC. The symbol shape represents a single dose and each data point represents one biological tissue sample.

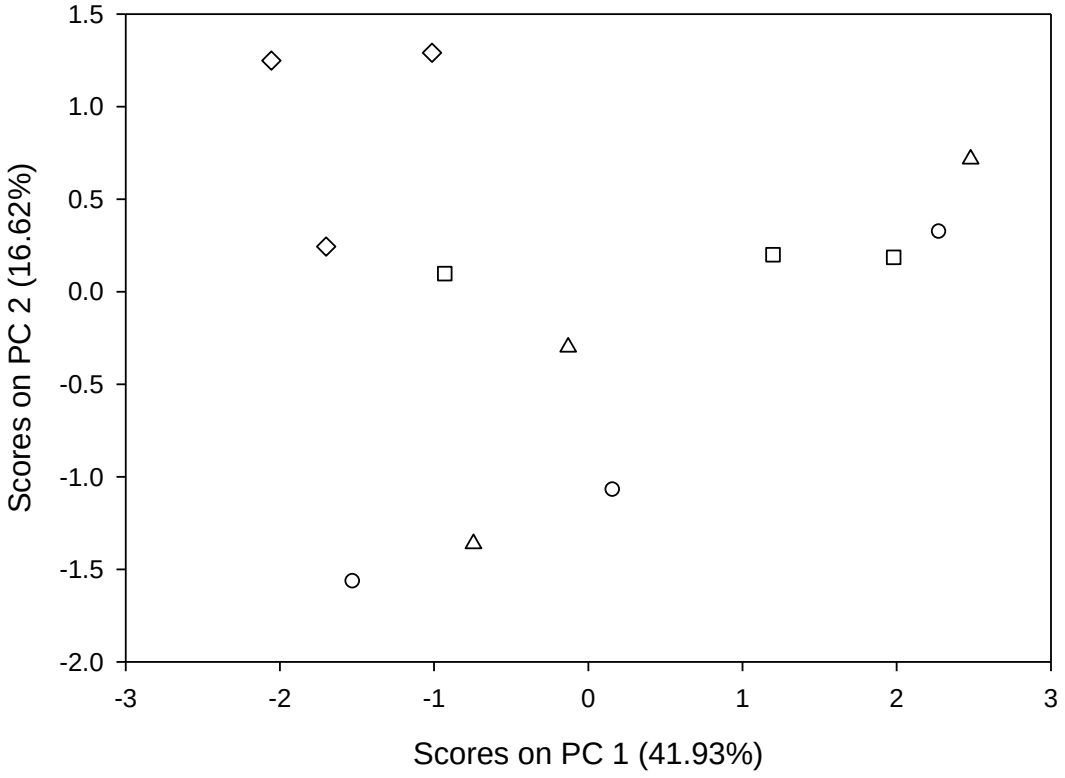


Figure 9. PCA scores plot of the ¹H NMR spectra of skeletal muscle extracts from adult topsmelt exposed for 96 h to Corexit 9500 dispersant. Controls and the three concentrations of Corexit 9500 are characterized as control (◊), 12.5 (○), 25 (△), 50 (□) mg/L. The symbol shape represents a single dose and each data point represents one tissue sample.

4.6 Specific Metabolic Changes in Adult Muscle after Acute Exposures

Well-resolved peaks, among the NMR spectra, were identified and integrated for comparison among each test. Metabolite changes were calculated by using the peak areas (Table 7) within the NMR spectra, specifically by dividing the average peak area of the treatment by that of the corresponding control. Therefore, a number below 100% represents a decrease in that metabolite within treated fish. Concentration-dependent changes and results of one-way ANOVAs from identified metabolites are presented in Table 8. Metabolic changes comparing the muscle tissues between WAF and CEWAF are provided

in Figure 10, whereas metabolic changes due to Corexit 9500 exposure are provided in Figure 11.

Specific changes for WAF include an increase in glutamine, taurine and valine, whereas CEWAF only had an increase in glutamine and valine. AMP decreased at the lower concentrations and increased at the higher concentrations, for WAF, and decreased for CEWAF, resulting in a change in ATP respectively. At intermediate WAF concentrations glycine and succinate decreased and at the low and high concentrations they increased, whereas the glycine continued to decrease at high CEWAF concentrations. Although there was variability in the changes among concentrations for majority of the metabolites, there is an indication that such changes are dose and metabolite dependent.

Following the exposures, there was a significant change (p<0.05) in lactate for WAF, even though it decreased at the intermediate concentrations, whereas phosphocreatine significantly (p<0.05) decreased for CEWAF. Following Corexit 9500 exposure, valine and succinate significantly decreased, whereas AMP significantly increased.

Table 7. Peak areas from the ^{1}H NMR spectra of muscle from adult topsmelt exposed to (a) WAF and (b) CEWAF of PBCO for 96-h.

a)

	WAF [a, b]					
Metabolites	**Control**	**0.685 mg/L**	**0.935 mg/L**	**1.21 mg/L**	**1.01 mg/L**	**3.39 mg/L**
Valine	4.11	4.44	4.59	4.18	3.99	4.31
Lactate	51.5	59.6	28.0	43.2	53.7	34.2
Alanine	11.6	11.2	11.7	13.1	11.3	13.5
Arginine/ Phosphoarginine	6.99	7.04	8.20	6.77	6.39	8.55
Glutamine	5.24	6.71	6.92	6.04	6.09	7.18
Succinate	1.87	1.91	2.07	1.73	2.05	2.31
Phosphocreatine	122	123	118	116	118	107
Taurine	66.7	82.7	96.8	88.4	82.1	64.2
Glycine	10.1	10.7	8.94	9.29	10.1	9.62
AMP	2.57	2.30	0.75	0.97	2.81	2.33
Histidine	4.79	3.38	4.03	6.15	5.06	4.28
ATP	1.60	1.94	2.95	2.45	1.44	2.32

b)

	CEWAF [a, b]					
Metabolites	**Control**	**9.63 mg/L**	**20.1 mg/L**	**26.1 mg/L**	**58.3 mg/L**	**270 mg/L**
Valine	3.81	4.03	3.85	4.10	4.32	3.80
Lactate	55.1	62.7	35.8	47.6	51.7	43.1
Alanine	11.5	10.5	10.8	12.1	12.1	14.0
Arginine/ Phosphoarginine	6.48	6.71	6.88	6.98	6.65	7.59
Glutamine	4.99	6.35	6.18	6.37	5.97	5.92
Succinate	1.81	1.94	1.85	1.94	2.00	1.94
Phosphocreatine	129	126	128	117	119	126
Taurine	58.8	61.5	69.9	66.0	77.0	64.6
Glycine	9.26	9.81	8.46	8.12	9.97	10.35
AMP	2.04	2.02	1.15	0.81	2.64	2.14
Histidine	3.97	2.83	4.52	4.99	4.28	3.63
ATP	2.29	2.15	3.07	3.18	2.21	2.32

[a.] All spectra were normalized by total spectral area.
[b.] WAF and CEWAF are characterized by total hydrocarbon content (mg/L).

Table 8. Metabolic changes in the muscle of adult topsmelt exposed to (a) WAF and (b) CEWAF for 96-h including one-way ANOVA p-values (significant $p<0.05$).

(a)

			WAF [a, b, c]			
Metabolites	**0.685 mg/L**	**0.935 mg/L**	**1.21 mg/L**	**1.01 mg/L**	**3.39 mg/L**	**(p)**
Valine	107	107	105	105	102	0.972
Lactate	111	59.5	82.9	100	68.4	*0.046
Alanine	96.0	101	110	103	120	0.906
Arginine/ Phosphoarginine	102	112	102	96.9	120	0.591
Glutamine	128	128	121	118	128	0.461
Succinate	104	106	99.7	110	115	0.499
Phosphocreatine	99.5	97.9	92.9	94.7	93.1	0.765
Taurine	115	132	123	127	103	0.126
Glycine	106	92.5	95.4	105	104	0.800
AMP	92.0	37.8	37.6	119	102	0.669
Histidine	70.9	97.5	127	107	90.2	0.880
ATP	105	155	145	93.7	119	0455

(b)

			CEWAF [a, b, c]			
Metabolites	**9.63 mg/L**	**20.1 mg/L**	**26.1 mg/L**	**58.3 mg/L**	**270 mg/L**	**(p)**
Valine	108	111	117	120	114	0.788
Lactate	86.4	68.2	86.0	77.9	68.8	0.308
Alanine	99.9	108	127	135	110	0.471
Arginine/ Phosphoarginine	86.8	122	106	111	105	0.611
Glutamine	114	100	101	116	150	0..377
Succinate	107	98.0	111	101	116	0.525
Phosphocreatine	93.3	109.0	93.5	90.4	92.5	*0.0010
Taurine	96.0	116	177	154	179	0.0659
Glycine	134	124	97.7	87.1	89.1	0.244
AMP	73.1	76.0	143	64.2	31.9	0.824
Histidine	109.0	96.5	93.5	96.5	93.6	0.851
ATP	126	121	75.3	117	143	0.367

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

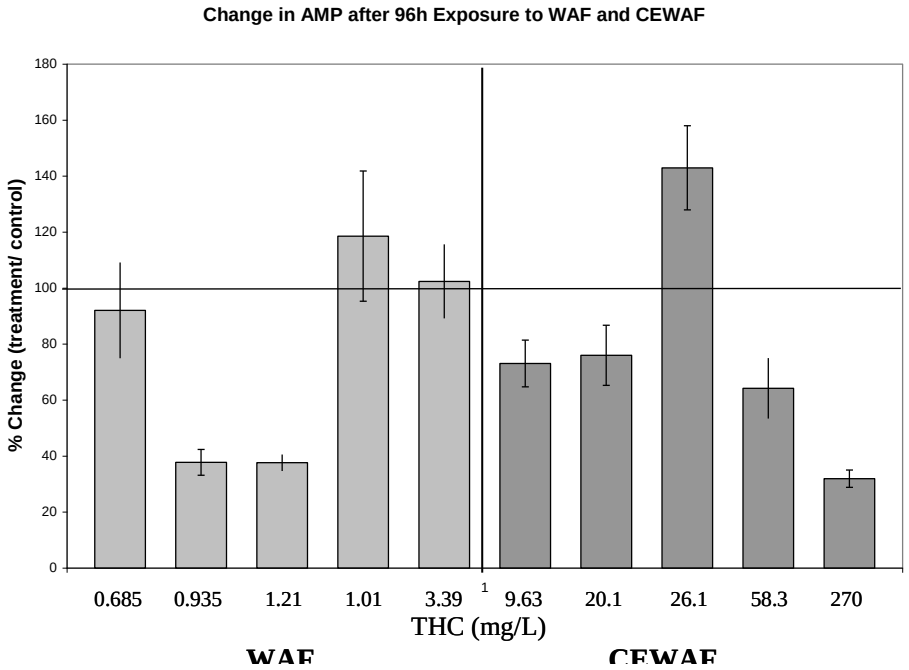
Table 9. Peak areas from the ^{1}H NMR spectra of muscle from adult topsmelt exposed to Corexit 9500 dispersant for 96-h.

		COREXIT [a, b]		
Metabolites	**Control**	**12.5 mg/L**	**25.0 mg/L**	**50.0 mg/L**
Valine	4.44	3.46	3.82	4.33
Lactate	96.9	95.0	108	83.2
Alanine	23.2	20.6	25.4	22.1
Arginine/ Phosphoarginine	9.50	6.59	8.58	10.3
Glutamine	5.46	4.96	5.44	5.31
Succinate	1.92	1.48	1.52	1.54
Phosphocreatine	103	93.7	90.5	94.1
Taurine	51.2	56.0	44.1	49.4
Glycine	16.6	18.2	19.6	18.8
AMP	4.21	5.51	5.70	5.82
Histidine	9.26	7.47	9.14	8.17

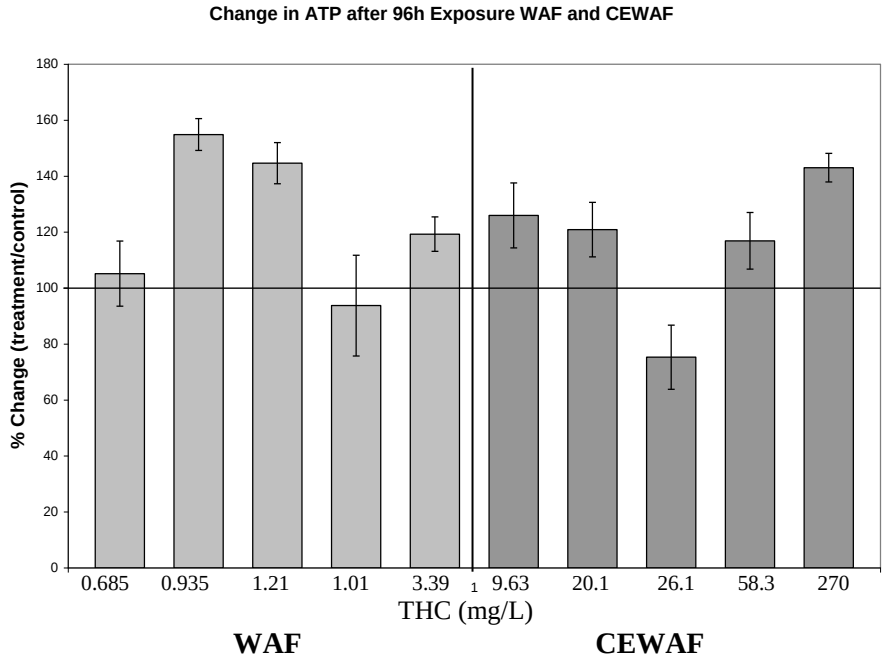
Table 10. Metabolic changes in the muscle of adult topsmelt exposed to Corexit 9500 dispersant for 96-h, including one-way ANOVA p-values (significant $p<0.05$).

		COREXIT [a, b]		
Metabolites	**12.5 mg/L**	**25.0 mg/L**	**50.0 mg/L**	**(p)**
Valine	77.9	86.1	97.5	*0.0272
Lactate	98	111	85.9	0.506
Alanine	88.8	109	95.4	0.111
Arginine/ Phosphoarginine	69.4	90.4	108	0.111
Glutamine	90.9	99.6	97.2	0.757
Succinate	76.9	78.9	80.1	*0.0414
Phosphocreatine	90.8	87.7	91.2	0.195
Taurine	109	86.1	96.5	0.642
Glycine	110	118	114	0.738
AMP	131	136	138	*0.0422
Histidine	80.6	98.6	88.2	0.677

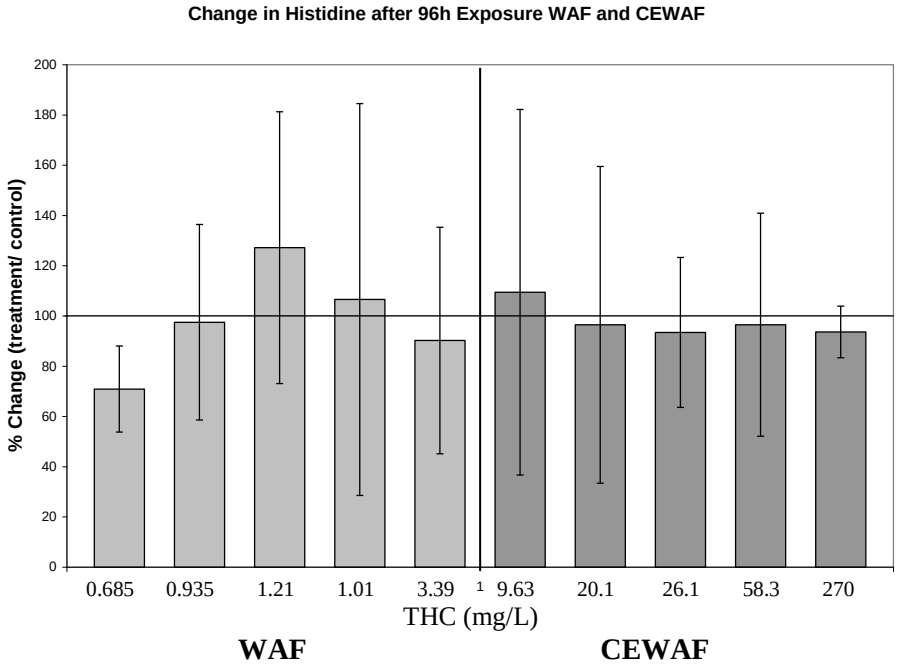
a)


Change in AMP after 96h Exposure to WAF and CEWAF
% Change (treatment/ control)
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

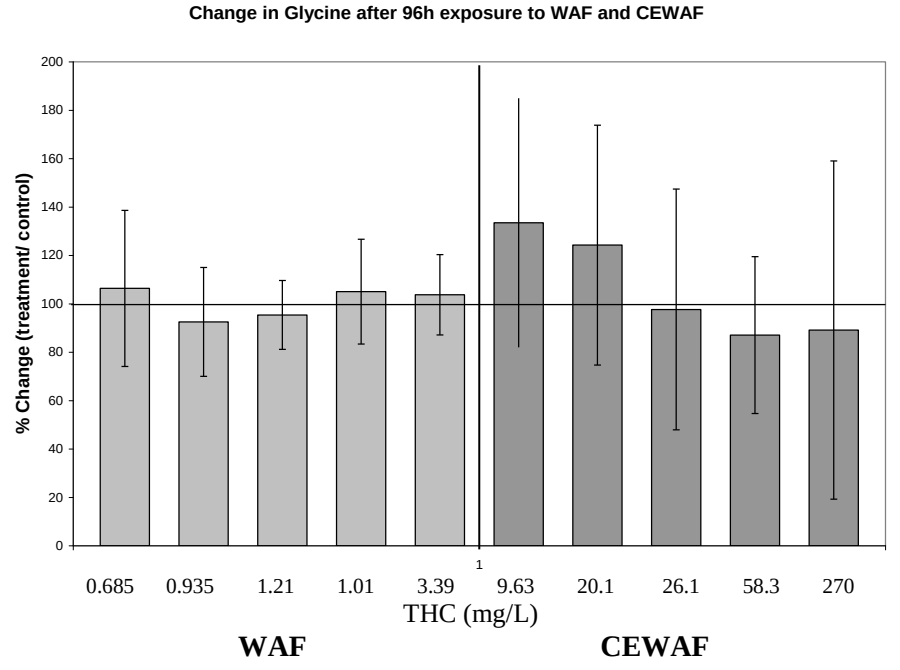
b)


Change in ATP after 96h Exposure WAF and CEWAF
% Change (treatment/control)
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

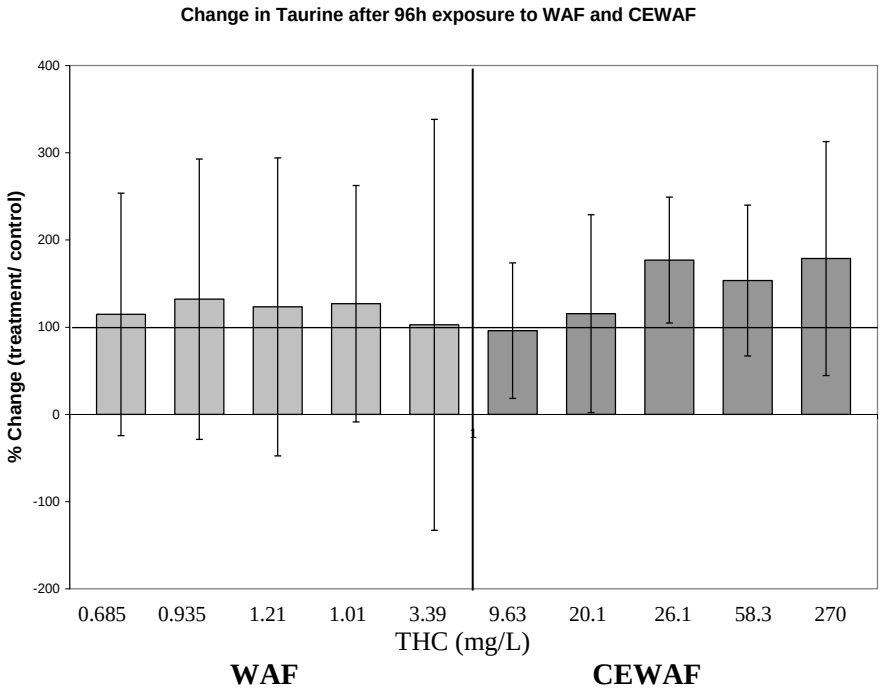
c)


Change in Histidine after 96h Exposure WAF and CEWAF
% Change (treatment/ control)
200
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

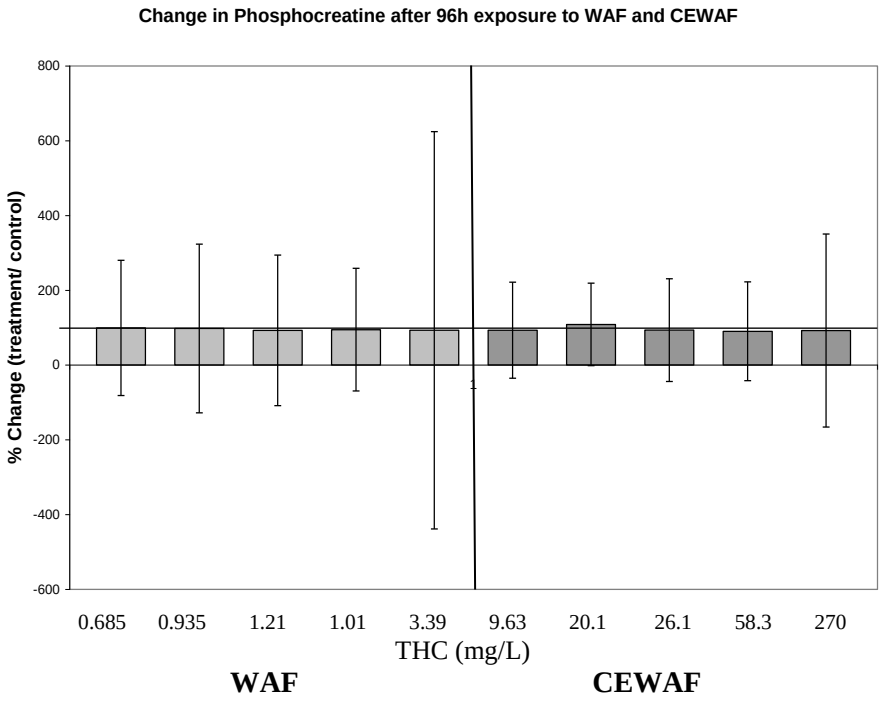
d)


Change in Glycine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
200
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

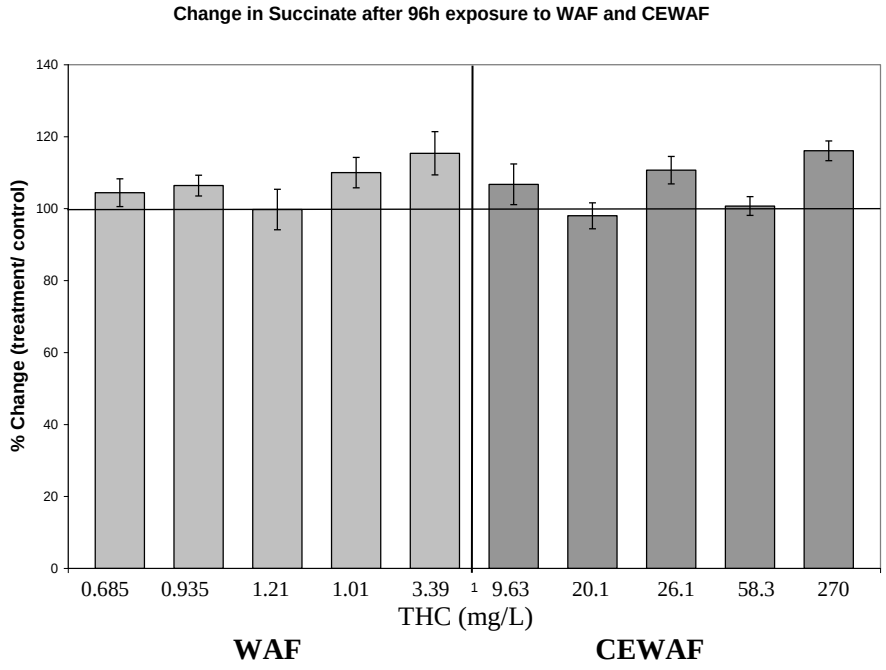
e)


Change in Taurine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
400
300
200
100
0
-100
-200
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

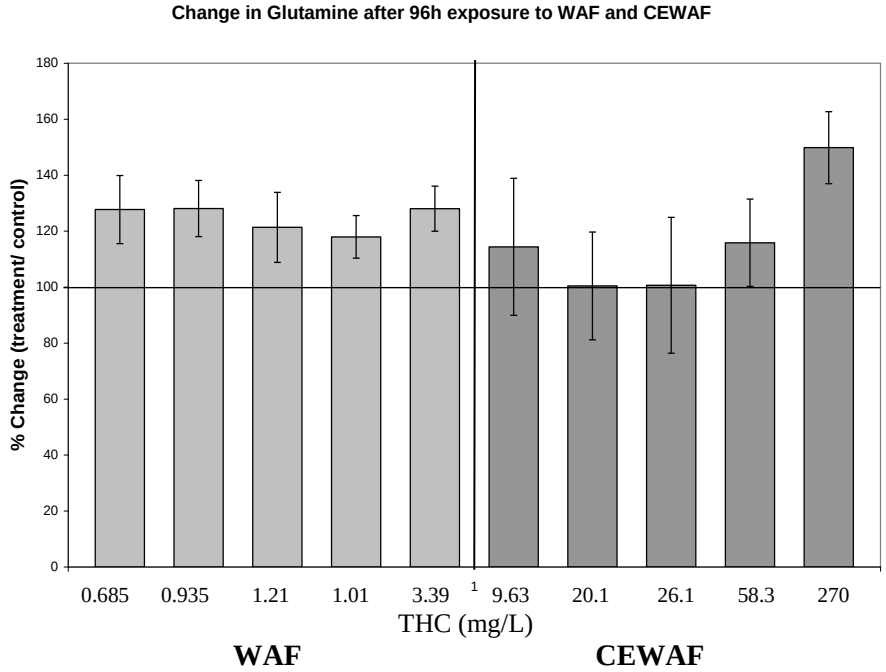
f)


Change in Phosphocreatine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
800
600
400
200
0
-200
-400
-600
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

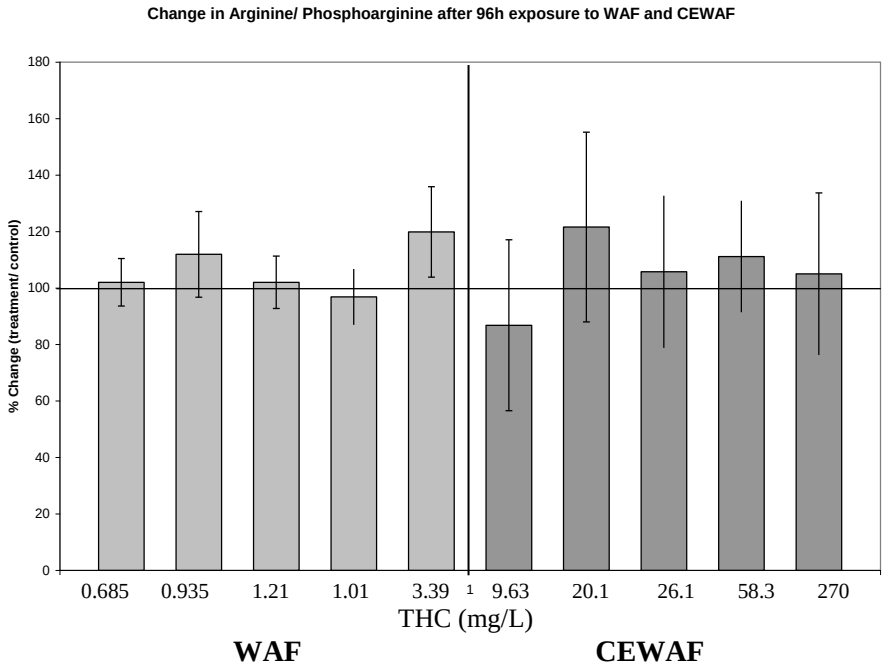
g)


Change in Succinate after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

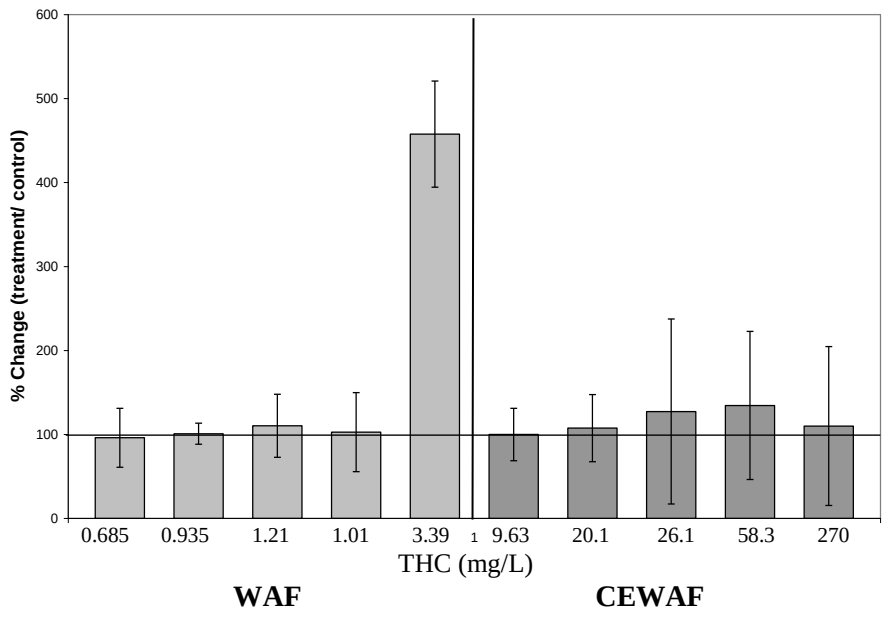
h)


Change in Glutamine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

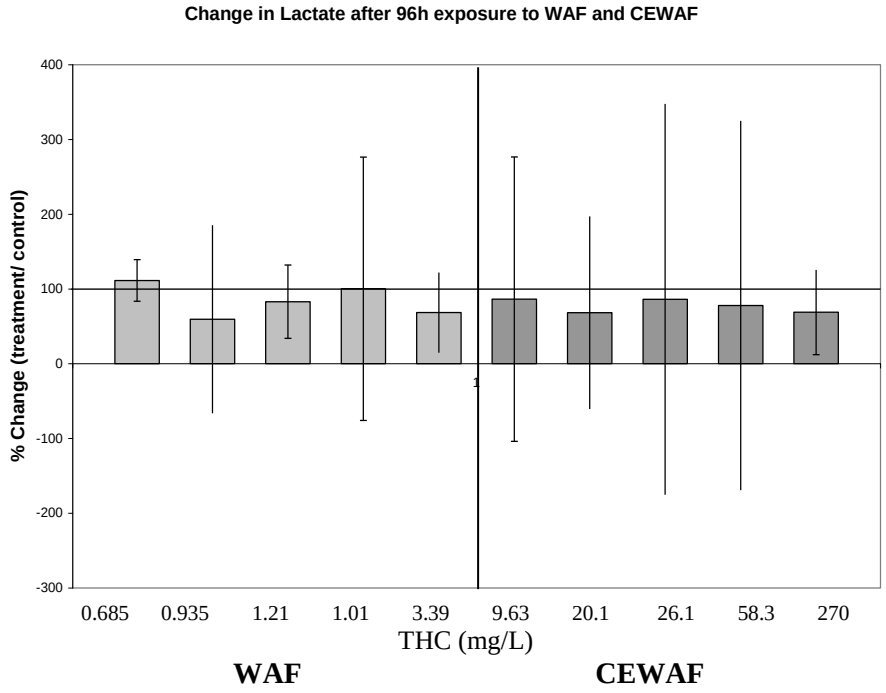
i)


Change in Arginine/ Phosphoarginine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

j)


Change in Alanine after 96h exposure to WAF and CEWAF
% Change (treatment/ control)
600
500
400
300
200
100
0
0.685
0.935
1.21
1.01
3.39
1
9.63
20.1
26.1
58.3
270
THC (mg/L)
WAF
CEWAF

k)



l)

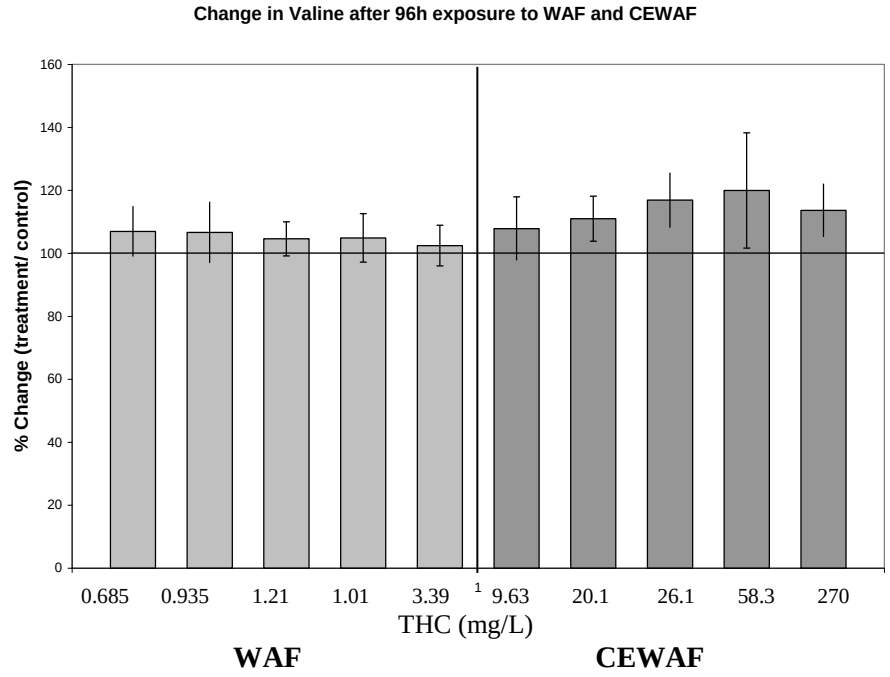
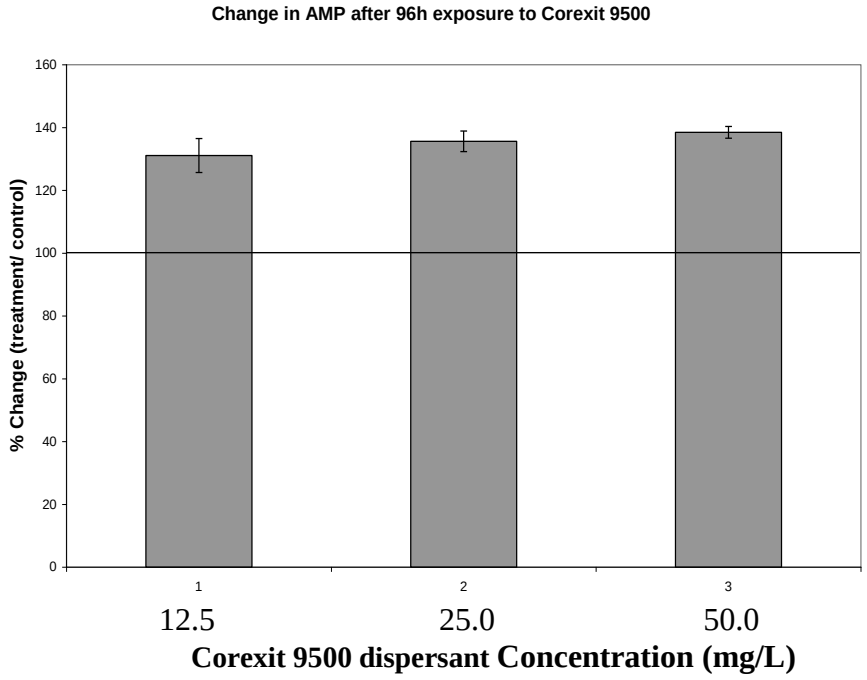
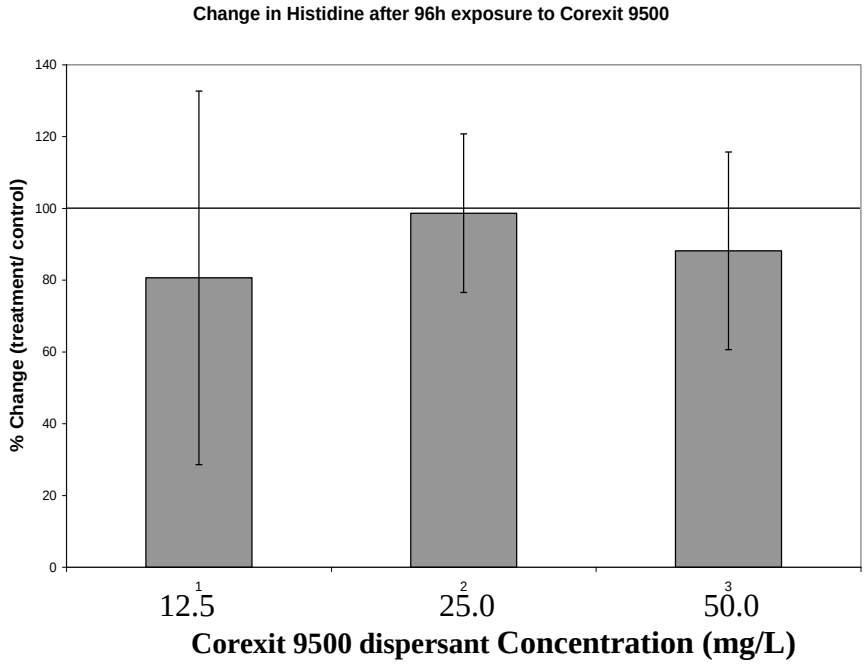


Figure 10. Changes in specific muscle tissue metabolites, after 96-h WAF and CEWAF exposure, include (a) AMP, (b) ATP, (c) histidine, (d) glycine, (e) taurine, (f) phosphocreatine, (g) succinate, (h) glutamine, (i) arginine/ phosphoarginine, (j) alanine, (k) lactate, and (l) valine.

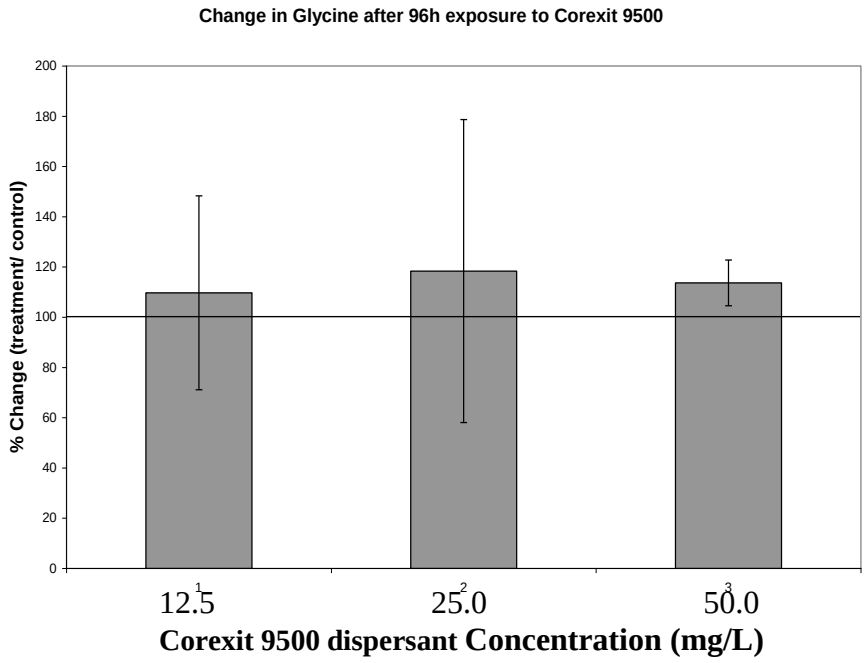
a)


Change in AMP after 96h exposure to Corexit 9500
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

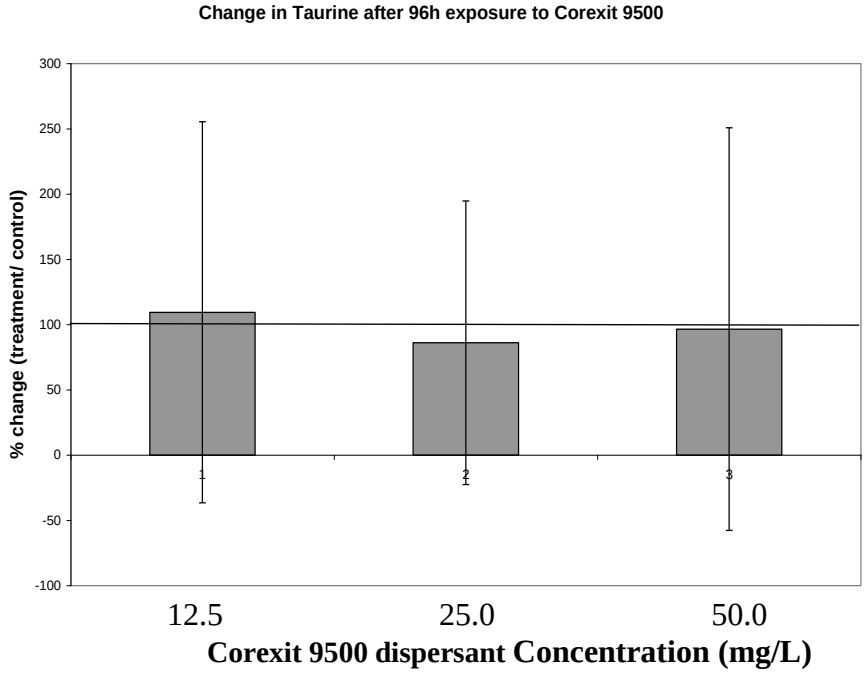
b)


Change in Histidine after 96h exposure to Corexit 9500
% Change (treatment/ control)
140
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

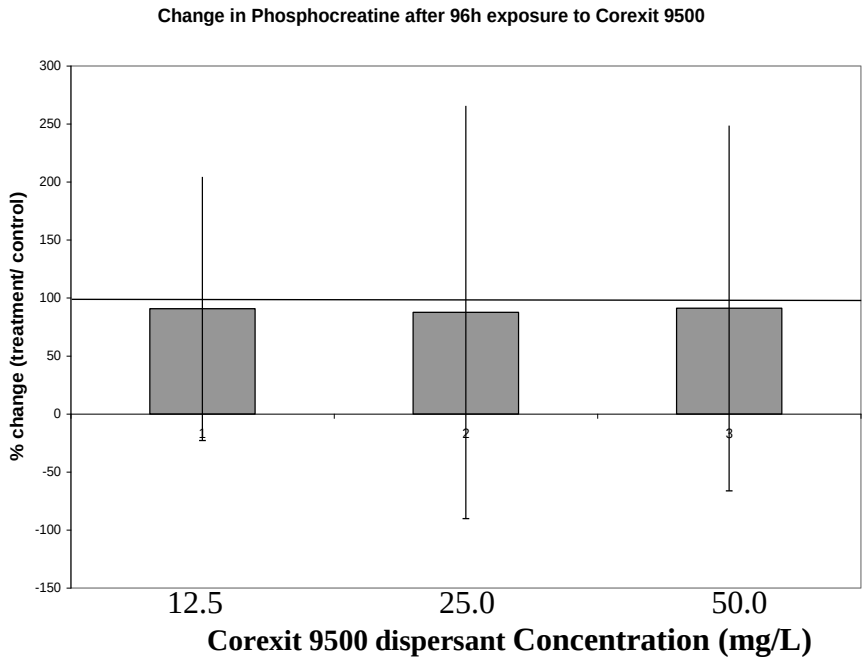
c)


Change in Glycine after 96h exposure to Corexit 9500
% Change (treatment/ control)
200
180
160
140
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

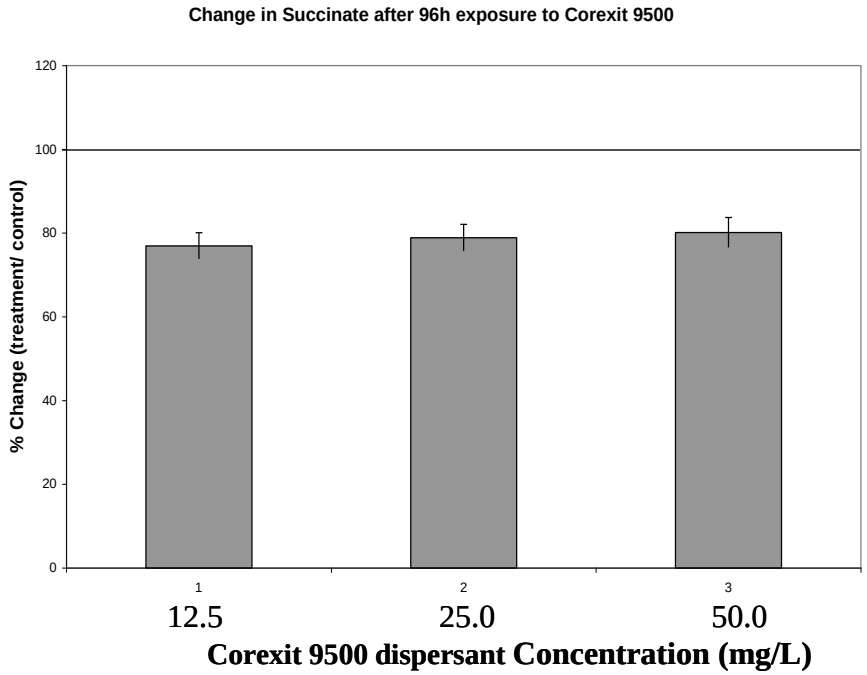
d)


Change in Taurine after 96h exposure to Corexit 9500
% change (treatment/ control)
300
250
200
150
100
50
0
-50
-100
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

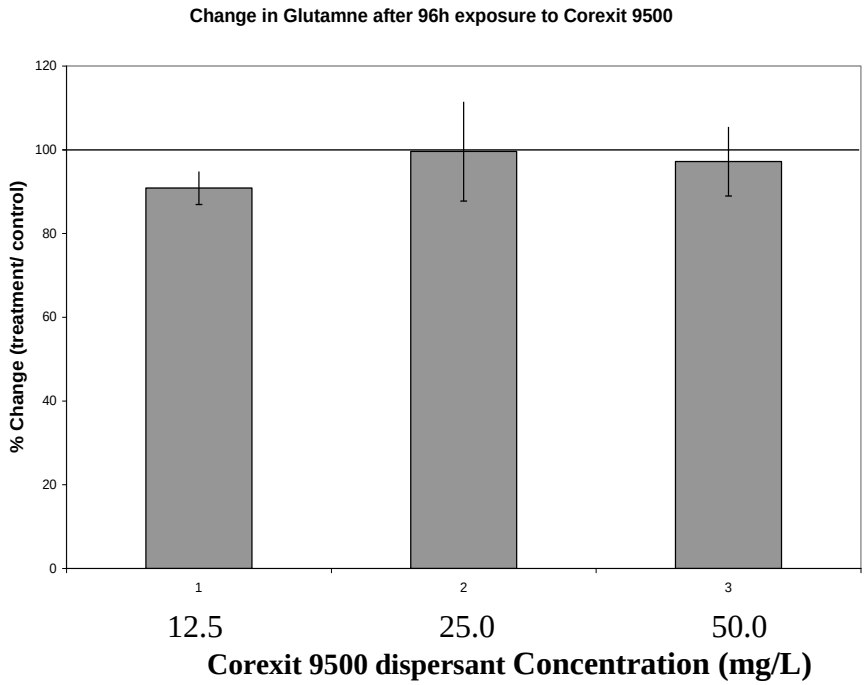
e)


Change in Phosphocreatine after 96h exposure to Corexit 9500
% change (treatment/ control)
300
250
200
150
100
50
0
-50
-100
-150
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

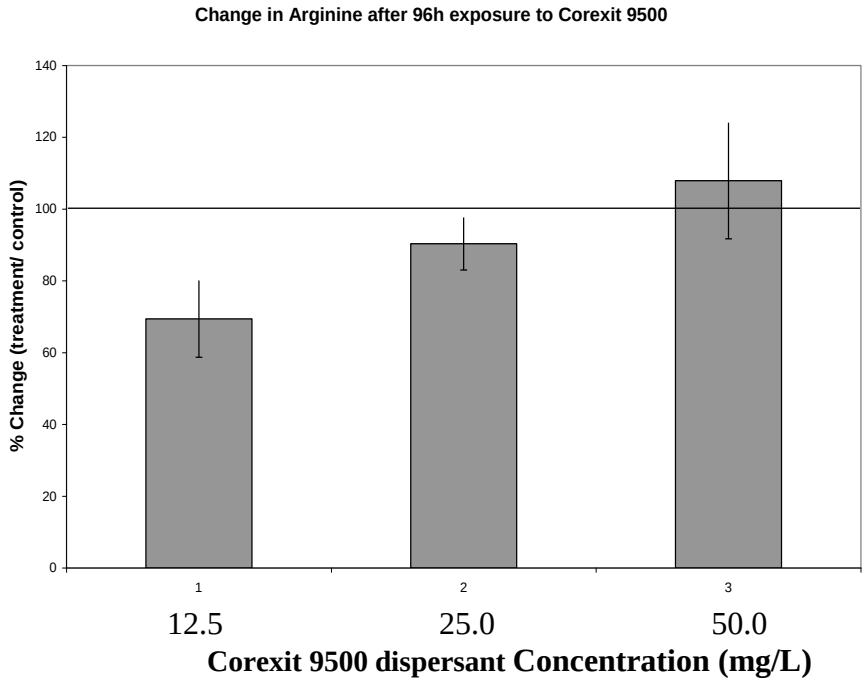
f)


Change in Succinate after 96h exposure to Corexit 9500
% Change (treatment/ control)
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

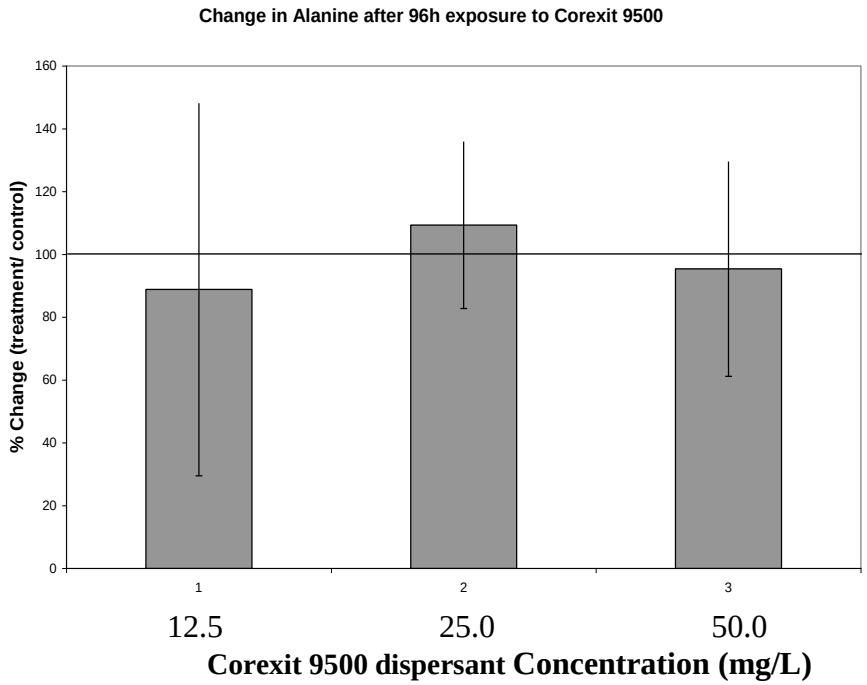
g)


Change in Glutamne after 96h exposure to Corexit 9500
% Change (treatment/ control)
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

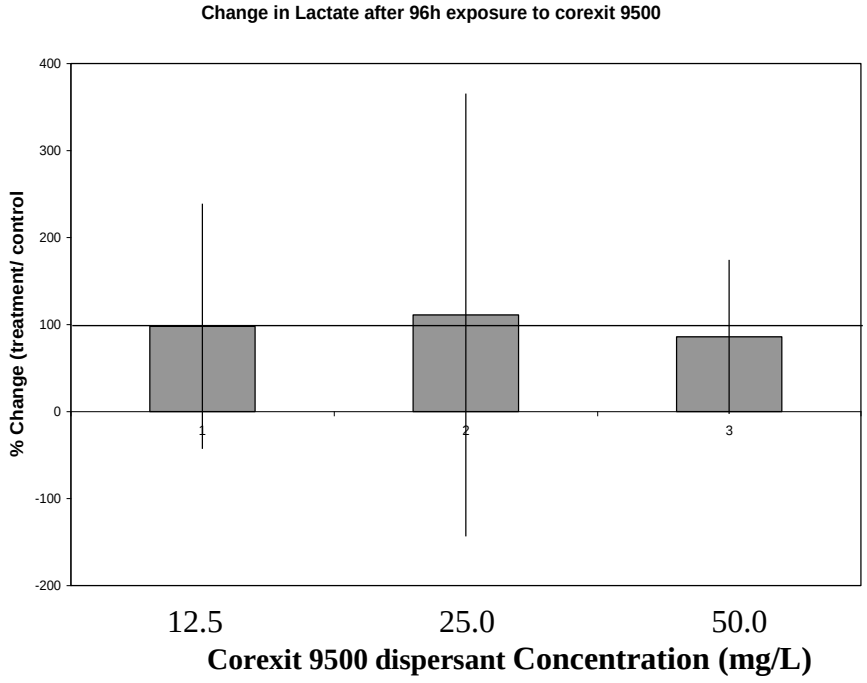
h)


Change in Arginine after 96h exposure to Corexit 9500
% Change (treatment/ control)
140
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

i)


Change in Alanine after 96h exposure to Corexit 9500
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

j)


Change in Lactate after 96h exposure to corexit 9500
% Change (treatment/ control
400
300
200
100
0
-100
-200
1
2
3
12.5
25.0
50.0
Corexit 9500 dispersant Concentration (mg/L)

k)

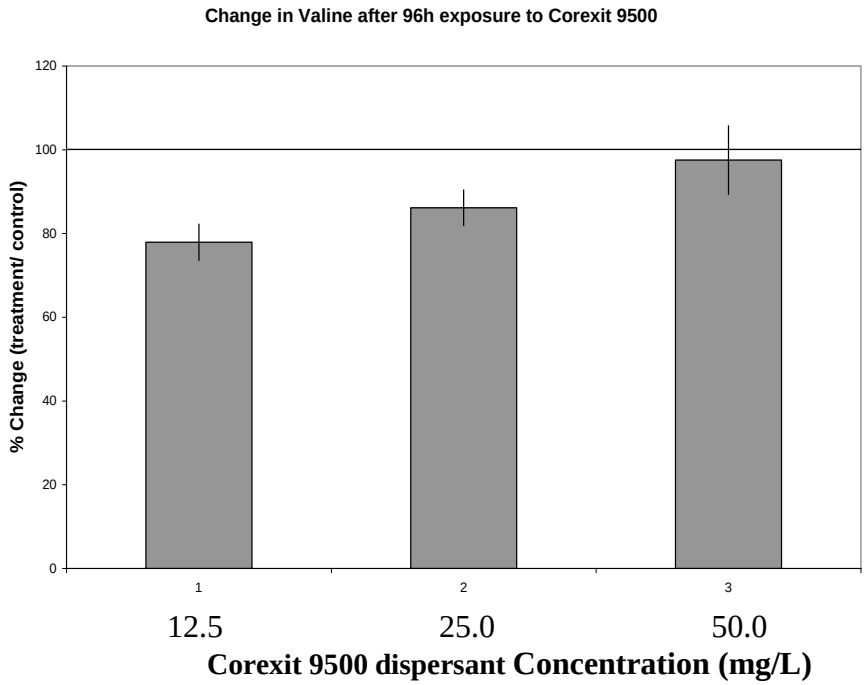


Figure 11. Changes in specific muscle tissue metabolites, after 96-h Corexit 9500 dispersant exposure, include (a) AMP, (b) histidine, (c) glycine, (d) taurine, (e) phosphocreatine, (f) succinate, (g) glutamine, (h) arginine/ phosphoarginine, (i) alanine, (j) lactate, and (k) valine.

4.7 Fecundity of WAF and CEWAF-Exposed Adults

Surviving fish from the adult WAF exposure were transferred to 4 foot diameter tanks at the end of the test. Fish from the three replicate exposure aquaria from each loading concentration were combined in one tank to provide sufficient numbers for spawning. After sacrificing one surviving fish from each replicate for metabolomic analysis, 30 to 37 fish were transferred to the holding tanks. The fish were allowed to acclimate for one week post exposure, and were held under a 16h light:8h dark light cycle to mimic spring/summer spawning conditions. After one week, water temperature was raised one degree per day from the ambient temperature (~15 ºC) to 18 ºC. Each tank was fitted with a re-circulating pump and water flow consisted of a combination of re-circulated water and ambient

seawater. Three spawning substrates consisting of dye-free yarn were added to each tank. To promote spawning, tank temperatures were increased to 20 ºC and spawning substrates were monitored daily. All eggs on each substrate were counted and the total number of eggs in each tank was divided by the total number of fish. The fish were held under these conditions for three weeks. While some spawning occurred in all but the control tanks during this period, overall egg production was low, and was not sufficient to allow an adequate number of embryos for embryo viability and larval growth evaluations (data not shown). It is possible the WAF-exposed fish had not recovered from stress induced by the initial experiments, so the tank lighting was returned to a 12h light: 12h dark cycle and the water was allowed to cool to ambient temperature by turning off the recirculation pumps and immersion heaters, and increasing the flow of ambient seawater. All fish were held under these conditions for 5 months, during which time growth was monitored. After 5 months, the tank temperature and lighting conditions were returned to those described above to induce spawning. Spawning commenced immediately and embryo production was monitored. Spawning was monitored in three separate spawning events over a 2 week period. These data are provided below.

As in the WAF test, surviving adult fish from the CEWAF test were also transferred to spawning tanks at end of the exposure. After sacrificing one surviving fish from each replicate for metabolomic analysis, 38 to 40 fish from the four lowest concentrations were transferred to the holding tanks. Because of mortality, only 11 fish from the 0.5 g/L concentration were transferred. There were no survivors in the 1.0 g/L concentration. The fish were allowed to acclimate one week then tank lighting and temperature conditions were adjusted to promote spawning, as described above. The fish began spawning immediately and spawns were monitored for the three weeks.

During both the WAF and CEWAF spawning assessments, embryos from each of three spawns were transferred to scintillation vials containing 10 mL of seawater and held at 20 ºC. Twenty embryos were isolated from the first two spawns, and 100 embryos were isolated from the third spawn. Single embryos were placed in each scintillation vial, and embryo development and hatching success were assessed. As larvae hatched from the third

spawn, hatching success was recorded for the first 20 embryos, and all larvae were transferred to screen tubes fit within flowing seawater culture buckets. The larvae were fed newly hatched *Artemia* nauplii *ad libitum* twice daily and larval growth was monitored for 12 weeks. Growth was assessed by sacrificing 5 larvae every 4 weeks and length and dry weight of these larvae were recorded.

The mean number of eggs produced per WAF-exposed fish in each tank is given in Table 11. Egg production was 50 egg/fish in the control tank and declined progressively in all fish exposed to higher WAF concentrations. The lowest egg production was observed in fish exposed to the three highest WAF concentrations. Egg production was the most variable in the control fish. There were no apparent differences in embryo viability in these experiments; viability was 80% in the control embryos, and was 84 percent in the embryos hatched from adults which were exposed to 25g/L WAF. In addition, there were no apparent effects of WAF on embryo hatching success; 70% of the control embryos hatched, and 81% of the embryos hatched in the embryos spawned from adults which were exposed to 25g/L WAF.

As was observed in the WAF-exposed adults, adult topsmelt exposed to CEWAF produced fewer embryos than control fish. The numbers of embryos per fish declined 40% relative to the control in the lowest loading concentration (0.0625 g/L), and declined further as oil loading increased (Table 11). Embryo production at the two highest loadings (0.25 and 0.5 g/L) declined 84% and 72%, respectively, relative to the control. Between-spawn egg production was highly variable in all of the tanks in both WAF and CEWAF-exposed fish. High embryo production variability is reflected by very high standard deviations in the controls and in all WAF and CEWAF loadings, and because of this, no statistical analyses were conducted to compare embryo production in these experiments. Lower viability was observed in spawns from the CEWAF-exposed fish than was observed in the WAF-exposed fish. Control embryo viability was 58% in the CEWAF experiment, and viability was 69% at the highest loading. All embryos spawned from the fish exposed to 0.25 g/L CEWAF were non-viable. Hatching success of viable embryos was greater than 79% in all treatments except embryos spawned from fish exposed to 0.25 g/L CEWAF.

Many pollutants can elicit perturbations in components of reproduction. Specifically, changes in hormone production, synthesis of vitellogenin and changes in fertilization rates can affect reproductive capabilities (Kime, 1995). Studies on the effects PAHs have on fish reproduction have determined that PAHs cause a decrease in gametogenesis, gonad size and reduced production of eggs (Tuvikene, 1995). In particular, anthracene was found to bioconcentrate more in the ovaries and therefore the number of eggs produced was reduced (Tuvikene, 1995). According to Nicolas (1999), PAHs have been found to impair reproductive functions, specifically the vitellogenic cycle, leading to a reduction in reproductive success. Since hydrocarbons prefer lipid-rich organs, they may have accumulated in the ovaries during the WAF and CEWAF exposures, therefore affecting the overall spawning success. Energy is often allocated to gamete production and to maintain a constant number of eggs, while other physiological processes may be affected (Schreck et al., 2001), however the metabolomic results do not indicate a significant decrease in energy (ATP) reserves. Overall, the variation in the number of spawned eggs is potentially related to the amount of stress the topsmelt were experiencing at time of the exposures and the metabolic results cannot accurately determine the cause of reduced reproductive success without further experimental investigations which include observing specific reproductive processes and organs.

Table 11. Mean number of eggs per fish produced by WAF and CEWAF-exposed adults during 3 separate spawning events over a 2 week period. NA = Not Analyzed. PBCO concentrations are given as both loading (g/L) and Total Hydrocarbon Content (THC = mg/L).

WAF Definitive						
Loading g/L (THC mg/L)	Mean Eggs/Fish	SD	Mean % Viable	SD	Mean % Hatch	SD
0.00 (0.38)	50	49	80	33	70	18
1.56 (0.69)	40	38	91	9	83	12
3.13(0.94)	41	28	87	12	52	20
6.25 (1.21)	22	31	72	35	79	16
12.5 (1.01)	13	17	90	8	79	8
25.0 (3.39)	23	15	84	23	81	8

CEWAF Definitive						
Loading g/L (THC mg/L)	Mean Eggs/Fish	SD	Mean % Viable	SD	Mean % Hatch	SD
0.00 (0.38)	25	21	58	28	90	12
0.063 (9.63)	15	11	46	44	79	11
0.125 (20.1)	12	17	61	30	84	24
0.25 (26.1)	4	8	0	NA	NA	NA
0.50 (58.3)	7	11	69	32	81	7

Larvae hatched from the embryos spawned from WAF and CEWAF-exposed adults were cultured for approximately 12 weeks to assess growth. Larvae from all WAF-exposed adults doubled their size in length and weight between January and March, 2010, and there were no differences between control and WAF-exposed fish (Figures 12). Growth was also observed in larvae from the CEWAF-exposed adults, although the rate of growth of these larvae was less than that of the WAF-exposed larvae. Weight of all the CEWAF larvae doubled in the final three weeks of culture, and there were no differences in weight between the controls and CEWAF-exposed fish (Figure 13).

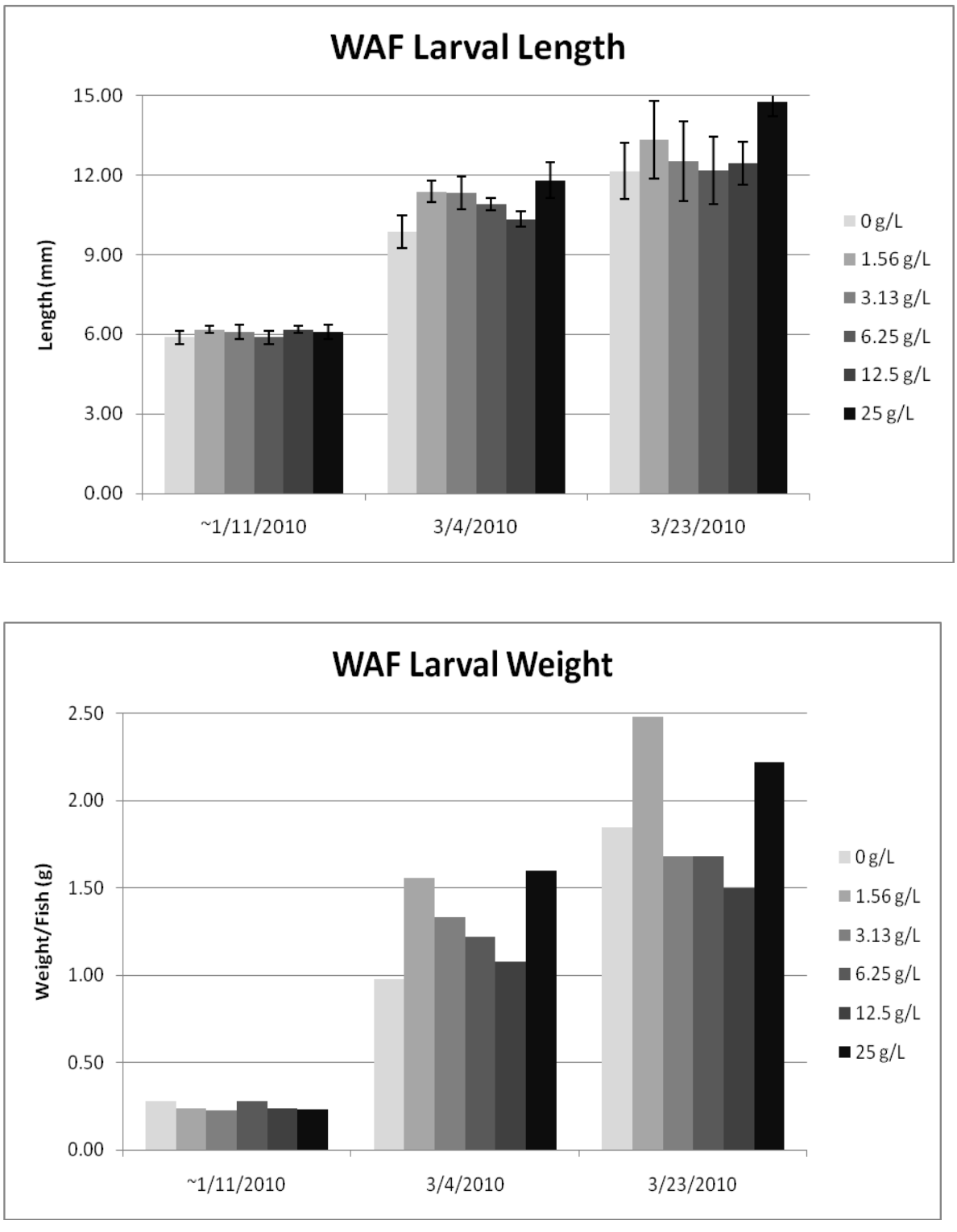
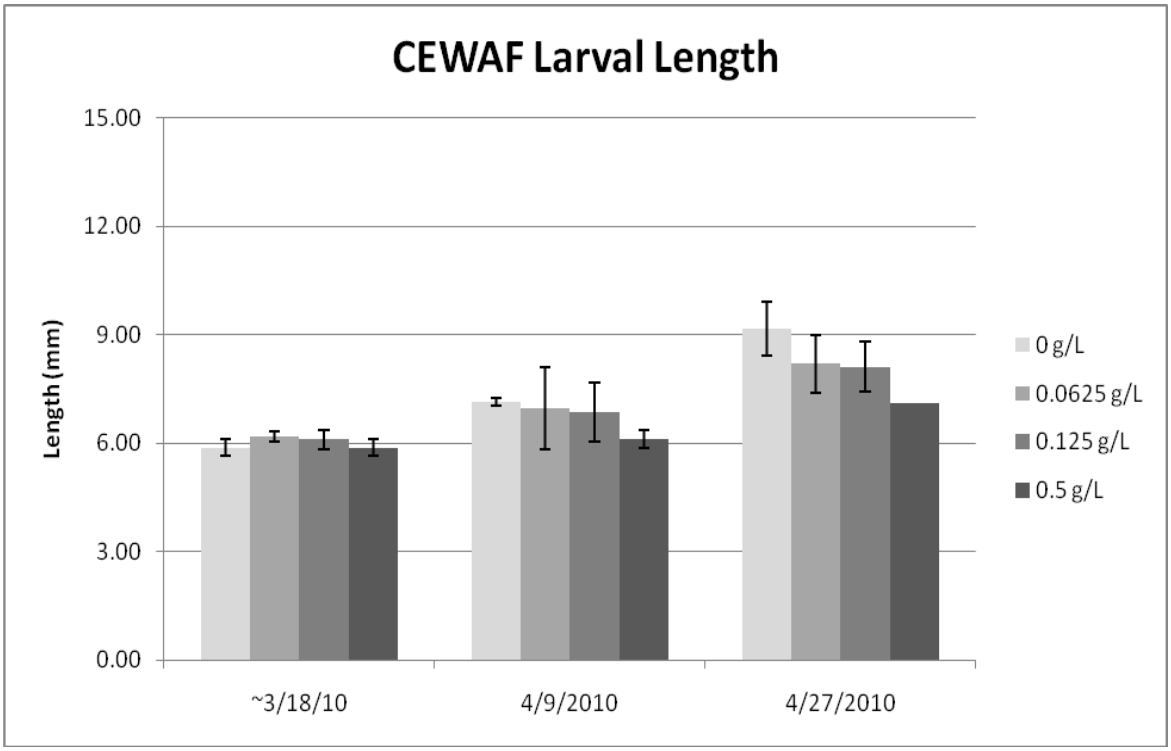


Figure 12. Growth of Topsmelt Larvae Hatched From WAF-Exposed Adults



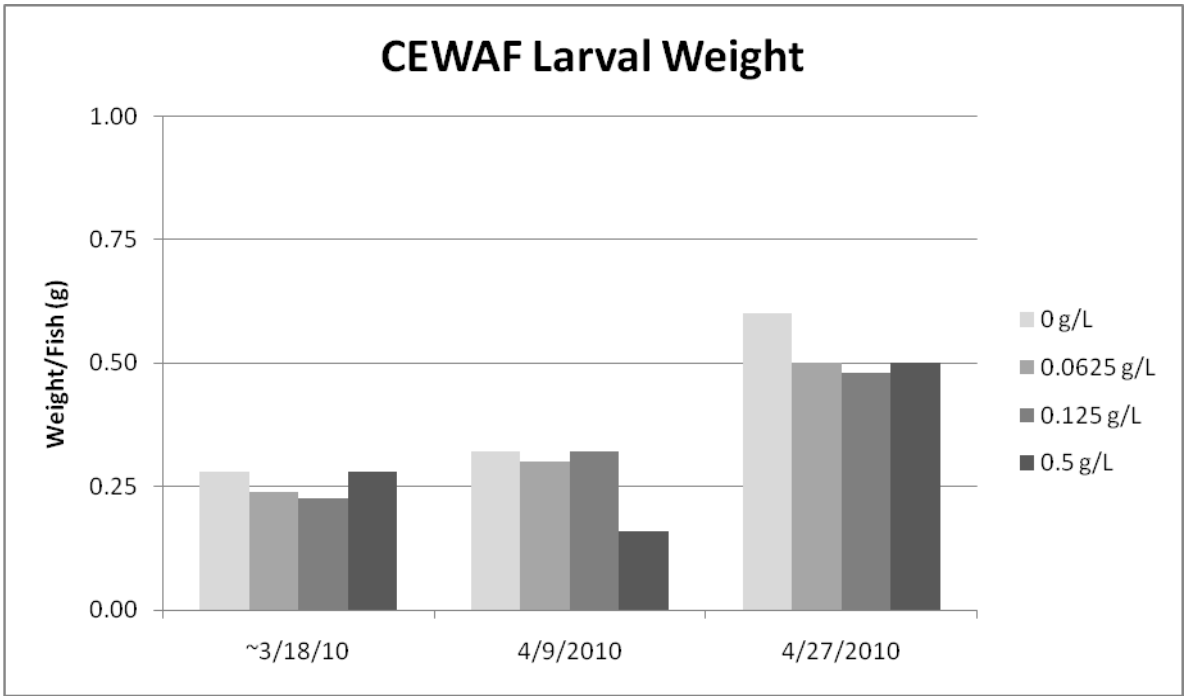


Figure 13. Growth of Topsmelt Larvae Hatched From CEWAF-Exposed Adults

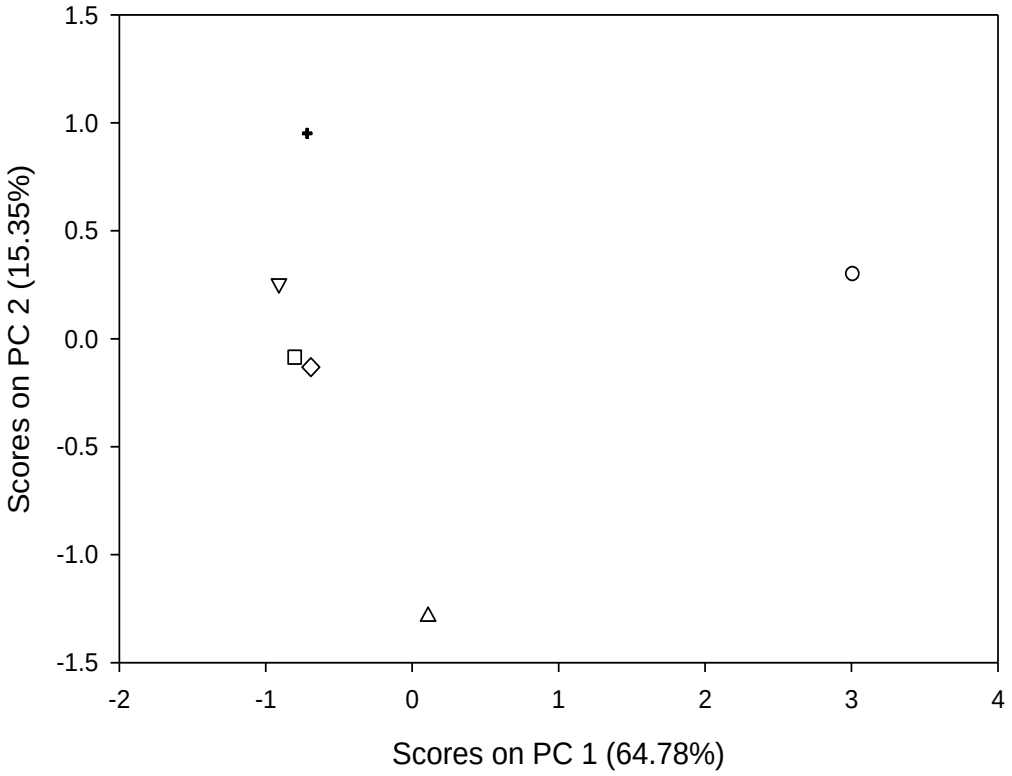
4.8 Dose-Response of Metabolic Profiles in Embryos Spawned from Adult Topsmelt Exposed to WAF and CEWAF

The ^{1}H NMR spectra were analyzed by PCA; providing a graphical output, comprising of individual samples, summarizing the similarity between the NMR spectra. Embryos from the control tanks and from each of the doses were analyzed individually to avoid variation from experimental conditions and possible physiological conditions. The WAF and CEWAF PCA score plots, represents the resultant metabolic dose-response for both tests.

Due to lack of replicates the WAF PCA scores plot, shows minimal clustering between control and dose 3 embryos, along the PC1 axis (containing 64.78% of variance; Figure 14a). Due to the lack of overlap among the metabolomes, a dose-response relationship between the individual WAF concentrations is apparent. Also due to lack of replicates, the CEWAF PCA scores plot lacks clustering between the individual samples along PC1 and PC2 (accounting for 85.58% and 9.98% of variance, Figure 14b); a metabolic dose-response relationship is also apparent

For embryos spawned from topsmelt following WAF and CEWAF exposure, a metabolic dose-response relationship is displayed among concentrations. Furthermore this indicates that the variation in metabolomes may be induced by the mother's hydrocarbon exposure, therefore possibly leading to maternal transfer of acute toxicity and hydrocarbon exposure.

(a)


1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
Scores on PC 2 (15.35%)
-2
-1
0
1
2
3
4
Scores on PC 1 (64.78%)

(b)

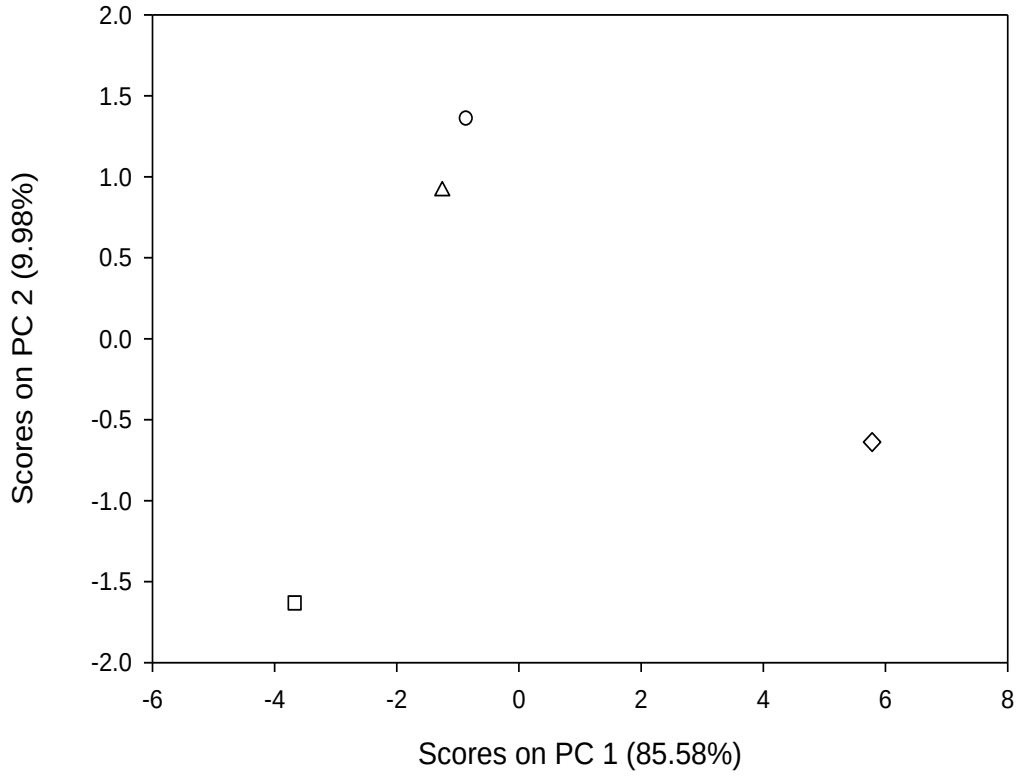


Figure 14. PCA scores plot of the ^{1}H NMR spectra of extracts from embryos spawned by topsmelt exposed to (a) WAF and (b) CEWAF of PBCO for 96-h. All analyses include an unexposed control. Controls and the five concentrations of WAF are characterized as control (◊), 2.32 (○), 2.66 (△), 3.87 (□), 4.49 (+) and 6.31 (▽) mg/L THC. Controls and the three concentrations of CEWAF are characterized as control (◊), 41.3 (○), 100 (△) and 168 (□) mg/L THC. The symbol shape represents a single dose and each data point represents one biological tissue sample.

4.9 Specific Metabolic Changes in Embryos Spawned from Adult Topsmelt Exposed to WAF and CEWAF

Well-resolved peaks were identified and integrated for comparison among each test. Metabolite changes were calculated, using peak areas (Table 12), by dividing the average peak area of the treatment by that of the corresponding control. A number below 100% represents a decrease in that metabolite within the treated embryos. Concentration-dependent changes from identified metabolites are presented in Table 13; due to lack of

replicates one-way ANOVAs could not be conducted for these samples. Metabolic changes comparing the spawned embryos between WAF and CEWAF are provided in Figure 15.

Specific changes for WAF include a decrease in succinate whereas, succinate increased for CEWAF. Lactate decreased at the lower concentrations of WAF and increased at the higher concentrations and steadily decreased for each concentration of CEWAF. At intermediate and high WAF concentrations metabolite changes varied between increasing and decreasing values. Majority of the identified metabolites for CEWAF either steadily increased or decreased except for glycerophosphocholine, which decreased at the intermediate CEWAF concentration. The variability among metabolic changes indicates that they are dose and metabolite dependent. Unfortunately, significant changes among the metabolites could not be determined for either WAF or CEWAF due to lack of replicates.

Table 12. Peak areas from the ^{1}H NMR spectra of extracts from embryos spawned by topsmelt exposed to (a) WAF and (b) CEWAF for 96-h.

(a)

			WAF [a, b]			
Metabolites	**Control**	**2.32 mg/L**	**2.66 mg/L**	**3.87 mg/L**	**4.49 mg/L**	**6.31 mg/L**
Valine	13.8	12.6	13.4	15.7	12.5	14.4
Lactate	24.7	22.3	23.1	25.9	31.7	27.4
Alanine	12.4	11.6	12.9	13.9	12.0	12.5
Glutamine	7.46	6.30	7.78	8.07	6.46	7.19
Glutamate	10.1	10.0	10.5	9.67	10.2	9.89
Succinate	3.66	3.07	3.54	3.20	3.59	3.49
Taurine	48.2	50.8	65.8	42.2	45.8	45.3
Glycine	29.5	29.3	31.3	32.2	30.7	29.5
Glycerophosphocholine	4.41	5.04	6.56	4.27	5.34	3.88

(b)

		CEWAF [a, b]		
Metabolites	**Control**	**41.3 mg/L**	**100 mg/L**	**168 mg/L**
Valine	18.1	16.2	16.2	17.8
Lactate	67.6	53.8	49.4	26.2
Alanine	17.8	17.4	16.9	14.3
Glutamine	6.72	9.10	8.32	6.94
Glutamate	6.59	11.3	11.1	9.73
Succinate	2.51	3.97	3.88	3.40
Taurine	24.9	35.2	37.1	49.7
Glycine	18.5	27.8	28.0	33.2
Glycerophosphocholine	1.81	2.32	1.80	2.67

[a.] All spectra were normalized by total spectral area.
[b.] WAF and CEWAF are characterized total hydrocarbon content (mg/L).

Table 13. Metabolic changes of extracts from embryos spawned by topsmelt exposed to (a) WAF and (b) CEWAF for 96-h.

(a)

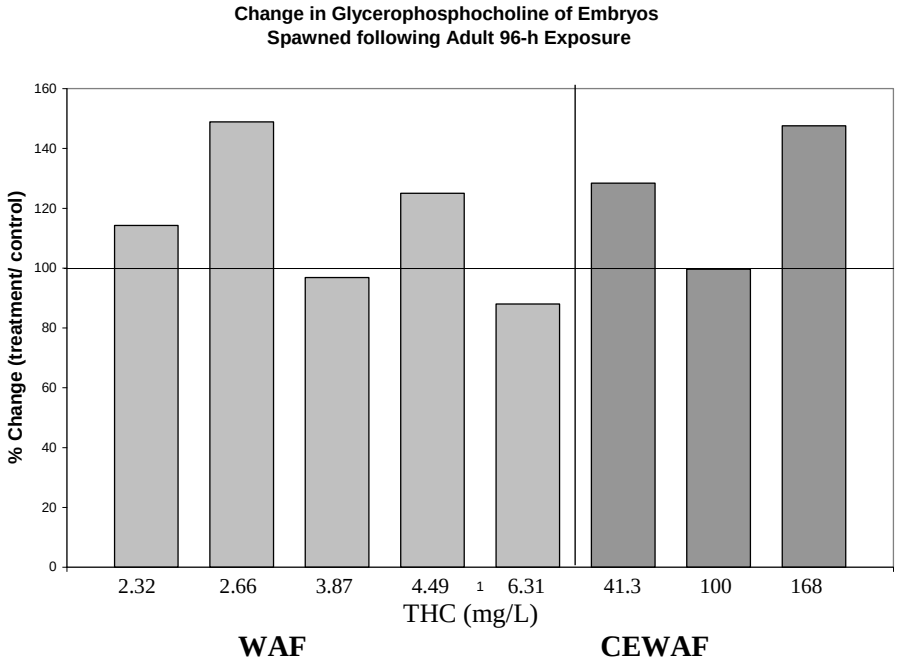
			WAF [a, b]		
Metabolites	**2.32 mg/L**	**2.66 mg/L**	**3.87 mg/L**	**4.49 mg/L**	**6.31 mg/L**
Valine	91.1	97.1	114	79.3	104
Lactate	90.6	93.7	105	123	111
Alanine	93.0	104	112	86.5	100
Glutamine	84.5	104	108	80.0	96.4
Glutamate	99.7	104	96.0	105	98.1
Succinate	84.0	96.7	87.5	112	95.4
Taurine	105	137	87.6	109	94.0
Glycine	99.3	106	109	95.3	100
Glycerophosphocholine	114	149	96.8	125	88.0

(b)

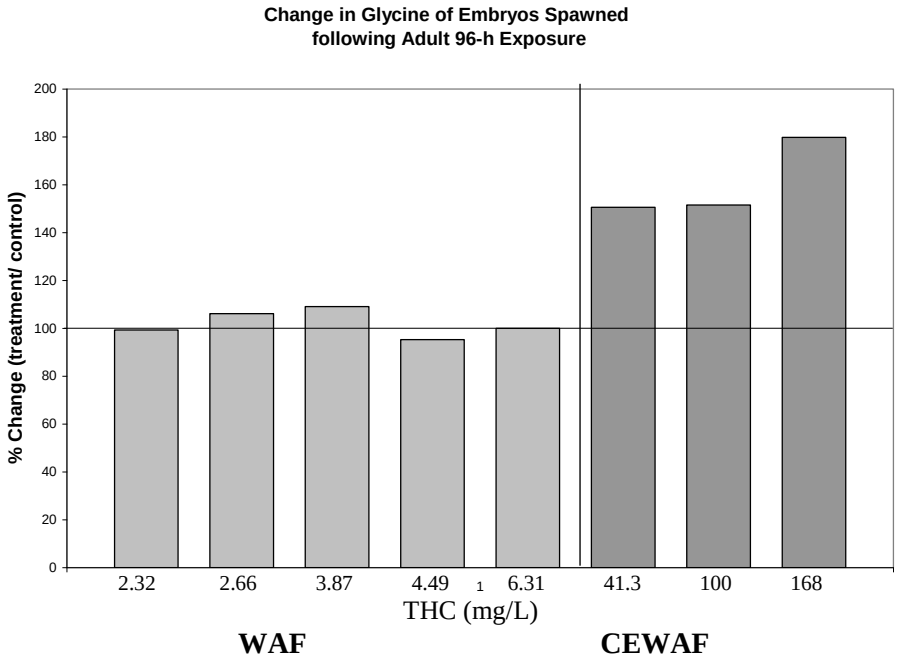
		CEWAF [a, b]	
Metabolites	**41.3 mg/L**	**100 mg/L**	**168 mg/L**
Valine	89.4	89.7	98.1
Lactate	79.6	73.1	38.7
Alanine	97.8	95.1	80.2
Glutamine	136	124	103
Glutamate	172	169	148
Succinate	159	155	136
Taurine	141	149	200
Glycine	151	152	180
Glycerophosphocholine	128	99.6	148

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

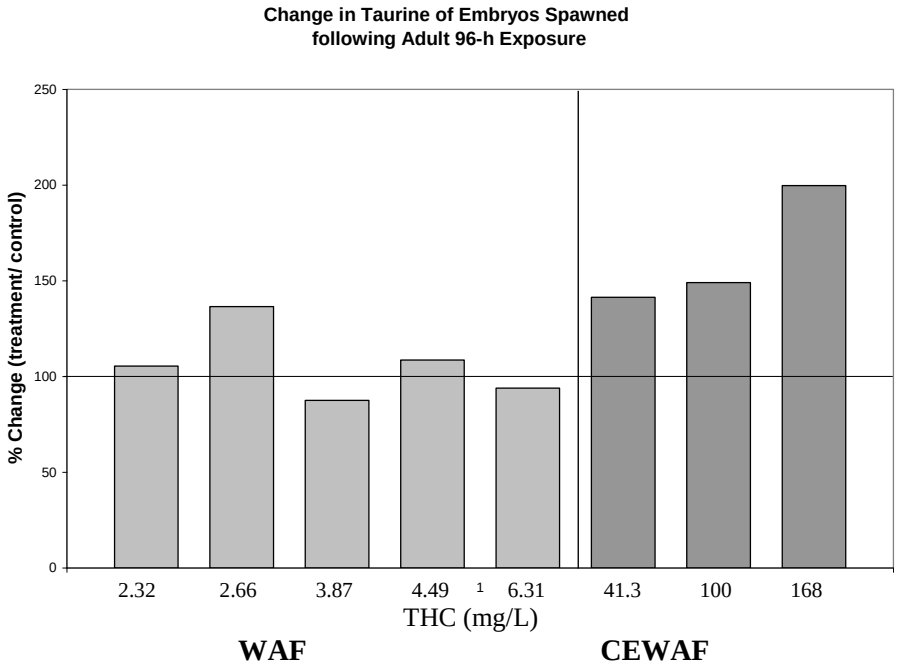
(a)


Change in Glycerophosphocholine of Embryos Spawned following Adult 96-h Exposure
% Change (treatment/ control)
0
20
40
60
80
100
120
140
160
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

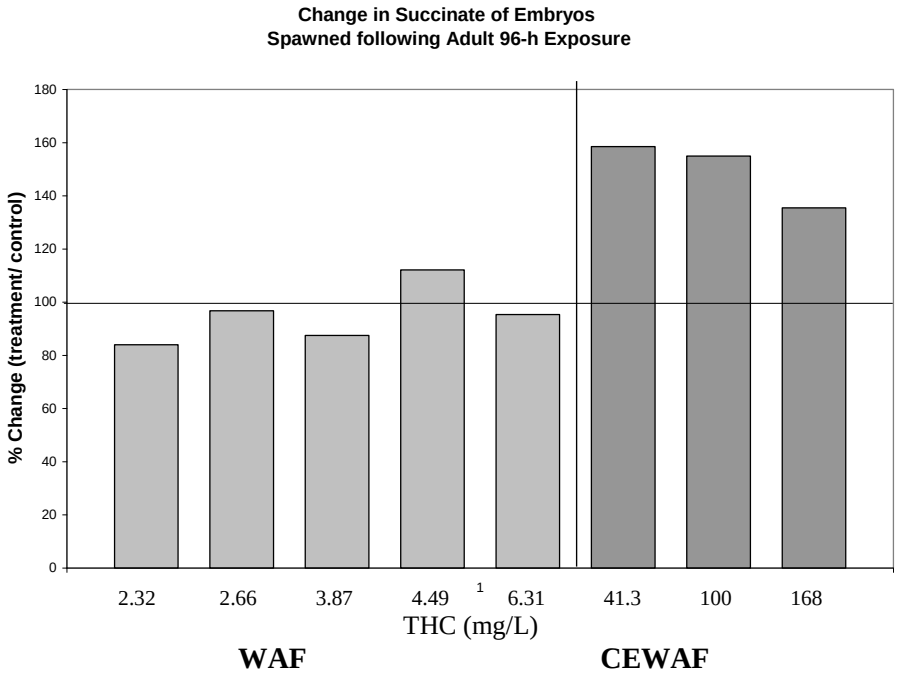
(b)


Change in Glycine of Embryos Spawned following Adult 96-h Exposure
% Change (treatment/ control)
0
20
40
60
80
100
120
140
160
180
200
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

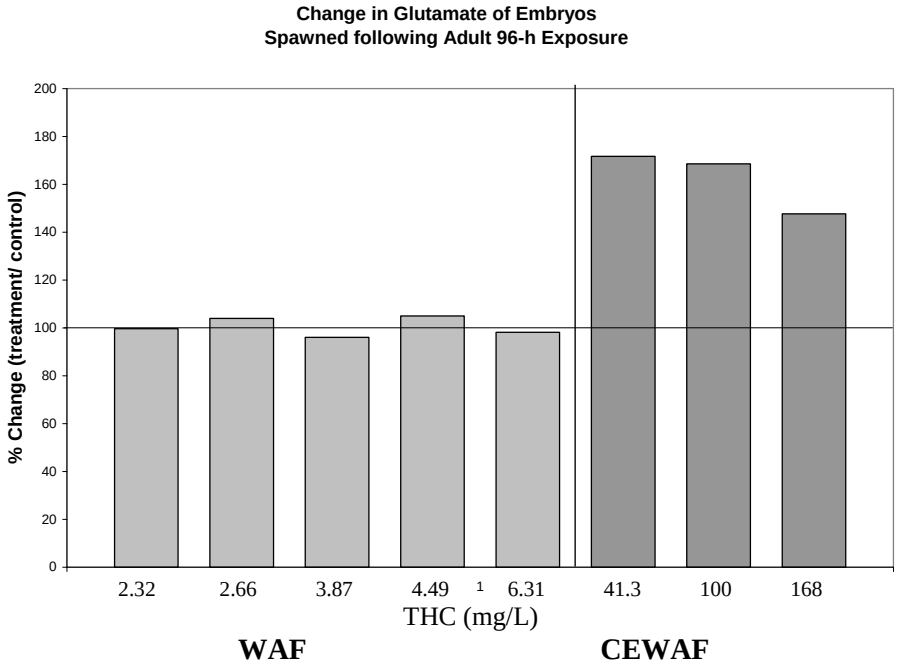
(c)


Change in Taurine of Embryos Spawned following Adult 96-h Exposure
% Change (treatment/ control)
250
200
150
100
50
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

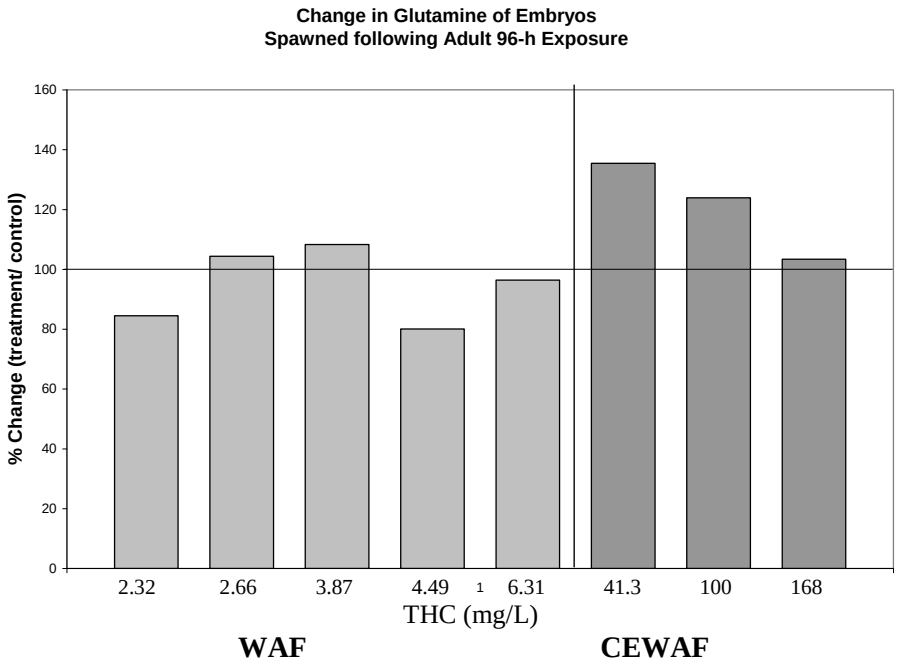
(d)


Change in Succinate of Embryos Spawned following Adult 96-h Exposure
% Change (treatment/ control)
180
160
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

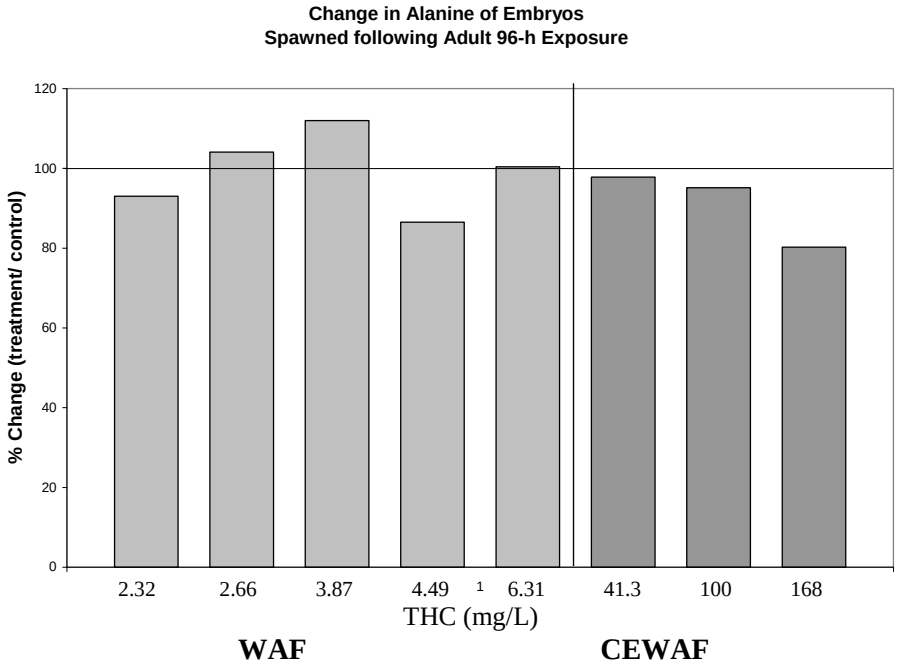
(e)


Change in Glutamate of Embryos
Spawned following Adult 96-h Exposure
% Change (treatment/ control)
200
180
160
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

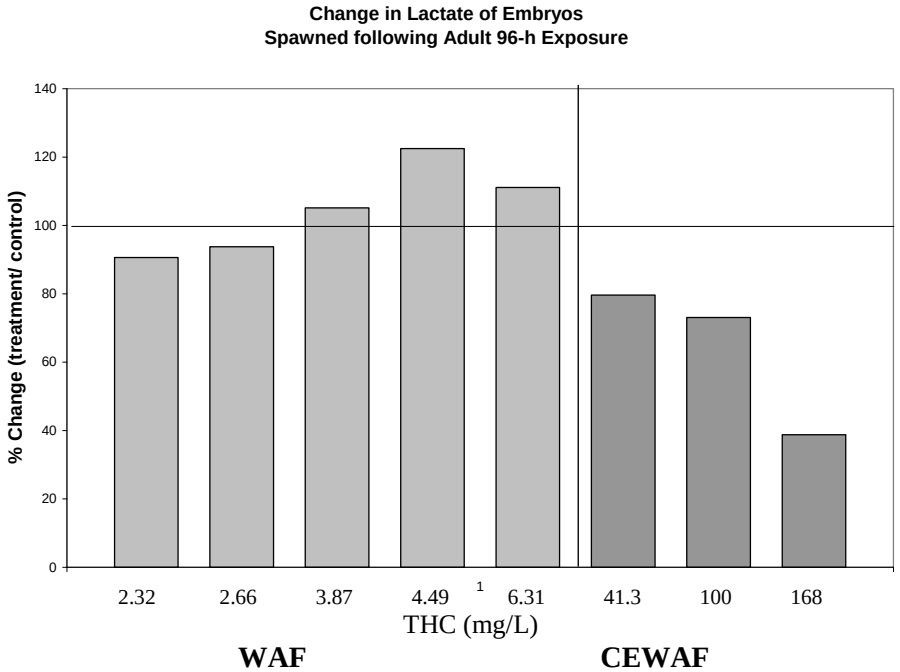
(f)


Change in Glutamine of Embryos
Spawned following Adult 96-h Exposure
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

(g)


Change in Alanine of Embryos
Spawned following Adult 96-h Exposure
% Change (treatment/ control)
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

(h)


Change in Lactate of Embryos
Spawned following Adult 96-h Exposure
% Change (treatment/ control)
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

(i)

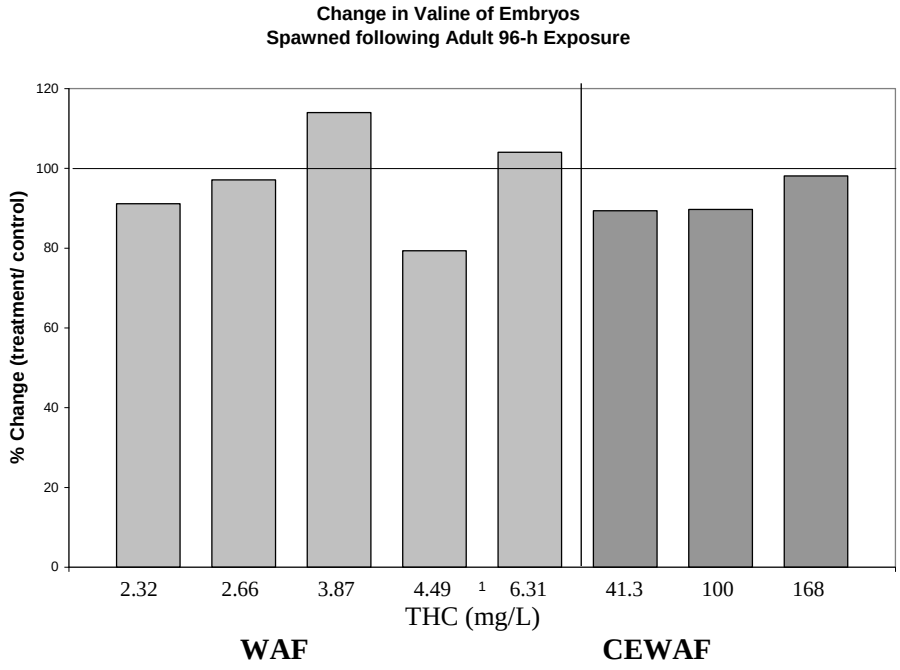


Figure 15. Changes in specific metabolites, from embryos spawned from adult topsmelt exposed to WAF and CEWAF for 96-h, include (a) glycerophosphocholine, (b) glycine, (c) taurine, (d) succinate, (e) glutamate, (f) glutamine, (g) alanine, (h) lactate, and (i) valine.

4.10 Long-Term Growth

Surviving topsmelt from the WAF and CEWAF tests were weighed upon test termination and placed in culture tanks for long-term growth. Data for Day 0, Day 40 and Day 78 growth measurements for the WAF test are presented in Figure 16.

Continual growth (as wet weight) was observed in the control and WAF-exposed fish over 78 days. Growth of was highly variable in control and exposed fish. This variability likely reflects variability in topsmelt size at the initiation of these experiments. Overall the growth data from the WAF test does not indicate any consistent residual effects of short-term WAF exposure on long-term growth of topsmelt.

Continual growth was also observed in the control and CEWAF-exposed fish (Figure 17), although the rate of growth in these fish was less than that observed in the WAF-exposed

fish. As discussed above, there were too few survivors of fish exposed to 270 mg/L THC to assess growth (= 1.0 g/L CEWAF loading). Fish exposed to THC concentrations ranging from 9.6 to 58.3 mg/L showed similar growth to the control in the first month of culture. Fish exposed to 26 mg/L THC (= 0.25 g/L loading) began showing signs of disease after the first month of culture (after the spawning assessment was completed). After consulting with Tresa Veek (California Department of Fish and Game pathologist) all fish (control fish + all CEWAF-exposed fish) were treated with a combination of antibiotic (oxytetracycline) and a low dose of formalin to treat for bacteria and the protozoan *Eurynema*. Antibiotic treatments were administered every day for three days (6 hours per day), and formalin was administered for 1 day (1 hour per day). The *Eurynema* progressed to a systemic infection in the 0.25 g/L tank, and all these fish died over the second month of growth. Infections were not observed in the other tanks and survival and growth was consistent in the remaining fish over the final three months of culture. There were no differences in growth between control fish and the CEWAF-exposed fish from the remaining 3 THC concentrations.

Variations in fish development can be linked to variations in biochemical effects (Heintz et al., 2000). Under stressful conditions, fish often experience a loss in body size and growth. However, growth can either be stopped or stunted due to a larger allocation of energy going to reproduction (Arukwe and Goksoyr, 1998). Since PAHs are known to activate the aryl hydrocarbon receptor (AhR) and subsequently induce CYP1A, hormone and signal production and possibly immune system functions may be affected, ultimately leading to changes in growth, health and behavior (Arukwe, 2001). Following 78 days after the exposure termination, fish from the CEWAF test had a decrease in ATP energy reserves, while fish from the WAF test had an increase in ATP; continual growth was observed for both tests despite the differences in energy reserves. Overall, the metabolic results do not specifically identify differences between control and exposed fish; therefore the growth and matabolomics data do not indicate effects from short-term WAF and CEWAF exposures on long-term growth in either test.

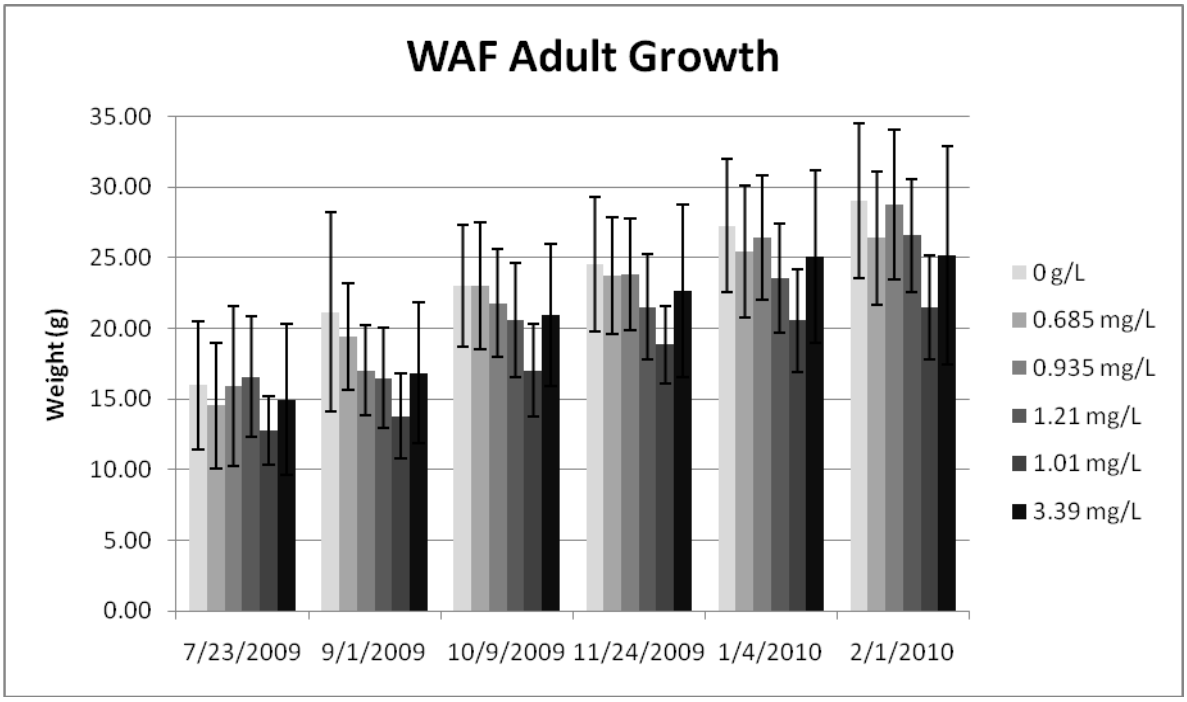


Figure 16. Growth of topsmelt for 78 days after 96 h exposure to WAF.

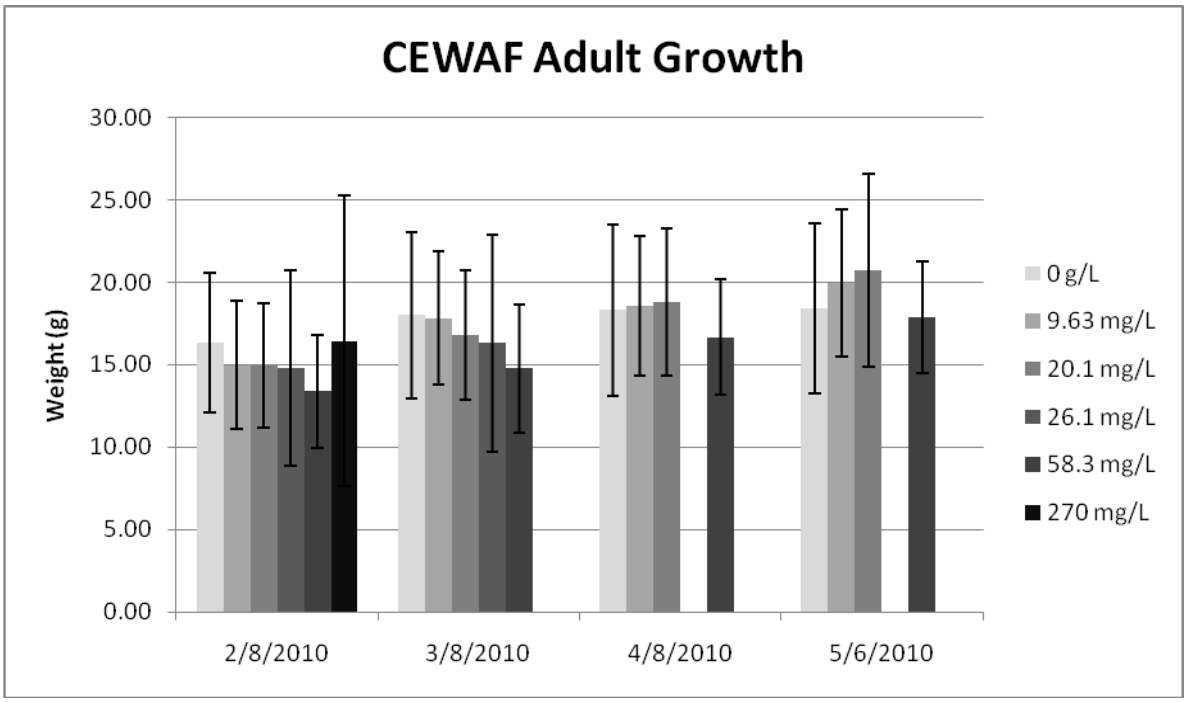


Figure 17. Growth of topsmelt 78 days after 96 h exposure to CEWAF.

4.11 Specific Metabolic Changes in Adult Muscle following 78 days of Growth

Well-resolved peaks were identified and integrated for comparison among each test. Metabolite changes were calculated, using peak areas (Table 14), by dividing the average peak area of the treatment by that of the corresponding control. A number below 100% represents a decrease in that metabolite within the treated fish. Concentration-dependent changes and results of one-way ANOVAs from identified metabolites are presented in Table 15. Metabolic changes comparing the muscle tissues between WAF and CEWAF are provided in Figure 18.

Specific changes for WAF include an increase in lactate and glycine, whereas CEWAF only had an increase of glycine. Phosphocreatine decreased at the lower concentrations of WAF and increased at the higher concentrations. At intermediate and high WAF concentrations glutamine, succinate and ATP decreased, but at the lowest concentration of CEWAF only ATP increased. Although a change in ATP should respectively result in a change in AMP, such change was not consistently observed in either WAF or CEWAF tests. It was seen that metabolic changes are dose and metabolite dependent resulting in the variability of metabolic changes observed.

Following 78 days of growth, there were no significant changes among metabolites for both the WAF and CEWAF tests.

Table 14. Peak areas from the ^{1}H NMR spectra of muscle from topsmelt following 78 days after exposure to (a) WAF and (b) CEWAF of PBCO for 96-h.

(a)

			WAF [a, b]			
Metabolites	**Control**	**0.685 mg/L**	**0.935 mg/L**	**1.21 mg/L**	**1.01 mg/L**	**3.39 mg/L**
Valine	4.24	3.58	4.20	3.97	3.71	3.58
Lactate	129	140	142	150	135	152
Alanine	39.0	27.1	29.3	27.2	33.6	31.3
Arginine/ Phosphoarginine	15.8	12.3	14.5	10.8	15.5	11.9
Glutamine	5.17	4.18	5.18	4.55	4.64	4.22
Succinate	1.64	1.51	1.83	1.60	1.65	1.50
Phosphocreatine	114	112	112	119	114	119
Taurine	20.5	20.1	15.9	11.9	17.0	15.3
Glycine	20.0	18.2	25.4	21.9	24.0	21.6
AMP	4.24	4.68	2.22	4.04	3.71	4.76
Histidine	14.1	14.4	17.6	15.0	13.7	12.6
ATP	1.06	0.81	1.49	0.99	1.10	0.68

(b)

		CEWAF [a, b]		
Metabolites	**Control**	**9.63 mg/L**	**20.1 mg/L**	**26.1 mg/L**
Valine	3.10	2.78	3.14	3.15
Lactate	160	161	157	166
Alanine	31.8	23.2	28.0	25.3
Arginine/ Phosphoarginine	7.46	10.2	8.17	7.17
Glutamine	3.86	3.51	3.92	3.58
Succinate	1.32	1.25	1.41	1.23
Phosphocreatine	109	107	108	106
Taurine	18.7	17.5	16.7	31.5
Glycine	27.4	37.7	36.3	28.8
AMP	5.59	5.31	5.46	5.36
Histidine	19.4	15.7	17.9	15.6
ATP	0.06	0.06	0.04	0.02

[a.] All spectra were normalized by total spectral area.
[b.] WAF and CEWAF are characterized by total hydrocarbon content (mg/L).

Table 15. Metabolic changes in the muscle of topsmelt following 78 days after 96-h exposure to (a) WAF and (b) CEWAF including one-way ANOVA p-values (significant $p<0.05$).

(a)

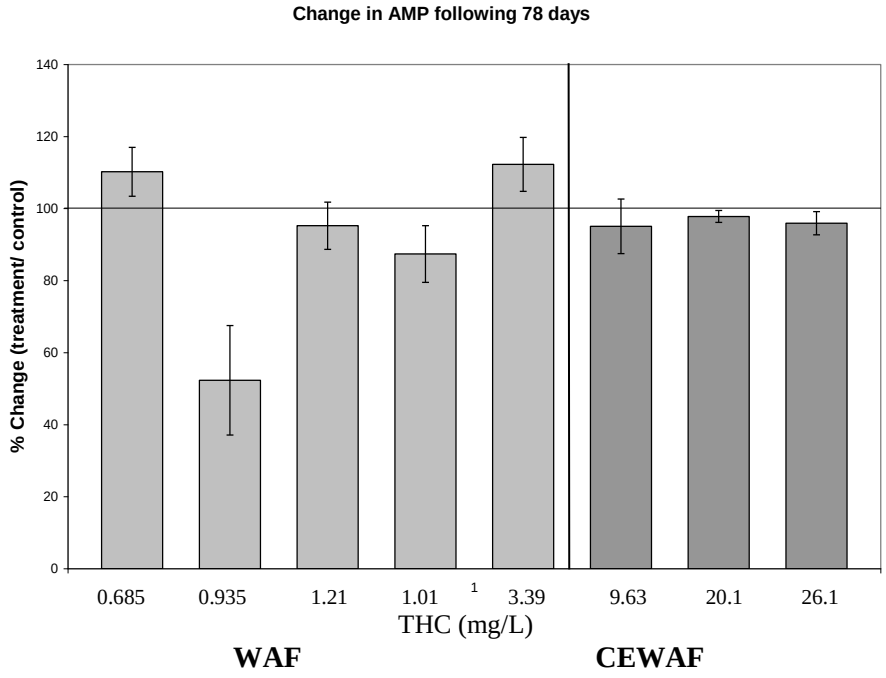
			WAF [a, b,,c]			
Metabolites	**0.685 mg/L**	**0.935 mg/L**	**1.21 mg/L**	**1.01 mg/L**	**3.39 mg/L**	**(p)**
Valine	84.4	99.2	93.8	87.5	84.5	0.924
Lactate	109	110	117	105	118	0.319
Alanine	69.4	75.0	69.7	86.1	80.2	0.330
Arginine/ Phosphoarginine	78.0	92.1	68.3	98.0	75.5	0.450
Glutamine	80.9	100	88.0	89.8	81.7	0.339
Succinate	92.2	112	97.7	101	91.5	0.138
Phosphocreatine	98.5	98.4	105	100	104	0.271
Taurine	97.9	77.5	58.1	82.7	74.4	0.121
Glycine	91.1	127	110	120	108	0.560
AMP	110	52.3	95.2	87.4	112	0.124
Histidine	102	125	106	96.7	89.4	0.054
ATP	76.4	140	92.8	104	64.0	0.612

(b)

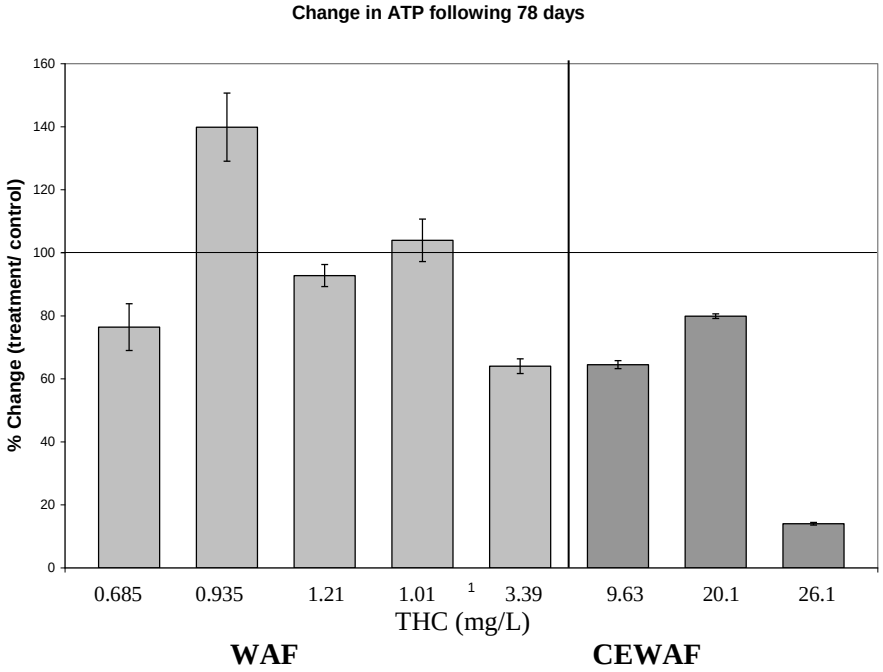
		CEWAF [a, b, c]		
Metabolites	**9.63 mg/L**	**20.1 mg/L**	**26.1 mg/L**	**(p)**
Valine	89.5	101	102	0.760
Lactate	101	98.4	104	0.973
Alanine	73.1	88.0	79.7	0.416
Arginine/ Phosphoarginine	137	109	96.1	0.239
Glutamine	90.9	102	92.8	0.847
Succinate	94.9	107	93.2	0.843
Phosphocreatine	98.2	98.7	97.0	0.953
Taurine	93.8	89.4	168	0.163
Glycine	137	133	105	0.369
AMP	95.1	97.8	95.9	0.785
Histidine	80.9	92.6	80.6	0.345
ATP	103	65.7	40.1	0.720

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

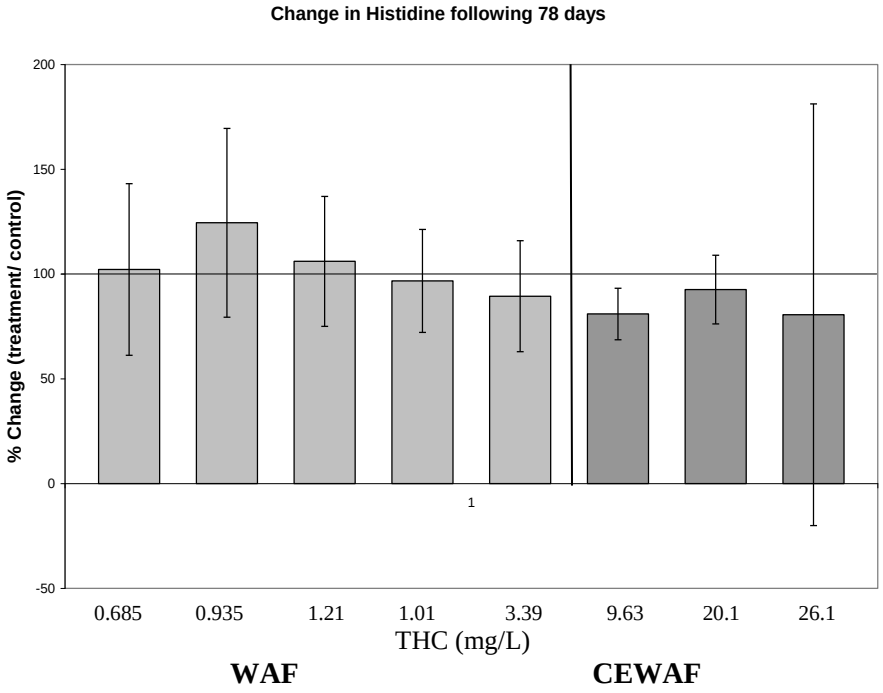
a)


Change in AMP following 78 days
% Change (treatment/ control)
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

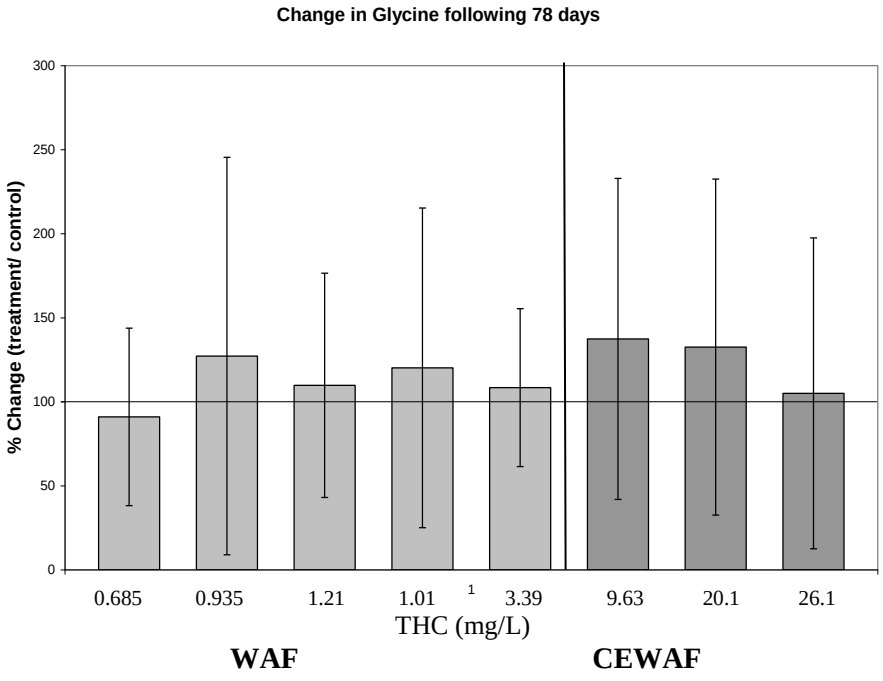
b)


Change in ATP following 78 days
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

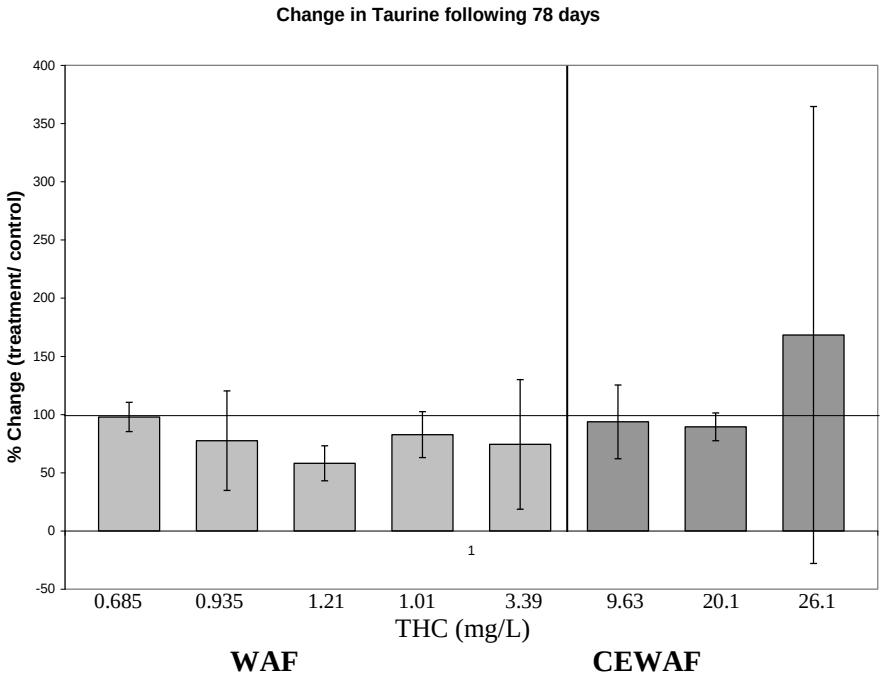
c)


Change in Histidine following 78 days
% Change (treatment/ control)
200
150
100
50
0
-50
1
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

d)


Change in Glycine following 78 days
% Change (treatment/ control)
300
250
200
150
100
50
0
0.685
0.935
1.21
1.01
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

e)


Change in Taurine following 78 days
% Change (treatment/ control)
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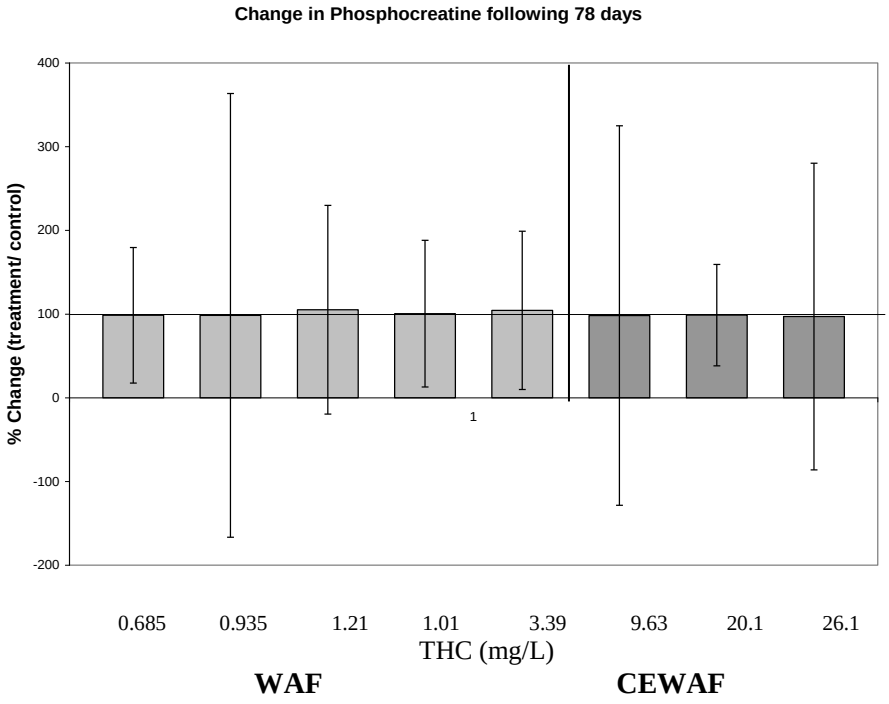
350
300
250
200
150
100
50
0
-50
1
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

f)


Change in Phosphocreatine following 78 days
% Change (treatment/ control)
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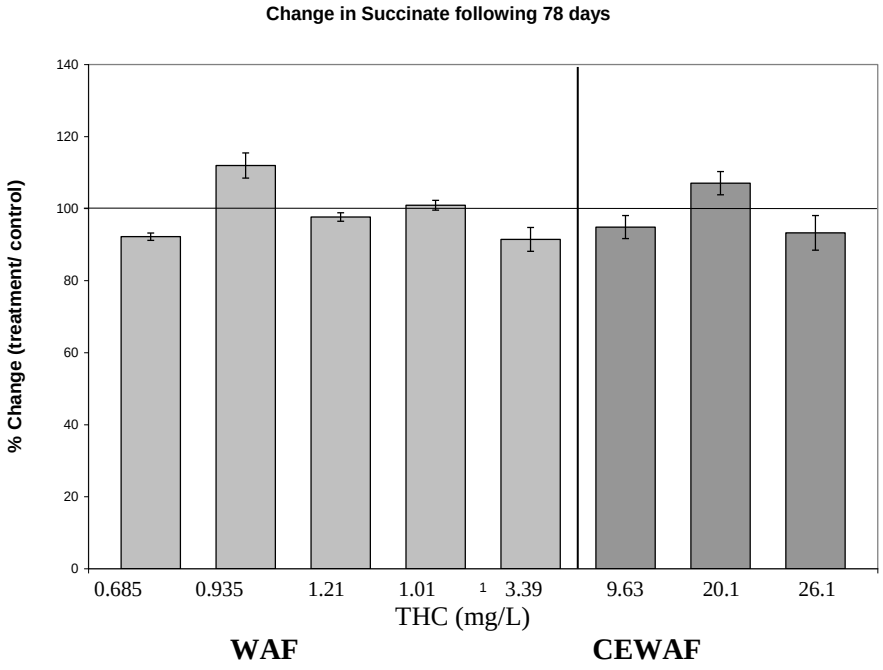
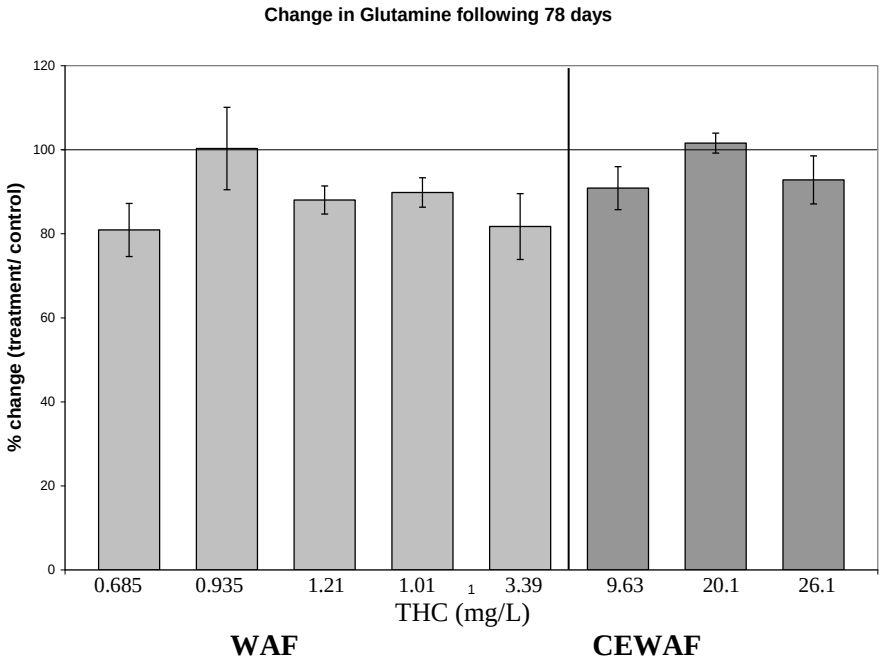
300
200
100
0
-100
-200
1
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

g)


Change in Succinate following 78 days
% Change (treatment/ control)
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

h)


Change in Glutamine following 78 days
% change (treatment/ control)
120
100
80
60
40
20
0
0.685
0.935
1.21
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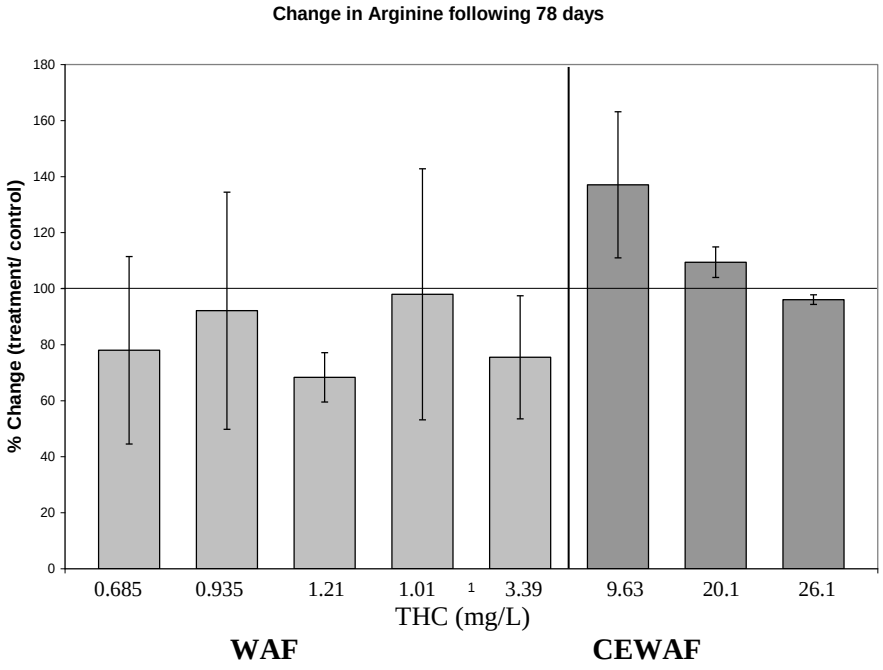
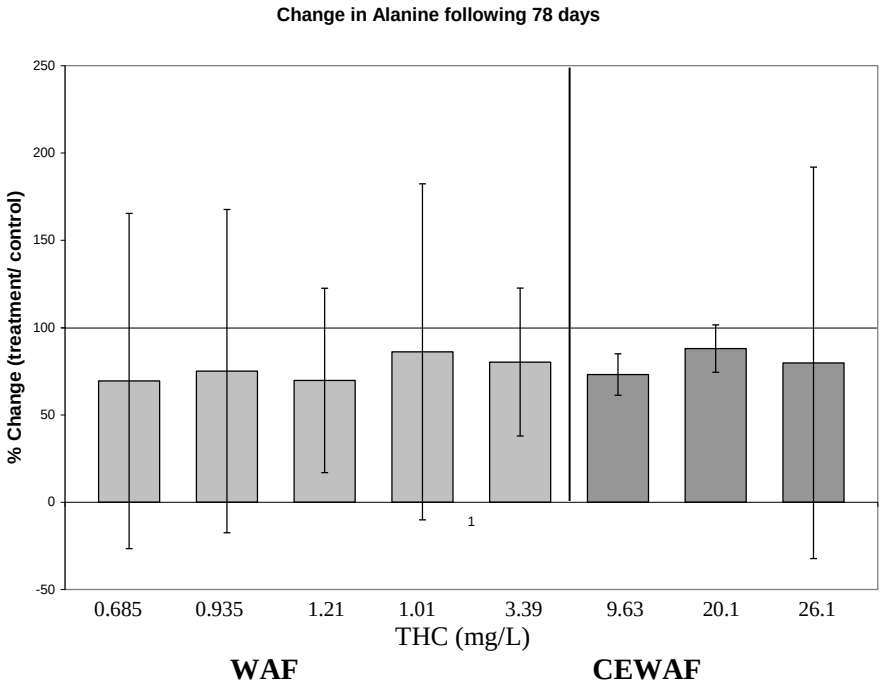
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

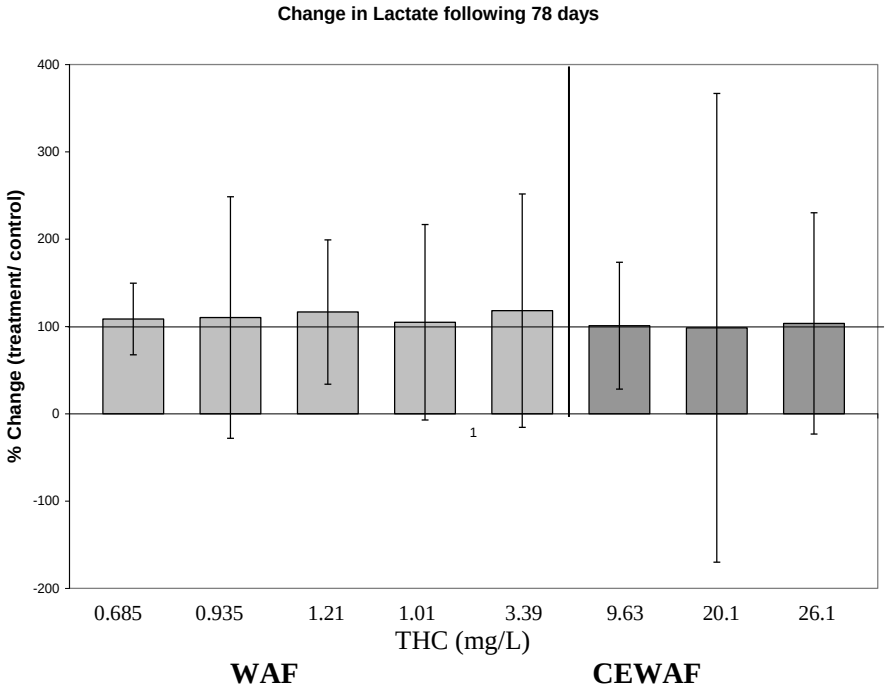
i)


Change in Arginine following 78 days
% Change (treatment/ control)
180
160
140
120
100
80
60
40
20
0
0.685
0.935
1.21
1.01
1
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

j)


Change in Alanine following 78 days
% Change (treatment/ control)
250
200
150
100
50
0
-50
1
0.685
0.935
1.21
1.01
3.39
9.63
20.1
26.1
THC (mg/L)
WAF
CEWAF

k)



l)

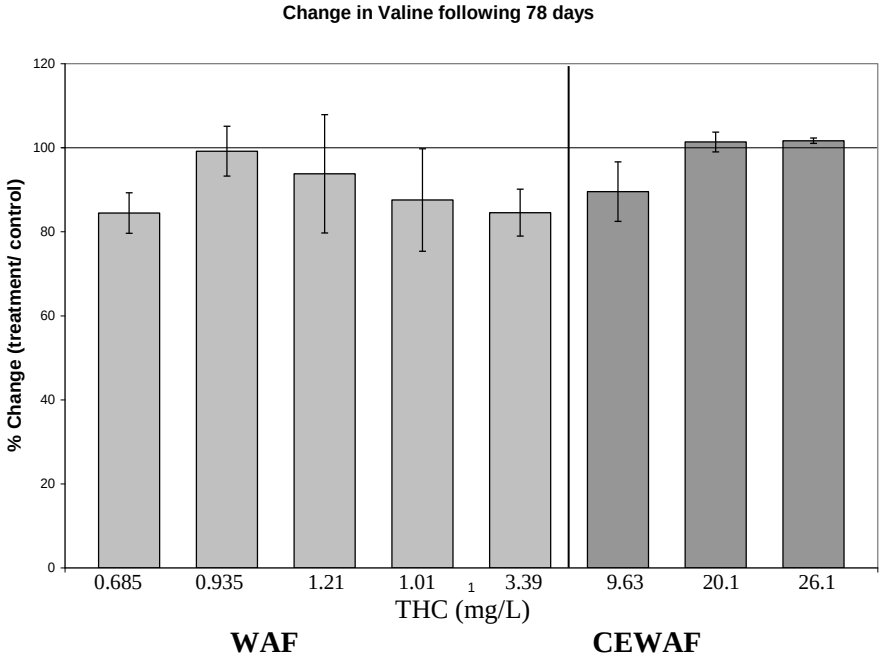


Figure 18. Changes in specific muscle tissue metabolites, 78 days after 96-h WAF and CEWAF exposure, include (a) AMP, (b) ATP, (c) histidine, (d) glycine, (e) taurine, (f) phosphocreatine, (g) succinate, (h) glutamine, (i) arginine/ phosphoarginine, (j) alanine, (k) lactate, and (l) valine.

4.12 Topsmelt Embryo Development after Direct Exposure to WAF and CEWAF

WAF Exposures

Topsmelt embryos were exposed to WAF created with weathered PBCO loadings as high as 25 g/L. In two range-finding tests there were no apparent effects of WAF on topsmelt embryonic development or hatching success. A definitive experiment was conducted with WAF concentrations from 0 to 25 g/L, and this test included analyses of THC and PAHs. In all experiments, embryos were observed microscopically on Day 5 of development when all had developed complete cardiovascular systems. The majority of embryos at all oil loading concentrations in these tests displayed normal development and no obvious evidence of hydrocarbon toxicity. Total hydrocarbon content was ~ 6.5 mg/L at the highest WAF loading concentration (25 g/L PBCO), and at this concentration 75% of the embryos hatched as normal larvae (Figure 19). Mean hatching success in the controls was 80% in the WAF tests. As was observed in the adult exposures to WAF, THC concentrations in the WAF embryo exposures were low relative to those in the CEWAF exposures (discussed below). In addition to relatively low THC, low concentrations of tricyclic PAHs were detected in the WAF solutions (discussed below).

WAF exposure had no effect on size of hatched larvae (Figure 20), but larvae from WAF-exposed embryos had smaller yolk sacs than control fish (Figure 21). Reduced larval yolk-sac area was also observed in CEWAF-exposed embryos (discussed below), and was the only apparent effect of WAF on topsmelt embryo-larval development. The physiological significance of reduce yolk sac size is unclear but may reflect subtle impacts of hydrocarbons on energy reserves in WAF-exposed embryos.

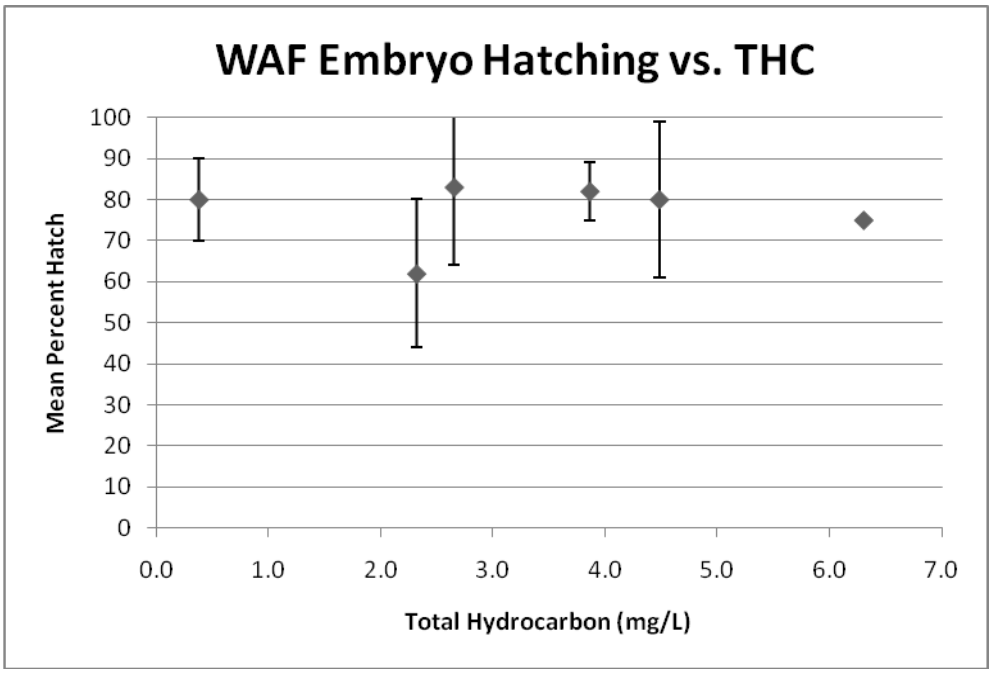


Figure 19. Topsmelt embryo development and hatching success in WAF of weathered PBCO.

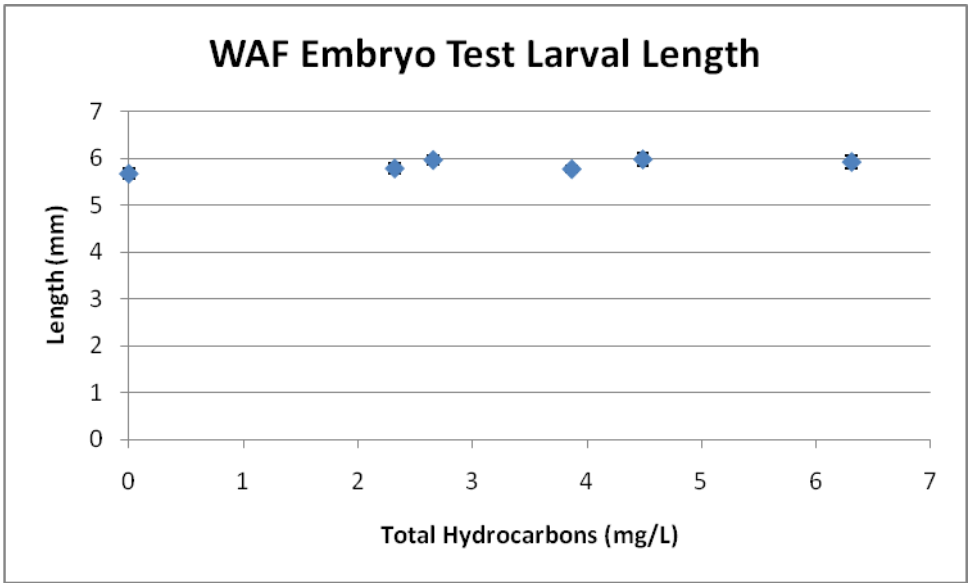


Figure 20. Length of topsmelt larvae hatched after embryonic development in WAF.

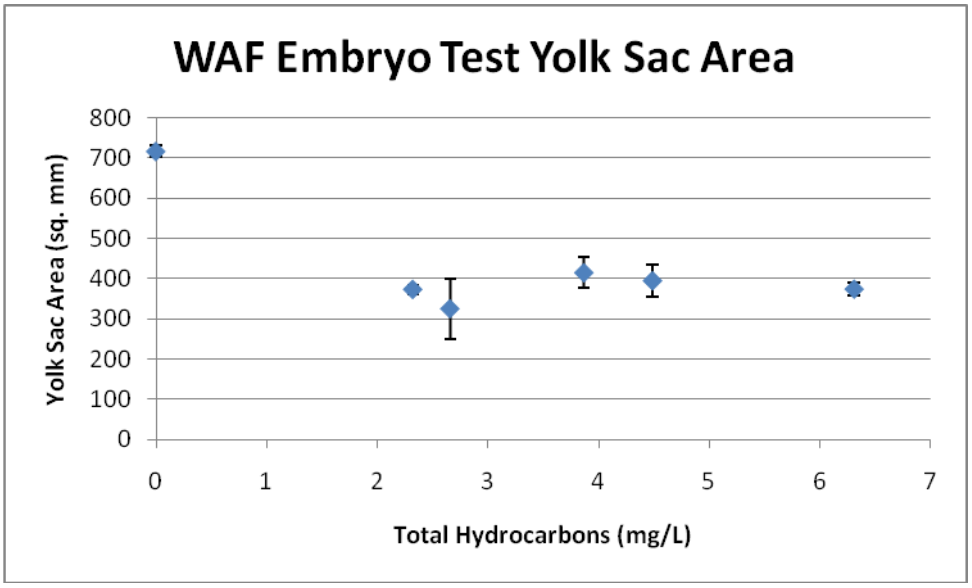


Figure 21. Yolk sac area (length x width) of larvae hatched after embryonic development in WAF.

Corexit 9500 Exposure

Topsmelt embryos were exposed directly to Corexit 9500 to assess toxicity of dispersant to embryonic development and larval hatching. The range of dispersant concentrations tested overlapped those used in the CEWAF exposure, and the methods duplicated those used in the WAF and CEWAF embryo tests. Twenty embryos per replicate were exposed in multi-well tissue culture plates submerged in polycarbonate plastic food trays containing dispersant in seawater. Each loading was replicated 3 times. After tissue culture plates and embryos were submerged, the trays were flushed for 24 hours (2 mL per minute) using the same declining exposure described for the WAF and CEWAF tests. At 24 hours, the embryos were transferred to scintillation vials to assess development and hatching.

There were no apparent effects of Corexit 9500 on topsmelt embryonic development at concentrations up to 1,000 mg/L (Table 16). Embryo hatching success declined to 63% at the highest dispersant concentration tested, but this was not significantly different from

hatching in the control (control = 88%). Lower hatching success and higher between replicate variability at the highest concentration was due to poor hatching success in one replicate.

Table 16. Topsmelt embryonic development and hatching success in Corexit 9500 dispersant-only test.

Corexit Concentration (mg/L)	PBCO Loading Equivalent (g/L)	Percent Normal Hatch	SD
0	0	88.0	2.9
25	0.25	95.0	0.0
50	0.50	97.0	5.8
100	1.00	93.0	7.6
500	5.00	87.0	11.5
1,000	10.0	63.0	25.7

CEWAF Exposures

In contrast to the apparent lack of WAF effects on topsmelt embryonic development, CEWAF affected development and hatching at the lowest concentration tested. Two range-finding tests were conducted with CEWAF prior to the definitive test. The range of oil loadings in the first test were 0, 0.25. 0.5, 1.0, 5.0 and 10.0 g/L, and the oil loadings in the second test were 0, 1.25, 2.5, 5.0, 10.0, and 20.0 g/L. Embryo-larval development and hatching success was highly variable at the highest loadings, and declined to 55% and 32%, respectively, at the 5.0 and 10.0 g/L loadings in the first test. Development and hatching declined significantly at all concentrations above the control in the second test (data not shown). Because of high variability in these tests, a relatively wide range of CEWAF concentrations was used in the definitive test.

The CEWAF LC50 based on oil loading concentrations for embryo-larval development and hatching success in the definitive test was less than the lowest loading concentration (0.5

g/L). Based on THC, the LC50 for larval hatching success in CEWAF was 17 mg/L (Figure 22). Embryos in the CEWAF test were observed microscopically on Day 5, when all embryos had fully developed cardiovascular systems. These fish were scored as normal or abnormal based on a suite of developmental endpoints described above. These included various degrees of craniofacial, cardiovascular or skeletal abnormalities. Based on these observations, 95%, 82%, 90%, 50%, 0% and 0% of embryos, respectively, were normal at 0, 0.5, 1.0, 2.5, 7.5, and 15.0 g/L oil on Day 5. Live embryos for metabolomic analyses were preserved at this time. The majority of abnormalities noted on Day 5 consisted of cardiovascular abnormalities, including hemostasis (lack of blood circulation), and tube heart formation at the lower loading concentrations. At higher oil loadings, embryos exhibited greater degrees of tube heart formation or a complete lack of cardiovascular development. Almost all of the embryos were dead by day 5 at the two highest loadings (7.5 and 15 g/L oil).

After 12 days of exposure to CEWAF, 75% of the control embryos had hatched as normal larvae. At the 0.5 g/L CEWAF loading concentration, 18% of the embryos hatched as normal larvae, and 55% were dead. The remaining fish hatched as abnormal larvae. Larval abnormalities consisted of pericardial edemas, and cardiovascular abnormalities ranging from hemostasis to tube heart formation. At loading concentrations above 0.5 g/L, the majority of fish that were alive at Day 5 were dead by Day 12.

In addition to cardiovascular abnormalities, CEWAF exposure reduced larval growth. Larvae hatched from embryos developing in the 0.5 g/L CEWAF loading concentration (=41.3 mg/L THC) were significantly smaller than control fish ($p < 0.05$; Figure 23). There was no apparent dose-response relationship between larval length and THC in this test; all larvae hatched from embryos exposed to CEWAF were smaller than control larvae. Larvae from CEWAF-exposed embryos also had smaller yolk sacs than control fish (Figure 24).

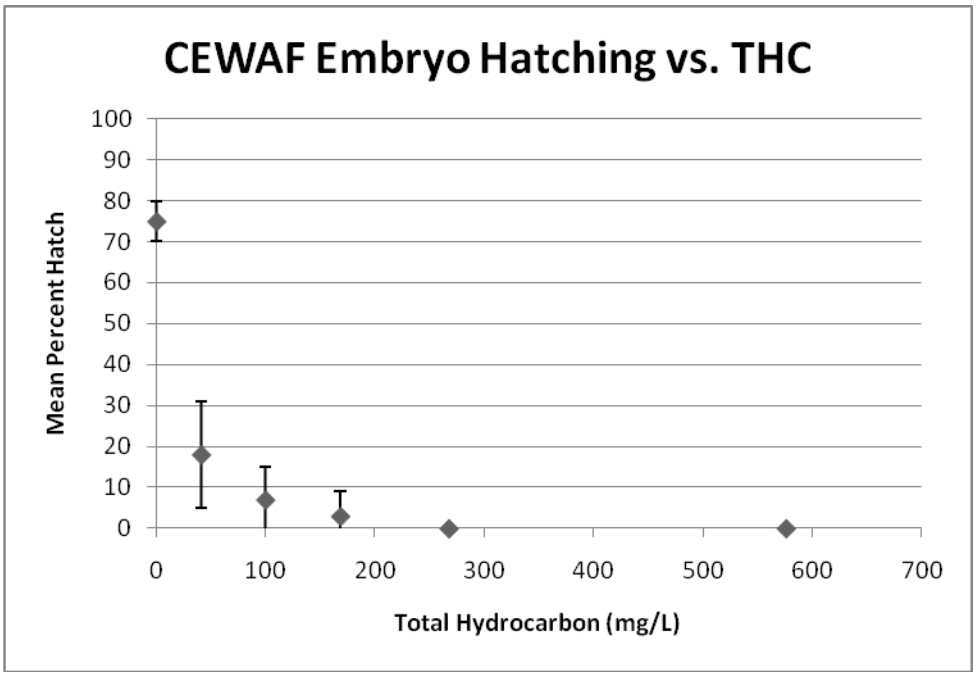


Figure 22. Topsmelt embryo development and hatching success in CEWAF of weathered PBCO.

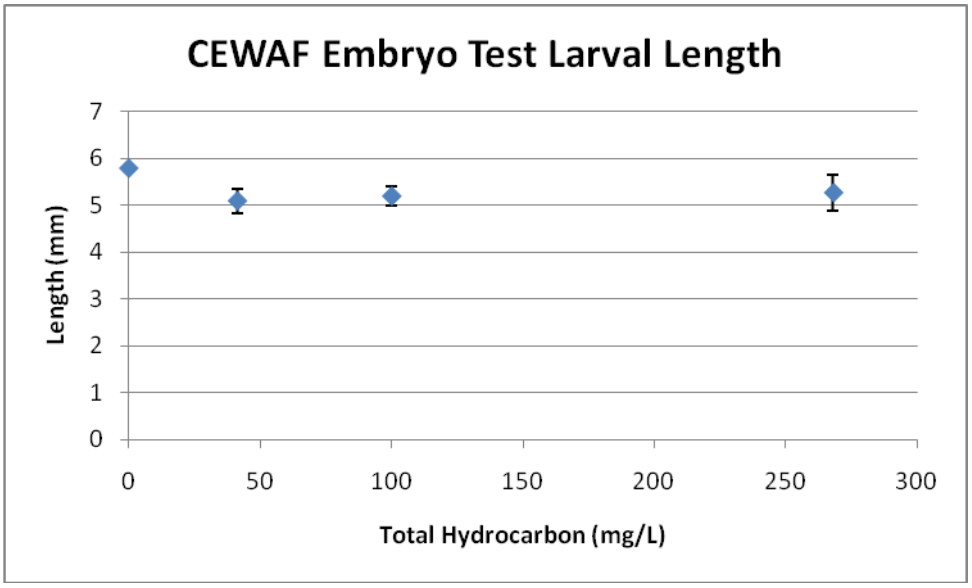


Figure 23. Length of topsmelt larvae hatched after embryonic development in CEWAF.

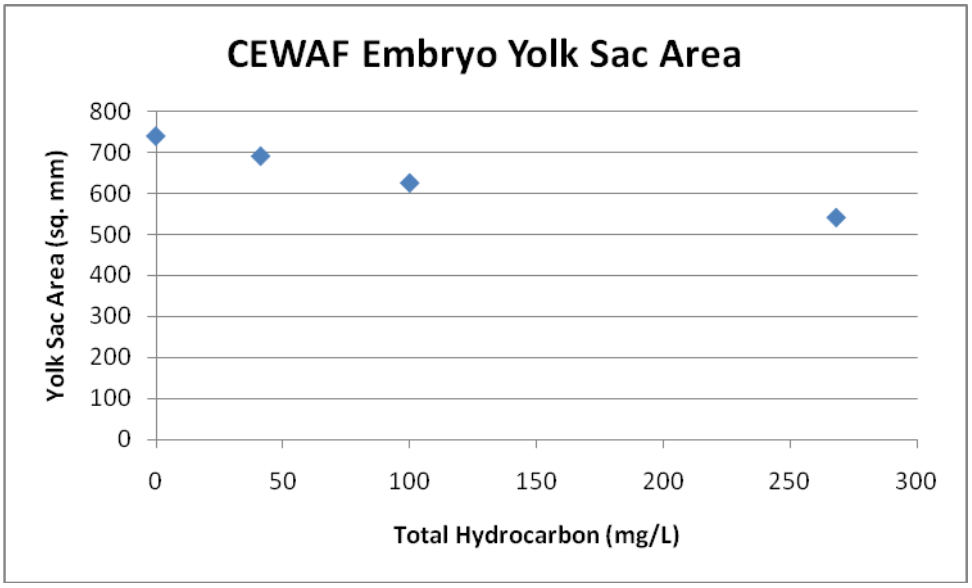


Figure 24. Yolk sac area (length x width) of larvae hatched from embryos exposed to CEWAF.

WAF vs. CEWAF THC chemistry

As was observed in the adult exposures, treating weathered PBCO with dispersant resulted in much higher concentrations of hydrocarbons in seawater relative to physical mixing of oil to create WAF. Total hydrocarbon content ranged from 2.3 to 6.3 mg/L at WAF loadings from 1.56 to 25 g/L. THC ranged from 41.3 to 576 mg/L at CEWAF loadings from 0.5 to 15 g/L. At comparable PBCO loadings (1.0 vs. 1.56 g/L oil in CEWAF and WAF, respectively), THC was 100 mg/L in CEWAF and 2.3 mg/L in the WAF.

Topsmelt embryos were also exposed to higher concentrations of PAHs in CEWAF relative to WAF. Despite the higher oil loadings in the WAF exposures, PAH concentrations other than naphthalene and biphenyl were at or below 2 µg/L in all treatments (Figure 25). High concentrations of naphthalene and biphenyl were measured in the CEWAF test, and there were relatively high concentrations of three ring PAHs in these solutions. Phenanthrene concentrations in CEWAF ranged from 11.1 to 96.8 µg/L in oil loadings from 0.5 to 15 g/L, respectively. Dibenzothiophene concentrations ranged from 8.9 to 72.7 µg/L (Figure

26). Concentrations of four ring PAHs were generally less than 1 µg/L, except at the higher oil loadings (Figures 26 and 27). Higher concentrations of tricyclic PAHs in the CEWAF solutions likely contributed to cardiovascular abnormalities observed in the developing topsmelt embryos. Incardona et al. (2005) found that the tricyclic PAH components of weathered Alaskan North Slope (ANS) crude oil comprised the major toxic fraction to developing zebra fish embryos. Their data suggested phenanthrene and dibenzothiophene were likely particularly important toxic constituents in weathered oil. As was observed in the current study, these authors reported that earliest and most pronounced effect of weathered ANS was impaired cardiac function during cardiac morphogenesis.

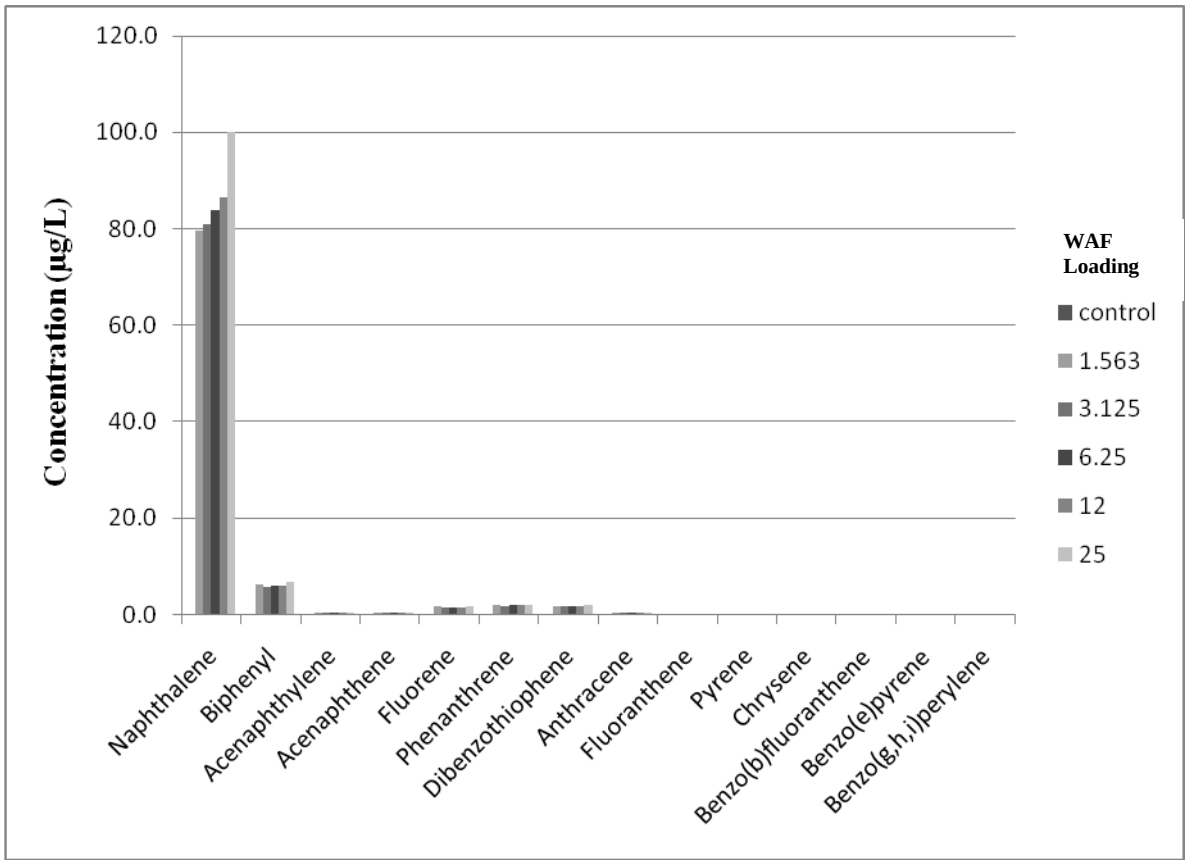


Figure 25. Concentrations of PAH parent compounds (µg/L) in WAF definitive test using topsmelt embryos

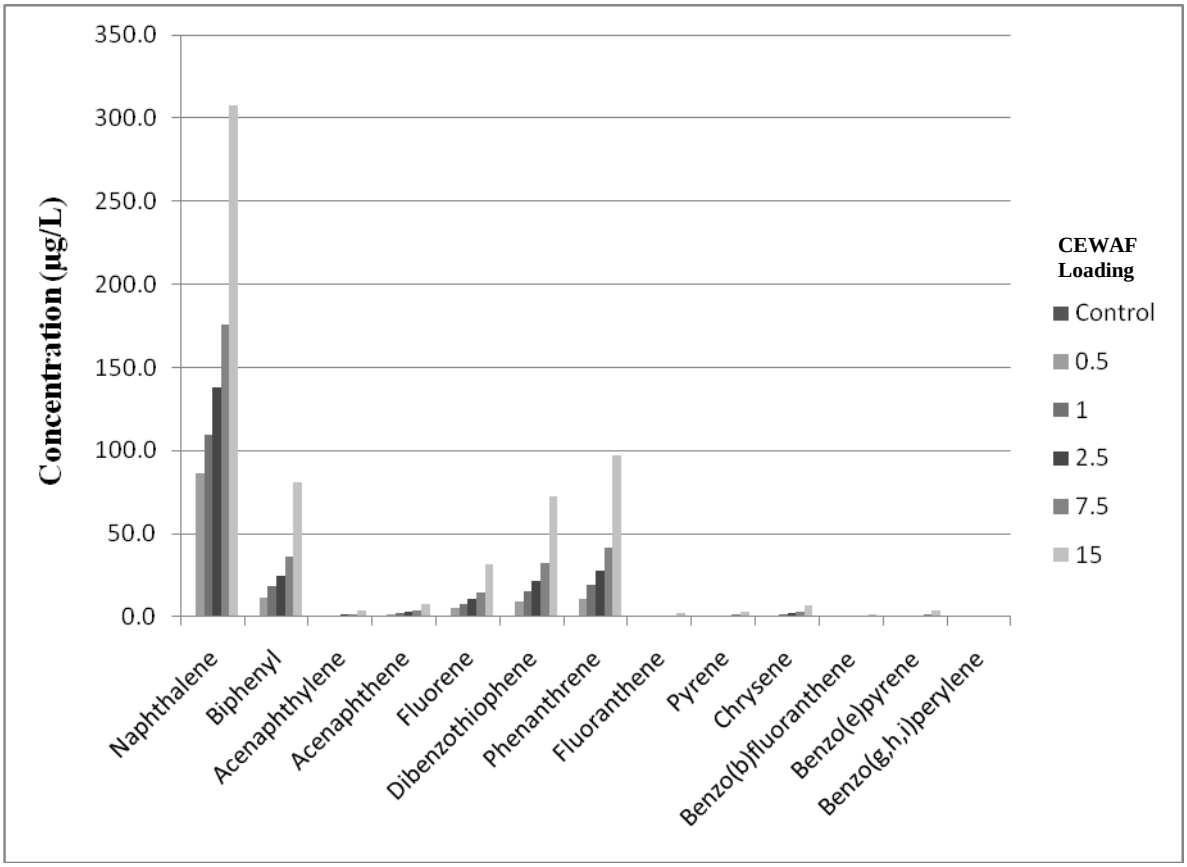


Figure 26. Concentrations of PAH parent compounds (mg/L) in CEWAF definitive test using topsmelt embryos.

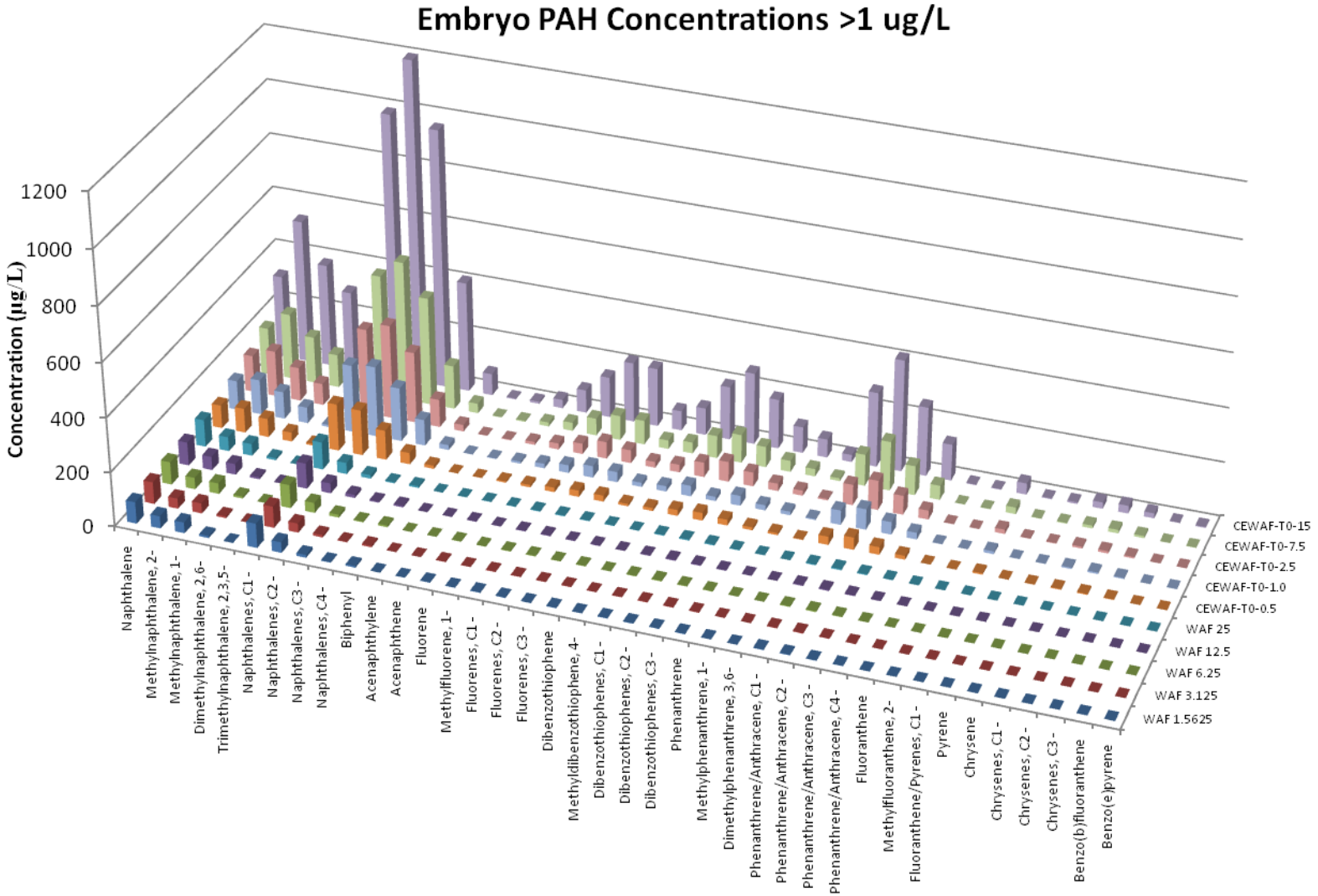


Figure 27. Relative concentrations of all PAHs detected at > 1 µg/L in WAF vs. CEWAF (Time 0 concentrations in µg/L).

These results show that CEWAF of weathered oil is more toxic to topsmelt embryonic development than it is to adult fish survival. The CEWAF 96h LC50 based on adult survival was 56.4 mg/L and the CEWAF LC50 based on embryo larval development was 17 mg/L. CEWAF toxicity to topsmelt embryos was under-estimated in this experiment because significant toxicity was observed at the lowest concentration tested. In addition, the majority of embryos used in these exposures had developed to stage 16 (after Lagler et al. 1962). This occurred because the embryos were shipped via overnight courier from Aquatic Bio Systems in Fort Collins Co, and though refrigerated, continued to develop in transit. Some of the embryos used in these experiments were less well developed (~ Stage 14) at the initiation of the exposures and evidence suggests that these embryos were more sensitive to CEWAF (data not shown). It is likely that exposures initiated with early-blastula stage embryos would be more sensitive since these would incorporate hydrocarbon effects on all aspects of development (McIntosh et al. 2010).

These results corroborate previous investigations of relative toxicity of dispersed and un-dispersed PBCO to topsmelt embryonic development. Using static exposures with un-weathered PBCO, Anderson et al. (2009) found that topsmelt embryo/larval development was affected at a THC concentration of 177 mg/L in CEWAF (loading = 0.25 g/L). Embryo/larval development was not affected at a THC of 47.7 mg/L CEWAF (loading = 0.125 g/L). Using un-weathered PBCO, Anderson et al. (2009) found topsmelt embryo development was unaffected by WAF solutions up to 12 g/L loading. Results from the current experiments suggest that chemical dispersal of weathered oil results in CEWAF that is more toxic to embryo development than CEWAF from un-weathered oil. For example, in the current experiments embryo/larval development was inhibited at a THC concentration of 41.3 mg/L (Note: This is the THC at Time 0, when the exposures were initiated. THC after 24 hours of flushing was 16% of the initial concentration). Thus, embryos in the 2009 experiments were continuously exposed to higher THC concentrations at any given oil loading. The difference in toxic response was likely due to weathering of PBCO prior to chemical dispersion. Incardona at el. (2005) showed that weathered Alaskan North Slope oil contained higher proportions of tricyclic PAHs, and these were the toxic components that affected zebra fish development. Although PAH concentrations

were not measured in the CEWAFs created from un-weathered PBCO in our previous experiments (Anderson et al. 2009), our current results demonstrate that CEWAF from weathered PBCO contain elevated concentrations of tricyclic PAHs.

While the cardiovascular abnormalities and inhibited larval growth observed in topsmelt embryos exposed to CEWAF of weathered PBCO were similar to those reported previously (Anderson et al. 2009), the lack of more severe manifestations of "blue sac disease" (i.e., yolk sac edema) was surprising. A number of previous studies have shown that blue sac disease is a common developmental response when fish embryos are exposed to hydrocarbons (Barron et al., 2004; Incardona et al. 2004 and 2005; McIntosh et al. 2010). While topsmelt larvae hatched from embryos that developed in CEWAF demonstrated pericardial edemas, the yolk sacs of these larvae were actually smaller than control fish (Figure 22). Yolk sac edema has been shown to be correlated with cytochrome P-4501A (CYP 1A) induction in embryos exposed to PAHs (Brinkworth et al. 2003). It is possible the lack of yolk sac edemas in the current experiments was related to the exposure of slightly more developed embryos (i.e., Stage 16). Thus, the initial stages of organogenesis may have progressed to the point where PAH exposure did not cause the fluid hemorrhaging associated with yolk-sac edema. Because the topsmelt embryo exposures incorporated cardiac development, the pericardial edemas associated with cardiac hemorrhaging were observed.

Embryos directly exposed to either WAF or CEWAF developed differently and had different resultant metabolic responses. Day 5 embryos exposed to WAF resulted in significant decreases in key metabolites (i.e. amino acids and osmolytes) and increase in energy (phosphocreatine), whereas the CEWAF-exposed embryos had elevated metabolites (i.e. amino acids) and a decrease in energy (phosphocreatine). The changes in metabolites alone show that the embryos from the two tests were experiencing different effects. During embryonic development, amino acids are often utilized as fuel, this correlates with the depletion of amino acids observed in the WAF exposed embryos, and thus these embryos were capable of normally developing. It is possible that the embryos exposed to CEWAF were not metabolically developed and therefore lacked normal developmental capabilities

which resulted in delayed hatching. Deb et al. (2000) note egg development, maturation of young fish and deformed young can result from PAH exposure. Although not many metabolites were identified, there still may have been further biochemical and physiological effects aiding in the formation of developmental abnormalities and ultimately decreasing hatchability.

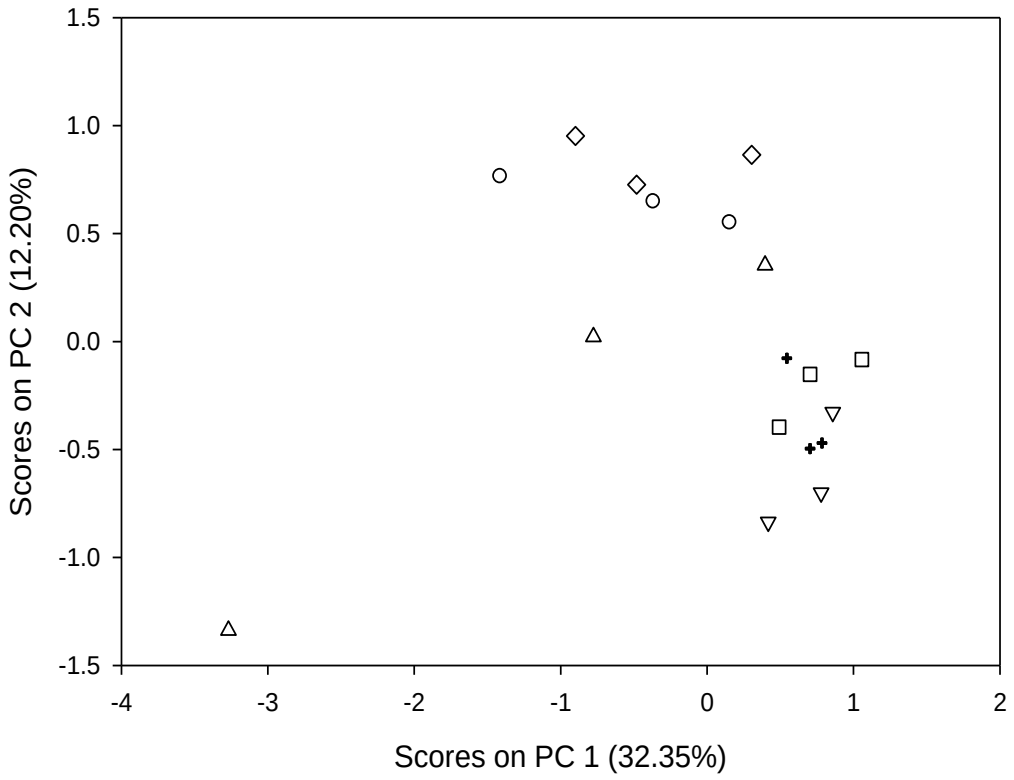
4.13 Dose-Response of Metabolic Profiles in Embryos Directly Exposed to WAF and CEWAF

The ^{1}H NMR spectra were analyzed by PCA; providing a graphical output, comprising of individual samples, summarizing the similarity between the NMR spectra. Embryos from the control tanks and from each of the doses were analyzed individually to avoid variation from experimental conditions and possible physiological conditions. The WAF and CEWAF PCA score plots, represents the resultant metabolic dose-response for both tests.

The WAF PCA scores plot shows minimal clustering between the control and dosed embryos, along the PC2 axis (containing 12.20% of variance; Figure 28a). Due to the minimal overlap among the metabolomes, there is a dose –response relationship between the individual WAF concentrations; therefore WAF doses are eliciting variations in metabolic responses. Due to lack of replicates, the CEWAF PCA scores plot lacks clustering between the individual samples along PC1 and PC2 (accounting for 85.58% and 9.98% of variance, Figure 28b); a metabolic dose-response relationship is also apparent.

For embryos exposed to WAF and CEWAF, a metabolic dose-response relationship is displayed between concentrations. Furthermore the WAF PCA scores plot indicates that the variation in metabolomes may be induced by similar concentrations of hydrocarbons within the water and not the dose concentrations themselves. Individual doses and controls are expected to result in similar metabolomes with minimal variation; however the control embryos for the WAF test do overlap with dosed embryos.

(a)


1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-4
-3
-2
-1
0
1
2
Scores on PC 2 (12.20%)
Scores on PC 1 (32.35%)

(b)

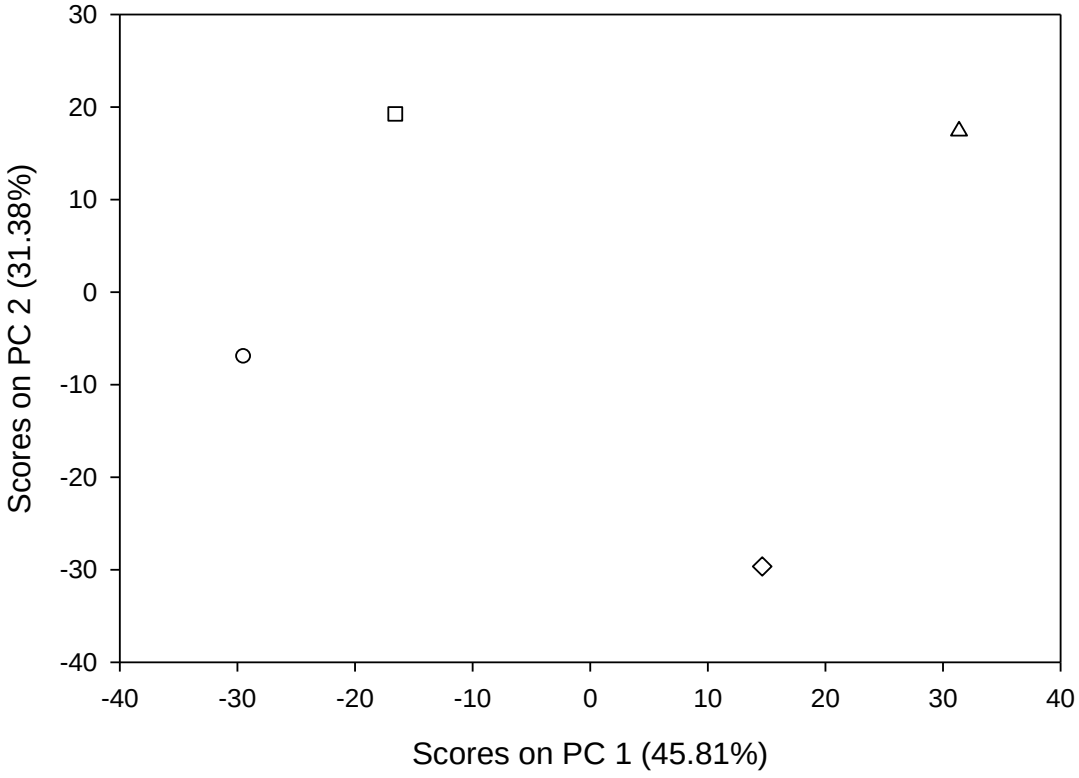


Figure 28. PCA scores plot of the ^{1}H NMR spectra of embryo extracts from embryos directly exposed to the (a) WAF and (b) CEWAF of PBCO. All analyses include an unexposed control. Controls and the five concentrations of WAF are characterized as control (◊), 2.32 (○), 2.66 (△), 3.87 (□), 4.49 (+) and 6.31 (▽) mg/L THC. Controls and the three concentrations of CEWAF are characterized as control (◊), 41.3 (○), 100 (△) and 168 (□) mg/L THC. The symbol shape represents a single dose and each data point represents one biological tissue sample.

4.14 Specific Metabolic Changes in Embryos Directly Exposed to WAF and CEWAF

Well-resolved peaks were identified and integrated for comparison among each test. Metabolite changes were calculated, using peak areas (Table 17), by dividing the average peak area of the treatment by that of the corresponding control. A number below 100% represents a decrease in that metabolite within the treated embryos. Concentration-

dependent changes from identified metabolites are presented in Table 18; due to lack of replicates one-way ANOVAs could not be determined for the CEWAF embryos. Metabolic changes comparing the exposed embryos between WAF and CEWAF are provided in Figure 29.

Specific changes for WAF include a decrease in lactate, alanine, glutamate, glutamine, and glycerophosphocholine whereas, glycerophosphocholine and glutamate decreased for CEWAF. Phosphocreatine and taurine decreased at the lower concentrations of WAF but increased at the intermediate and higher concentrations; however phosphocreatine decreased following the intermediate and higher CEWAF concentrations. At low WAF concentrations metabolite changes were decreasing, but at high WAF concentrations metabolic changes were increasing. Majority of the identified metabolites for CEWAF either steadily increased or decreased. The variability among the metabolic changes indicates that the changes are dose and metabolite dependent.

Following the 96-h embryo exposures, there was a significant change ($p<0.05$) in majority of the metabolites (valine, lactate, alanine, glutamine, glutamate, succinate and taurine) for WAF. Unfortunately significant changes of metabolites following CEWAF exposure could not be determined.

Table 17. Peak areas from the ^{1}H NMR spectra of embryos directly exposed to (a) WAF and (b) CEWAF for 96-h.

(a)

			WAF [a, b]			
Metabolites	**Control**	**2.32 mg/L**	**2.66 mg/L**	**3.87 mg/L**	**4.49 mg/L**	**6.31 mg/L**
Valine	8.83	9.47	8.05	9.07	8.83	8.12
Lactate	76.1	36.2	37.6	37.1	33.7	34.1
Alanine	16.0	15.0	14.0	16.2	15.4	15.3
Glutamine	11.9	12.7	10.5	11.9	11.8	8.87
Glutamate	13.0	12.8	12.0	10.8	11.1	16.8
Succinate	3.18	3.62	3.34	3.69	3.39	3.50
Phosphocreatine	18.9	17.0	15.6	19.5	19.3	19.6
Taurine	23.8	22.6	18.7	24.9	25.1	24.0
Glycine	21.7	22.0	23.7	21.8	21.9	23.1
Glycerophosphocholine	2.85	2.22	2.08	2.48	2.49	2.29

(b)

		CEWAF [a, b]		
Metabolites	**Control**	**41.3 mg/L**	**100 mg/L**	**168 mg/L**
Valine	10.4	11.8	11.4	11.6
Lactate	32.2	32.6	35.9	37.7
Alanine	13.8	15.0	14.7	15.5
Glutamine	15.1	15.6	16.7	18.8
Glutamate	11.3	11.1	10.8	11.4
Succinate	4.66	4.49	5.34	5.60
Phosphocreatine	17.9	18.1	17.6	17.7
Taurine	27.5	29.1	26.4	27.7
Glycine	20.3	20.9	22.6	21.4
Glycerophosphocholine	2.62	2.49	2.45	2.45

[a.] All spectra were normalized by total spectral area.
[b.] WAF and CEWAF are characterized by total hydrocarbon content (mg/L).

Table 18. Metabolic changes in of embryos directly exposed to (a) WAF and (b) CEWAF for 96-h. including one-way ANOVA p-values (significant $p<0.05$).

(a)

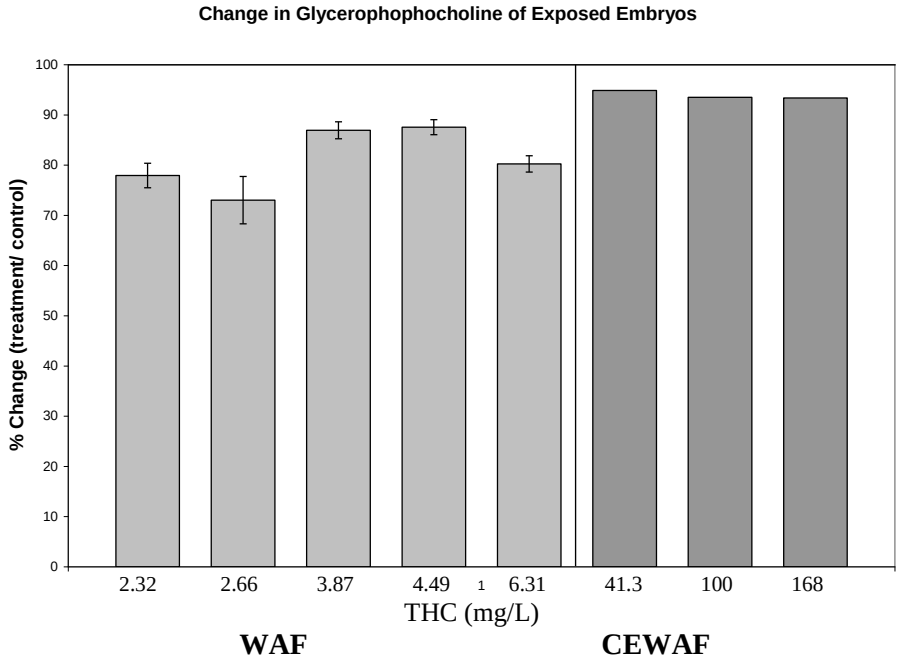
			WAF [a, b, c]			
Metabolites	**2.32 mg/L**	**2.66 mg/L**	**3.87 mg/L**	**4.49 mg/L**	**6.31 mg/L**	**(p)**
Valine	107	91.2	103	100	91.9	*0.018
Lactate	47.6	49.4	48.7	44.3	44.8	*<.0001
Alanine	93.6	87.9	101	96.5	95.8	*0.008
Glutamine	107	87.9	99.9	99.0	74.5	*<.0001
Glutamate	97.9	92.2	83.1	85.2	129	*<.0001
Succinate	114	105	116	107	110	*0.032
Phosphocreatine	89.8	82.7	103	102	104	0.358
Taurine	95.0	78.6	105	106	101	*0.035
Glycine	101	109	100	101	107	0.272
Glycerophosphocholine	77.9	73.0	86.9	87.5	80.2	0.387

(b)

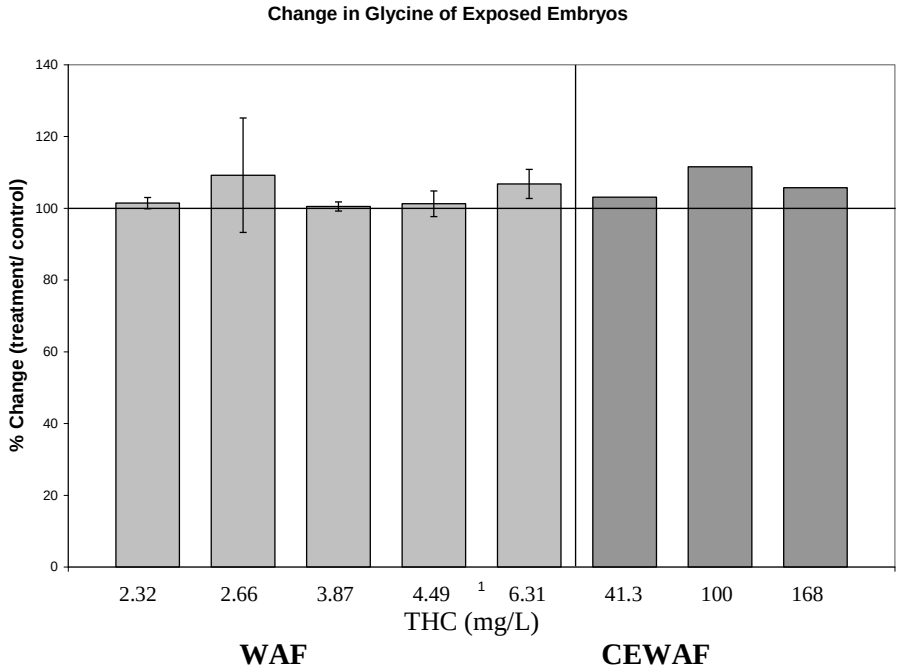
		CEWAF [a, b]	
Metabolites	**41.3 mg/L**	**100 mg/L**	**168 mg/L**
Valine	113	109	111
Lactate	101	112	117
Alanine	108	106	112
Glutamine	103	111	124
Glutamate	98.6	96.0	101
Succinate	96.4	115	120
Phosphocreatine	101	98.5	98.9
Taurine	106	96.2	101
Glycine	103	112	106
Glycerophosphocholine	94.9	93.5	93.4

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

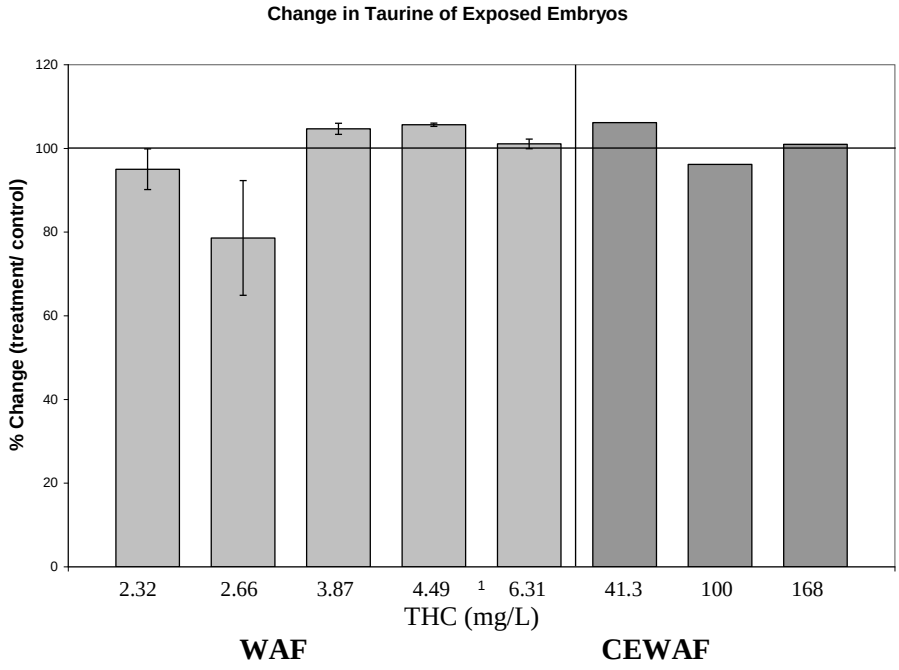
(a)


Change in Glycerophophocholine of Exposed Embryos
% Change (treatment/ control)
100
90
80
70
60
50
40
30
20
10
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

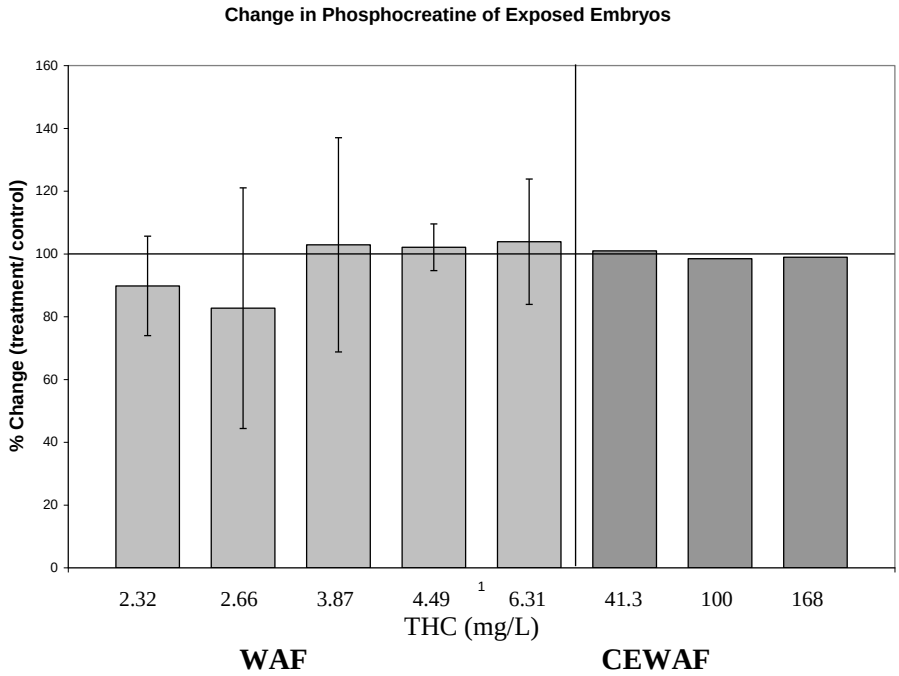
(b)


Change in Glycine of Exposed Embryos
% Change (treatment/ control)
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

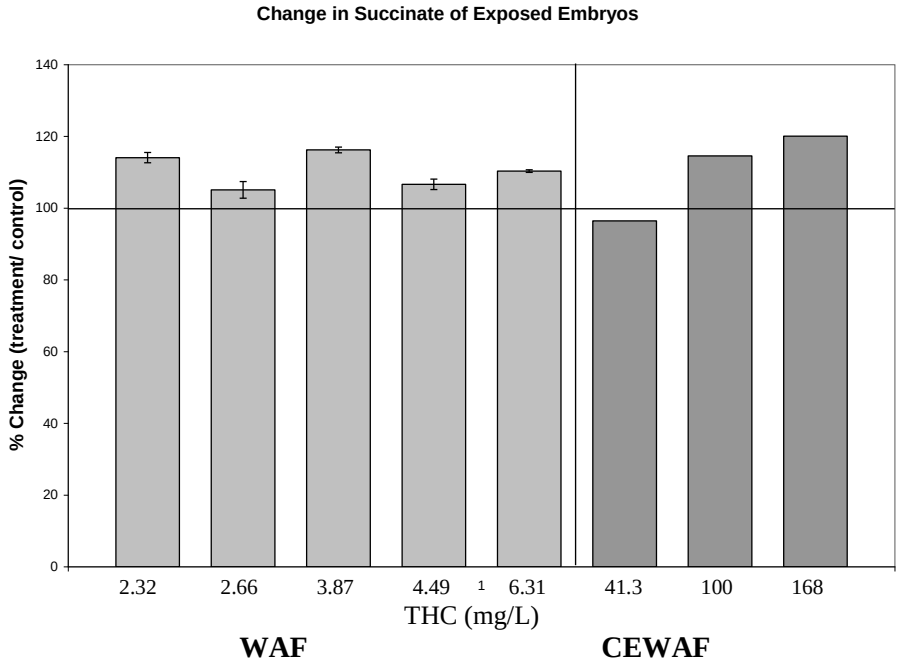
(c)


Change in Taurine of Exposed Embryos
% Change (treatment/ control)
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

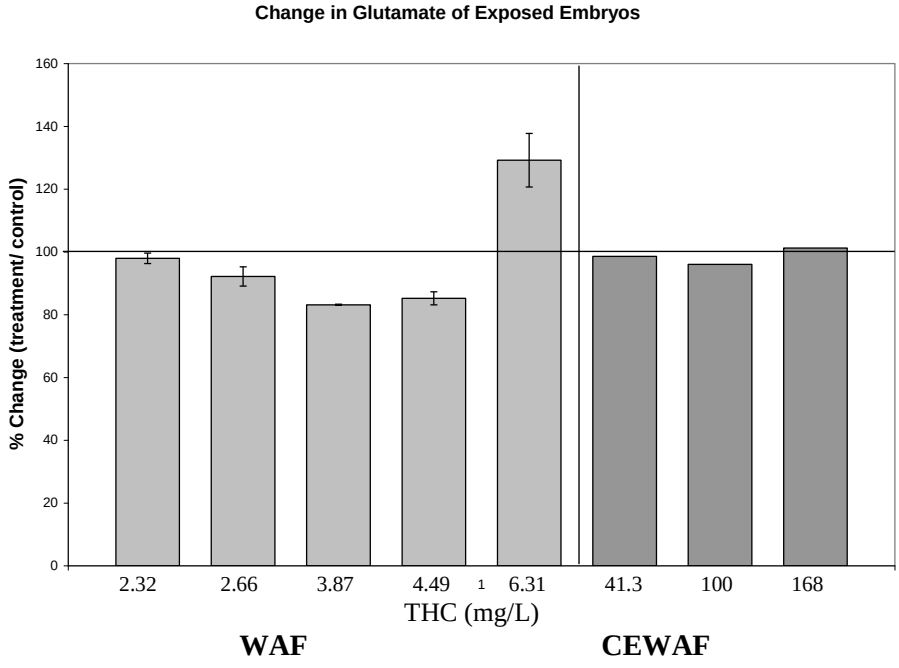
(d)


Change in Phosphocreatine of Exposed Embryos
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

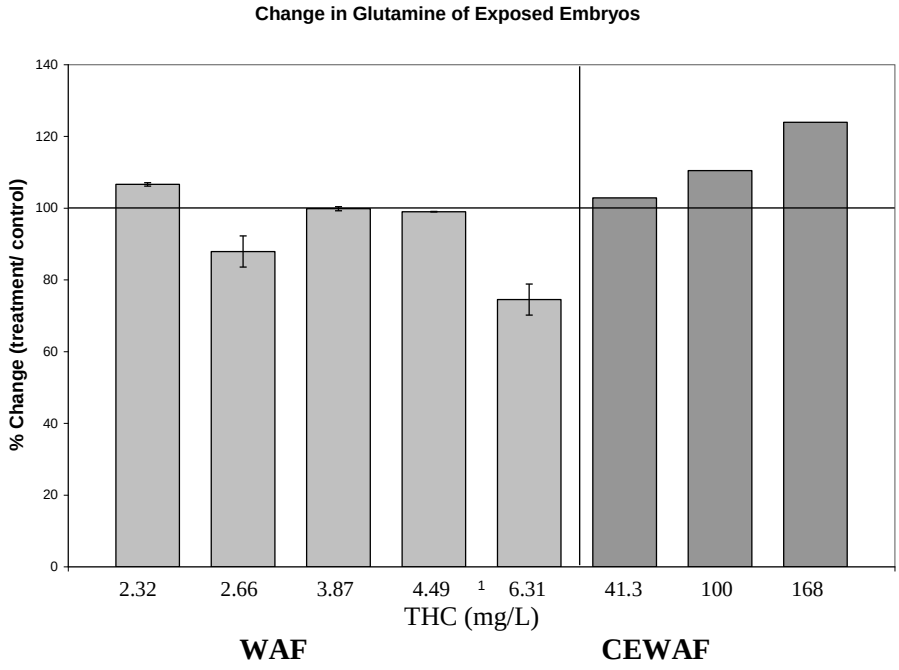
(e)


Change in Succinate of Exposed Embryos
% Change (treatment/ control)
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

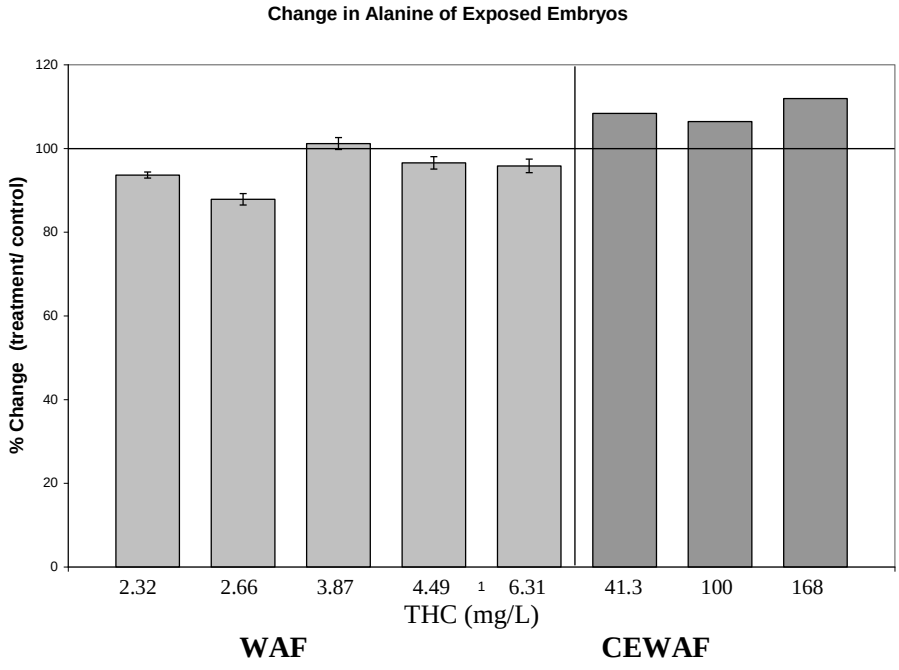
(f)


Change in Glutamate of Exposed Embryos
% Change (treatment/ control)
160
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

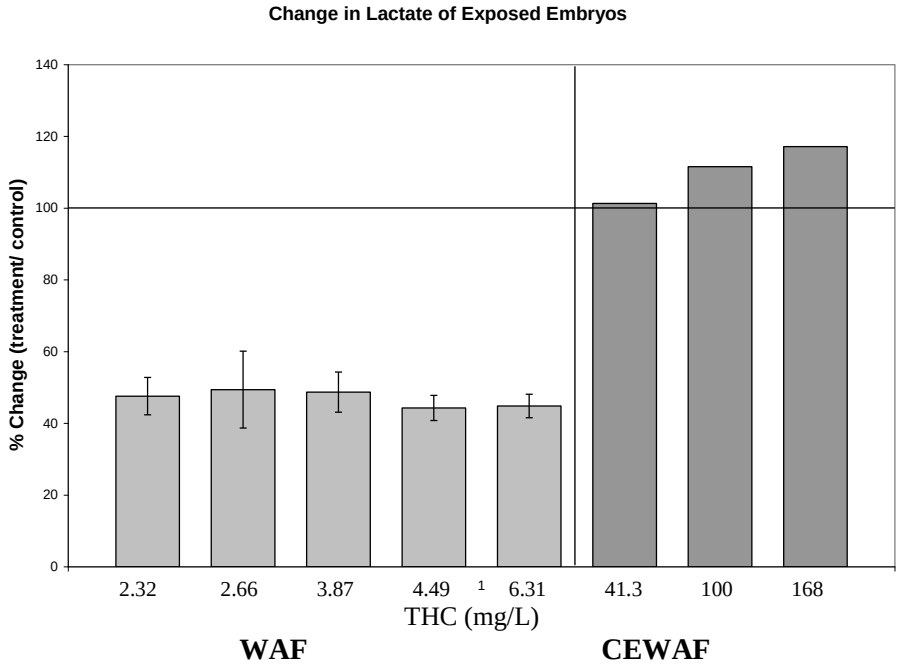
(g)


Change in Glutamine of Exposed Embryos
% Change (treatment/ control)
140
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

(h)


Change in Alanine of Exposed Embryos
% Change (treatment/ control)
120
100
80
60
40
20
0
2.32
2.66
3.87
4.49
1
6.31
41.3
100
168
THC (mg/L)
WAF
CEWAF

(i)



(j)

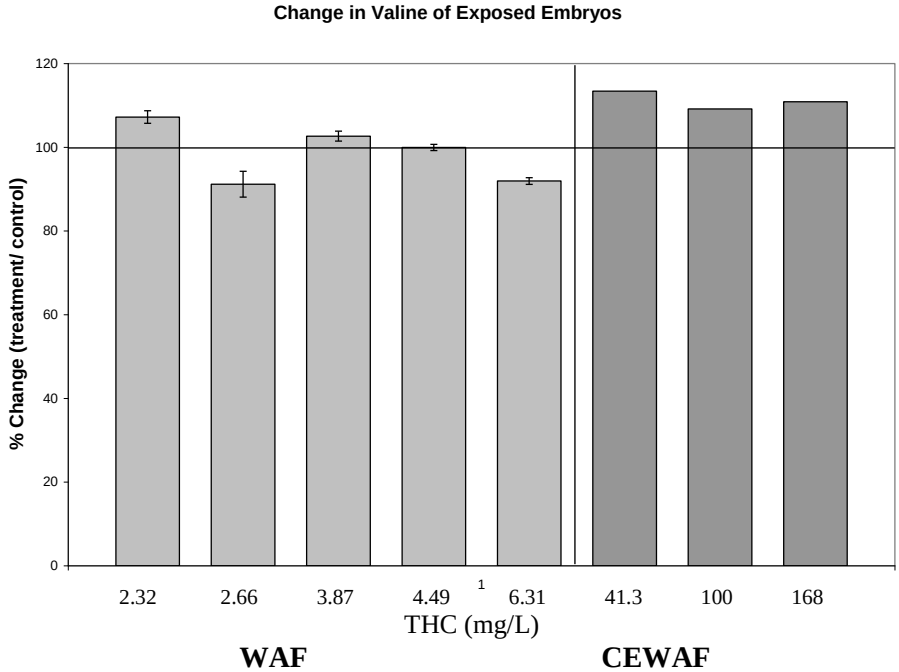


Figure 29. Changes in specific metabolites, from embryos exposed to WAF and CEWAF for 96-h, include (a) glycerophosphorylcholine, (b) glycine, (c) taurine, (d) phosphocreatine, (e) succinate, (f) glutamate, (g) glutamine, (h) alanine, (i) lactate, and (j) valine.

5.0 Discussion

5.1 Relative Toxicity of Dispersed and Non-Dispersed Oil

These experiments demonstrate that chemical dispersion of weathered PBCO resulted in greater hydrocarbons in seawater relative to physical dispersion of weathered oil. Despite the fact that the CEWAF solutions were created with much lower PBCO loading concentrations, addition of Corexit 9500 resulted in much higher concentrations of THC and higher concentrations of PAHs in CEWAF solutions relative to WAF solutions. Higher THC and PAHs resulted in greater toxicity of CEWAFs to adult and embryonic topsmelt. In addition, Corexit 9500 by itself was completely toxic to adult topsmelt at 100 mg/L.

Adult topsmelt exposed to sublethal concentrations of WAF and CEWAF survived and grew after the initial 96-h exposures. There were no differences in growth between control fish and those exposed to WAF and CEWAF after long-term culture. However, lower fecundities were observed after the 96 h exposure of adult fish to WAF and CEWAF. In both cases, there was a dose-dependent reduction in the number of eggs per fish in cohorts of adult fish that survived sublethal exposures to WAF and CEWAF. This relationship warrants additional research. There was however, no apparent relationship between embryo viability and *in vivo* exposure. Embryo viabilities were similar in control embryos and embryos hatched from WAF and CEWAF-exposed adult fish. In addition, larvae from these embryos developed and grew similar to control larvae.

Physical dispersion of weathered PBCO at loadings as high as 25 g/L oil did not result in sufficient THC or PAH concentrations to inhibit topsmelt embryos exposed directly to WAF. Chemical dispersion of weathered PBCO resulted in much higher concentrations of THC and select PAHs, and these inhibited embryo development at the lowest oil loading tested. In contrast to adult topsmelt, Corexit 9500 was not highly toxic to embryo development. CEWAF solutions created from dispersion of PBCO inhibited embryo cardiovascular development and resulted in high rates of embryo mortality. The current experiments extend results of previous experiments conducted with topsmelt embryos (Anderson et al. 2009), by using environmentally-relevant exposure conditions. Topsmelt

embryos were exposed to weathered crude oil in declining concentrations designed to mimic those that would naturally occur under *in situ* spill conditions. These results suggest that dispersion of weathered oil with Corexit 9500 could affect fish embryo development in situations where embryos were subjected to elevated hydrocarbons during critical stages of development. This would most likely be in response to spills in the near-shore environment or in enclosed bays and estuaries where this species spawns during the spring and summer months.

5.2 Application of Metabolomics

The research on metabolic responses of topsmelt to physically and chemically dispersed oil provides useful information on the potential mechanisms of oil toxicity. This approach provides a sensitive indicator of environmental stress and observed metabolites may be used as bio-indicators of the health of aquatic organisms when exposed to oil spills. Overall, the WAF and CEWAF tests resulted in similar metabolic profiles of muscle extracts, for both the control and treated adult fish at different concentrations, in which amino acids, lactate and nucleotides comprised the highest intensity signals. Metabolic profiles and changes observed for embryos were variable amongst the WAF and CEWAF tests, ultimately defining a difference between direct exposure and an *in vivo* exposure. Since embryos contain many compartments, it is not possible to specifically determine which compartment the identified metabolites were located; therefore the results have been interpreted as the metabolites from whole embryos. Differences between muscle and embryo extracts, and identified metabolites are apparent and also indicate that metabolic changes are organ and life-stage 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 individual definitive experiments were conducted at the same time it is unlikely that the physical state of the fish changed during the test, but since the adults were gravid it is possible that changes in physiology may have occurred; this may have contributed to the variation in metabolic responses of the tissue at different WAF and CEWAF concentrations, within the PCA scores plots.

The PCA score plots of each sample from the WAF exposed fish slightly represented a dose-response relationship. Although there is overlap among the metabolomes, for these samples, there is indication of a dose –response relationship for at least two of the three metabolomes for each dose. Therefore, the acute 96-h exposures did result in a clustering between two metabolomes at each dose. Similarly the PCA score plots of each sample from the CEWAF exposed fish displayed a similar dose-response trend. The WAF and CEWAF PCA scores plots, for embryos spawned following adult exposure, and those exposed to WAF and CEWAF, show minimal clustering among metabolomes; a dose –response relationship between the individual concentrations is apparent. Overall, results of the metabolic changes do indicate dependence for various doses and for each of the different life stages.

5.4 Role of Endogenous Metabolites

Metabolites and metabolic profiles in these experiments appear to be similar for both the control and dosed fish, and the embryos. Metabolites that were abundant within the muscle tissue included amino acids, lactate and ATP. Although changes did occur among the metabolites and corresponding treatments, significant changes were only observed among two metabolites. Abundant metabolites observed among the embryos also included amino acids and lactate. Unfortunately due to the lack of replicates among the embryo tests, significant changes were only observed for one WAF test.

Lactate decreased in muscle tissue following adult exposure to WAF and CEWAF, but a significant change was only observed for WAF. The presence of lactate may indicate that the muscle tissues were experiencing varied oxygen concentrations. Variations in oxygen ultimately lead to a disruption of energy production (i.e., ATP) via the tricarboxylic acid cycle (TCA), but due to an increase in ATP, disruption of energy production could not be possible. Although, lactate was present, its decrease indicates that it possibly is being used for energy or for the formation of glucose via gluconeogenesis. Embryos exposed *in vivo* and those directly exposed also experienced varied changes in lactate, resulting in a change in succinate, therefore influencing the production of energy via the TCA cycle.

Although not significant, results also indicate an increase in ATP for WAF and CEWAF exposed muscle tissue, subsequently a significant decrease in phosphocreatine was observed for CEWAF. Since phosphocreatine is dependent on ATP concentrations, this decrease after exposure to CEWAF indicates a lack of use of the emergency energy source during the exposures and therefore indicates lack of energy consumption as a whole. Phosphocreatine was not detected in extracts from embryos exposed *in vivo*, but it was observed in embryos directly exposed to WAF and CEWAF, although ATP was not identified among the metabolic profiles. This may indicate that at an early life-stage phosphocreatine is in a more abundant form of energy than ATP; therefore it is possibly the main energy source.

Organic osmolytes such as taurine accumulate within cells during hyperosmolarity and cell shrinkage, in order to adapt to changes in cell volume, but this process is metabolically expensive (Sperelakis, 1998). The presence of taurine, in the adult fish samples, indicates cell volume alteration and the attempt to regulate natural cell osmosis, otherwise this metabolite may indicate cell damage. Furthermore, Ronnestad (1993) notes that the presence of taurine within embryos is due to its incorporation into the eggs prior to spawning; therefore it is constant throughout development. Taurine remained elevated for both embryos exposed in vivo and directly to WAF and CEWAF.

Insignificant changes in amino acids were also observed in muscle tissue. Glutamine increased after WAF and CEWAF exposures but in both embryo tests, glutamine only increased following CEWAF exposure. As a precursor to the non-essential amino acid glutamate, glutamine participates in cellular metabolism by reacting with α-ketoglutarate (von Bohlen und Halbach, 2002). Increased glutamine concentrations may also be from glutamate collecting toxic ammonium ions within the cells resulting in glutamine formation; decreasing glutamate should occur respectively however a decrease in glutamate only occurred in embryos exposed directly to CEWAF. Podrabsky et al., (2007) note that glutamate and glutamine accumulate, under normal oxygen conditions, in embryos during their development; therefore the increase in glutamine may indicate that the embryos were

not experiencing anoxic exposure conditions and therefore the dispersed oil was not affecting their oxygen consumption.

Observed metabolic responses from WAF and CEWAF adult and embryo exposures may indicate metabolic changes are dependent on treatments and/or exposure concentrations. However, it is unknown which individual hydrocarbons are influencing such metabolic responses.

5.5 Potential Biomarkers of WAF and CEWAF Exposures

Several endogenous metabolites identified within muscle tissue and embryos may serve as potentially beneficial bio-markers. Identified amino acids, TCA cycle intermediates, osmolytes, and energy metabolites may be bio-indicators of protein degradation, cellular repair, change in cell structure, cellular stress or development. Some of the metabolites show consistent changes at various doses which suggest these metabolites can be used as endpoints for WAF and CEWAF toxicity. Possible bio-indicators include amino acids such as glycine and glutamine.

5.6 Concluding Remarks

Our results with spawning topsmelt provide toxicological information for oil spill response teams. Because these experiments used weathered PBCO, the results represent real-world conditions where the oil weathers considerably after a spill, before dispersant application. Overall, topsmelt growth data does not indicate any significant effects of short-term WAF exposure on long-term growth. Although no effects were detected by growth, WAF and CEWAF exposed adults produced fewer embryos compared to the control fish.

Embryos exposed to WAF and CEWAF elicited different effects, although reduced yolk-sac area was observed in WAF-exposed embryos, developmental abnormalities affecting hatching success were only observed in embryos directly exposed to CEWAF; dispersed PBCO contained considerably higher hydrocarbon concentrations and was more toxic to embryos than non-dispersed PBCO.

Metabolomics can be used for determining the subtle impacts of oil on spawning topsmelt and exposed embryos. Metabolic results provide clues to the potential mechanisms of toxicity that hydrocarbons pose towards spawning fish and their offspring, but further biochemical and physiological analyses are important to fully understand the impacts crude oil pose towards these life-stages. Overall, the results from the metabolomic analysis do not indicate that the short-term 96-h exposures led to any sublethal effects to either the adult or embryonic stages spawned from these fish.

6.0 Technology Transfer

The results from this 2-year project will provide resource managers and spill responders with basic toxicological information on the acute and chronic effects of oil spills on spawning topsmelt and their embryos; additionally providing data to support decisions regarding the advisability of applying dispersants under spill conditions where topsmelt spawning grounds are present. 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 have provided matching funds for this project.

7.0 Achievement and Dissemination

7.1 Publication

Anderson, BS, Arenella-Parkerson D, Phillips BM, Crane DL and Tjeerdema RS. 2009. Effects of dispersed and un-dispersed oil on developing topsmelt embryos (*Atherinops affinis*). *Environ. Pollut. 157: 1058-1061*.

Lin C-Y, B.S. Anderson, B.M. Phillips, A.C. Peng, S. Clark, J. Voorhees, H-D. I. Wu, M.J. Martin, J. McCall, C.R. Todd, F. Hsieh, D. Crane, M.R. Viant, M.L. Sowby and R.S. Tjeerdema. 2009. Characterization of the metabolic actions of crude versus dispersed oil in salmon smolts via NMR-Based metabolomics. *Aquat. Toxicol*. 95: 230-238.

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7.2 Presentation

Van Scoy, A.R., Lin CY, Anderson BS, Phillips BM, Viant MR and Tjeerdema RS. "Comparison of Metabolic Responses Produced by Crude Versus Dispersed Oil in California Fishes via NMR-Based Metabolomics." SETAC North America 2009 Annual Meeting, New Orleans, LA. (2009).

Van Scoy, A.R., Lin CY, Anderson BS, Phillips BM, Viant MR and Tjeerdema RS. "Impacts of Crude Versus Dispersed Oil in Salmon as Characterized by NMR-Based Metabolomics". NorCal SETAC 19th Annual Meeting, Davis, CA. (2009).

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

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 Pre-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 Pre-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).

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APPENDIX I. Flow measurements from the individual Topsmelt WAF experiment.

Tank	7/22/2009	Mean	SD
1	40	38.67	1.15
2	38		
3	38		
4	42	40.67	2.31
5	42		
6	38		
7	38	40.67	3.06
8	40		
9	44		
10	38	39.33	1.15
11	40		
12	40		
13	38	38.67	1.15
14	38		
15	40		
16	44	40.67	3.06
17	40		
18	38		

Tank	7/23/2009	Mean	SD
1	40	39.33	1.15
2	40		
3	38		
4	44	41.33	2.31
5	40		
6	40		
7	40	41.33	2.31
8	40		
9	44		
10	40	40.00	0.00
11	40		
12	40		
13	40	40.00	0.00
14	40		
15	40		
16	44	41.33	2.31
17	40		
18	40		