The 2015 Refugio Beach Oil Spill: Assessment of Surfperch (Embiotocidae) Exposure

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Bernadita Anulacion, Gina Ylitalo, National Oceanic and Atmospheric Administration Northwest Fisheries Science Center, Seattle, WA

Regina Donohoe, Ph.D., California Department of Fish and Wildlife, Office of Spill Prevention and Response, Monterey, CA

David Witting, Ph.D., National Oceanic and Atmospheric Administration, Fisheries Restoration Center, Long Beach, CA

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INTRODUCTION

On May 19, 2015, an underground pipeline (Line 901), owned and operated by Plains All American Pipeline, L.P., sustained a release of crude oil near Refugio State Beach in Santa Barbara County, California. Oil released from the pipeline pooled, then overflowed into a nearby culvert, across land and other drainage systems, and entered the Pacific Ocean in the surf zone. The surf zone in this area supports relatively large populations of fish, such as silversides, surfperches, croakers, flatfishes and rays (Allen and Pondella, 2006). These fish would have been exposed by direct contact with floating or submerged oil, uptake from oil dissolved or suspended in the water column and the food chain. Additionally, the spill occurred during the spawning season of several surf zone fish species, such that sensitive early life stages may have been exposed to oil. For example, the barred surfperch (*Amphistichus argenteus*) and walleye surfperch (*Hyperprosopon argenteum*) give birth to live young from March to July in this area (Carlisle et al., 1960; California Department of Fish and Game, 2001).

Crude oil contains hundreds to thousands of chemicals that are potentially toxic to fish. Exposure to one class of chemicals, polycyclic aromatic hydrocarbons (PAHs), has been associated with developmental abnormalities, immunosuppression, hepatic lesions and altered growth in fish (Myers et al. 1994; Heintz et al. 2000; Arkoosh et al. 2001; Meador et al. 2006; Reynaud and Deschaux 2006; Incardona et al. 2004; Incardona et al. 2012). Fish rapidly take up PAHs present in their food and the environment and quickly metabolize these compounds to more polar compounds. The more polar PAH metabolites are then secreted into fluids such as bile and urine for elimination via the gastroenteric tract or kidneys (Roubal et al., 1977; Varanasi et al., 1989; Krahn et al., 1984). Therefore, assessment of bile for PAH metabolites provides information on recent uptake and exposure to these compounds. Elevated biliary PAH metabolites have been measured in fish following oil spills (Krahn et al., 1986; Sol et al., 2000; Murawski et al, 2014; Snyder et al., 2015). Additionally, fish living near the Coal Oil Point oil seeps in Santa Barbara have been shown to have elevated levels of PAH metabolites in bile, compared to nearby reference locations (Spies et al., 1996; Roy et al., 2003).

The primary objective of this assessment was to obtain a quantitative estimate of PAH exposure in fish by measuring bile and muscle tissue concentrations in an indicator fish, as well as concentrations in the water. Surfperches (Embiotocidae) were selected because they are relatively resident and occupy the surf zone and shallow subtidal areas where significant oiling occurred (Carlisle et al, 1960). Barred surfperch and walleye surfperch were the two species evaluated. Exposure at Refugio State Beach, a heavily oiled area, was compared to a lesser oiled area, Gaviota State Beach, and an area near the Coal Oil Point oil seep, Campus Point, using samples from all three sites collected at both four days and approximately one year after the oil spill.

METHODS

Field Sampling Procedures

Sampling locations were selected based on shoreline oiling observations on 22 May 2015 (Figure 1). Gaviota State Beach was not reported as being oiled at that time. Refugio State Beach was adjacent to the spill location and was heavily oiled. Campus

Figure 1. *Sampling locations for surfperch bile on 23 May 2015 and 18 May 2016.*

Point was not reported as oiled but may have had some oil exposure from Line 901 oil and adjacent Coal Oil Point seeps. On 23 May 2015, surfperches were caught by hook and line at Refugio State Beach due to safety limitations of entering oil contaminated water. A beach seine was used at Campus Point and hook and line was used at Gaviota State Beach due to wind and surf conditions. On 18 May 2016, a beach seine was used at the same three locations to collect surfperch. Fish were identified to species and maintained alive until sample processing within one to two hours. Total length was measured and the fish were killed by cervical dislocation. The gall bladder was immediately extracted and bile was collected in 4 milliliter Sun-Sri™ amber vials and stored on ice in the field. Bile samples were then frozen at -20°C until analysis at the Northwest Fisheries Science Center, Seattle, WA. The sex was determined by examining gonadal tissue. The remaining carcass was wrapped in foil, placed in a zip-top bag, stored on ice in the field, and then frozen at -20°C until analyses were conducted at the California Department of Fish and Wildlife, Water Pollution Control Laboratory (CDFW-WPCL), Gold River, CA. As part of the fisheries closure assessment (OEHHA, 2015), barred surfperch were collected by hook and line at Gaviota State Beach and Refugio State Beach on 10 June 2015. No fish were collected from Campus Point. Fish were wrapped in foil, placed in a zip-top bag, stored on ice in the field, and then frozen at -20°C until analyses were conducted at the CDFW-WPCL.

Triplicate surf water samples were collected on 27 May 2015 and 17 May 2016 at Gaviota State Beach, Refugio State Beach and Campus Point. Samples were collected in one-liter amber glass bottles by submerging the bottle in the surf zone until filled with minimal headspace. Samples were immediately placed on ice and transported to the CDFW-WPCL for analysis.

Bile Analysis

Bile samples were analyzed using a high-performance liquid chromatography fluorescence (HPLC-F) method described in Krahn et al. (1984). This method results in the determination of the concentrations of classes of PAH metabolites fluorescing in the regions typified by naphthalene (NPH), phenanthrene (PHN) and benzo[a]pyrene (BaP). Bile was injected directly onto a Waters HPLC-F system equipped with a C-18 reverse-phase column (Phenomenex Synergi Hydro). The PAH metabolites were eluted with a linear gradient from 100% water (containing a trace amount of acetic acid) to 100% methanol at a flow of 1.0 mL/min. Chromatograms were recorded at the following wavelength pairs: 1) 292/335 nm where many 2-3 benzene ring aromatic compounds (e.g., NPH) fluoresce, 2) 260/380 nm where several 3-4 ring compounds (e.g., PHN) fluoresce and 3) 380/430 nm where 4-5 ring compounds (e.g., BaP) fluoresce. Peaks eluting after 9 minutes were integrated and the areas of these peaks were summed. The concentrations of fluorescent PAHs in the bile samples of the fish were determined using NPH, PHN or BaP as external standards and converting the fluorescence response of bile to PHN (ng PHN equivalents/g bile), NPH (ng NPH equivalents/g bile) or BaP (ng BaP equivalents/g bile) equivalents on a wet weight basis. In addition, protein analysis as described in da Silva et al. (2006) was completed for all bile samples as previous laboratory contaminant exposure studies on fish have shown that normalization of biliary PAH metabolite concentrations to protein values may help account for variation in metabolite levels based on feeding status (Collier and Varanasi 1991).

To ensure that the HPLC-F system was operating properly, a NPH/PHN/BaP calibration standard was analyzed numerous times ($n \geq 5$) until a relative standard deviation < 15% was obtained for each PAH. As part of the laboratory quality assurance plan (Sloan et al. 2006), a method blank and a fish bile positive control sample (bile of Atlantic salmon exposed to 25 mg/L of Monterey crude oil for 48 hours) were analyzed with each batch of fish bile samples. All sample batches met the laboratory quality assurance criteria.

Fish Tissue and Water Analysis

For the 23 May 2015 collection, skinless filets of individual barred surfperch were composited into one sample for each site: Gaviota (n=6 fish), Refugio (n=9 fish), and Campus Point (n=6 fish). For the 10 June 2015 collection, skinless filets of individual barred surfperch were again composited into one sample for each site: one sample for Gaviota (n=9 fish), and one for Refugio (n=4 fish). Tissues were extracted by pressurized fluid extraction, followed by gel permeation chromatography and silica clean-up. Water samples and tissue extracts were analyzed for PAHs by GC/MS-SIM (USEPA Method 8270 mod). Results for these 45 individual PAHs and alkylated homologue groups were summed to estimate total PAHs ($TPAH_{45}$): naphthalene; C1-naphthalenes; C2-naphthalenes; C3-naphthalenes; C4-naphthalenes; acenaphthylene; acenaphthene; fluorene; C1-fluorenes; C2-fluorenes; C3-fluorenes; phenanthrene; anthracene; C1-phenanthrene/anthracene; C2-phenanthrene/anthracene; C3-phenanthrene/anthracene; C4-phenanthrene/anthracene: pyrene; fluoranthene; C1-fluoranthene/pyrenes; C2-fluoranthene/pyrenes; C3-fluoranthene/pyrenes; C4-fluoranthene/pyrenes; benz[a]anthracene; chrysene; C1-chrysenes; C2-chrysenes; C3-

chrysenes; C4-chrysenes; benzo(a)pyrene; perylene; benzo(e)pyrene; benzo(b)fluoranthene; benzo(k)fluoranthene; benzo(g,h,i)perylene; indeno(1,2,3-c,d)pyrene; dibenz(a,h)anthracene; C1-dibenz(a,h)anthracene; C2-dibenz(a,h)anthracene; C3-dibenz(a,h)anthracene; dibenzothiopene; C1-dibenzothiophenes; C2-dibenzothiophenes; C3-dibenzothiophenes and biphenyl. When calculating $TPAH_{45}$, non-detects were assumed to be zero. Tissue results are reported on a dry weight basis.

RESULTS

Field Observations

Total lengths of surfperch caught on 23 May 2015 ranged from 153 to 297 mm at Gaviota, 130 to 230 mm at Refugio and 142 to 205 mm at Campus Point. At each location, one female barred surfperch was observed to contain live young upon dissection. For the 18 May 2016 sampling, total lengths of surfperch ranged from 115-190 mm at Gaviota, 145-225 mm at Refugio and 145-195 mm at Campus Point. Two female with live young were observed at Campus Point and Refugio. In 2016, other species caught in the surf zone via beach seine included: shiner surfperch (*Cymatogaster aggregate*), kelp surfperch (*Brachyistius frenatus*), corbina (*Menticirrhus undulates*), topsmelt (*Atherinops affinis*), Pacific sardine (*Sardinops sagax*), jacksmelt (*Atherinopsis californiensis*), black perch (*Embiotoca jacksoni*), sargo (*Anisotremus davidsoni*), opaleye (*Girella nigricans*), white croaker (*Genyonemus lineatus*); giant kelpfish (*Heterostichus rostratus*), and diamond turbot (*Hypsopsetta guttulata*).

Biliary PAH Metabolite Concentrations

Concentrations of biliary PAH metabolites (based on wet weight or biliary protein) are provided in Appendices 1 and 2. The mean (± SD) biliary NPH, PHN and BaP equivalent concentrations measured in surfperch collected at Gaviota State Beach (n=6 for barred), Refugio State Beach (n=21 for barred) and Campus Point (n=5 for barred; n=9 for walleye) in 2015 are shown in Figure 2 (ng/g bile, wet weight) and Figure 3 (ng/mg protein). Significant differences (ANOVA $p < 0.05$; Tukey-Kramer HSD test) in mean NPH, PHN and BaP equivalent concentrations (based on wet weight or biliary protein) were found among collection sites. For each PAH metabolite, barred surfperch from Refugio State Beach, adjacent to the oil release site, had a significantly higher mean level than those determined in fish from Campus Point or Gaviota. Mean PAH metabolite concentrations measured in bile of barred surfperch from Campus Point, adjacent to offshore oil seeps, were significantly higher than the same metabolites measured in barred surfperch from Gaviota, a lesser oiled site. At the Campus Point site, mean levels of biliary NPH and PHN equivalents (based on wet weight only) were significantly higher (ANOVA $p < 0.05$; t-test) in barred surfperch than in those measured in walleye surfperch. Mean concentrations of NPH and PHN were higher than BaP equivalents.

Mean (± SD) NPH, PHN and BaP equivalent concentrations based on wet weight (Figure 4) or biliary protein (Figure 5) for each species collected at Gaviota (n=7 for barred; n=1 for walleye), Refugio State Beach (n=8 for barred; n=20 for walleye) and

Campus Point (n=1 for barred; n=13 for walleye) were not significantly different (ANOVA $p > 0.05$; log_{10} transformed data) in fish collected one year after the spill except PHN equivalent levels (wet weight only) in barred surfperch from the Refugio Beach site and Gaviota ($p = 0.0487$).

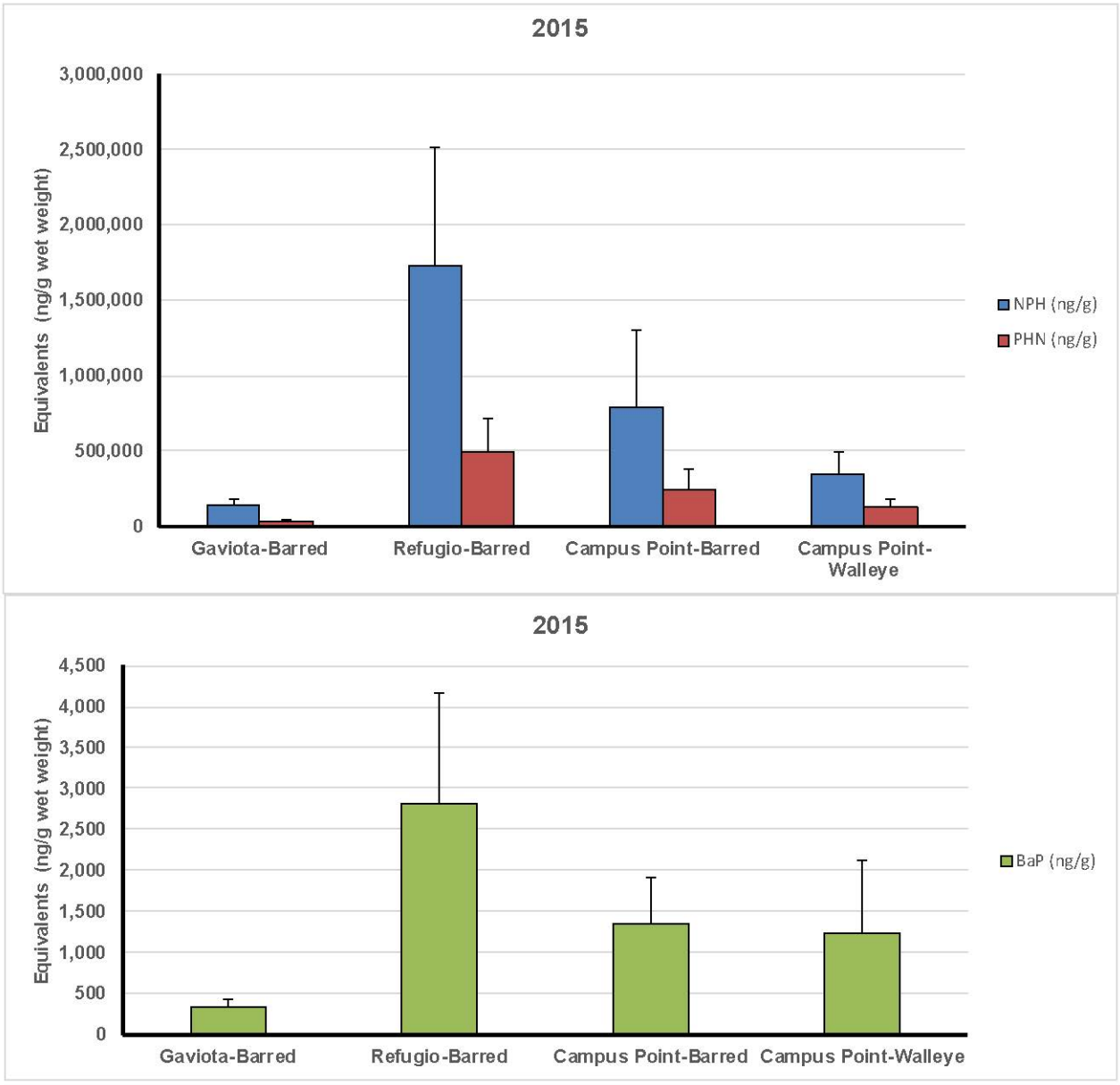

Figure 2. *Mean (±SD) concentrations of bile equivalents (ng/g bile wet weight) measured in barred and walleye surfperch collected in 2015: naphthalene (NPH), and phenanthrene (PHN; Top) and benzo[a]pyrene (BaP; Bottom).*

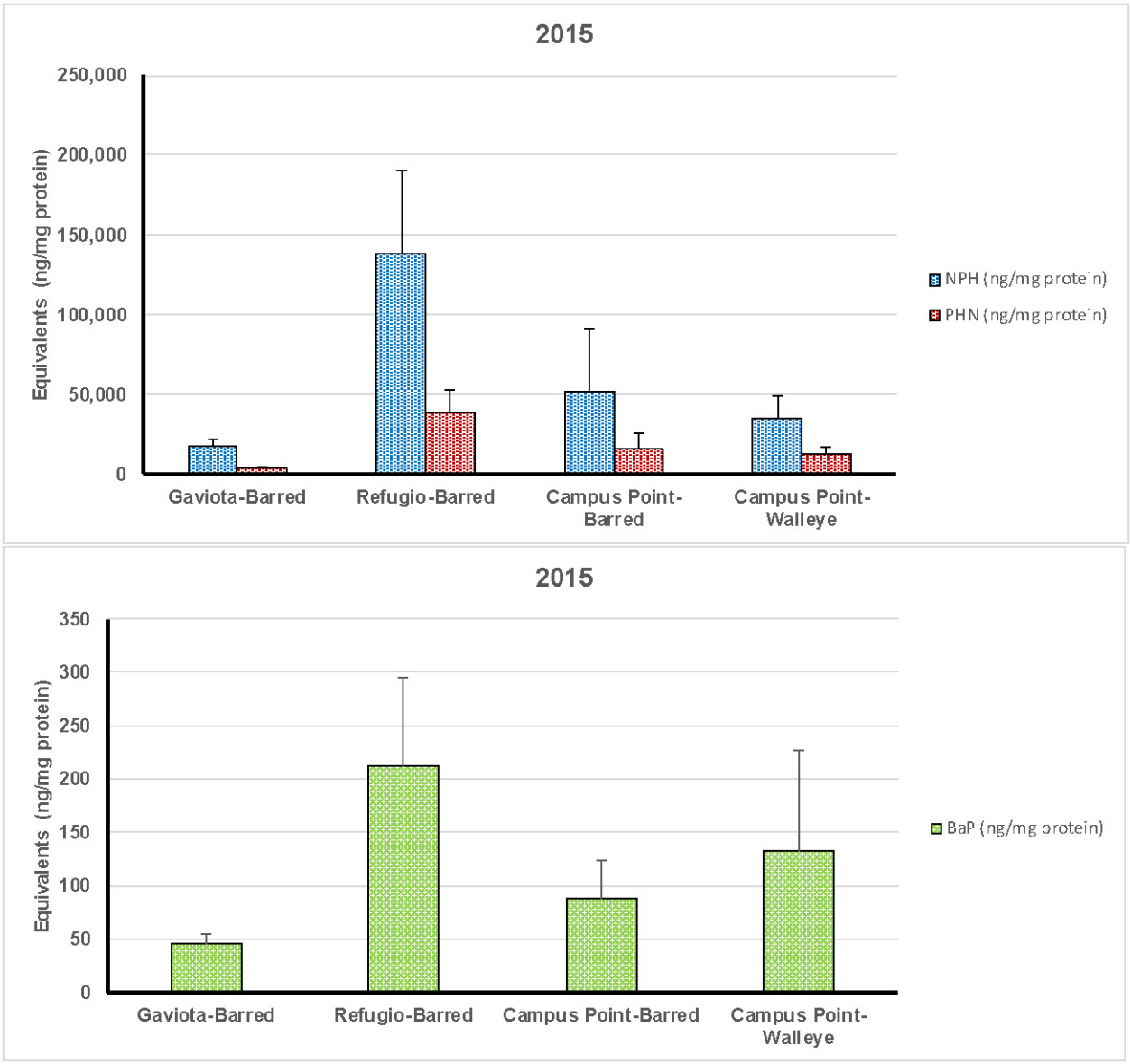

Figure 3. *Mean (±SD) concentrations of bile equivalents (ng/mg bile protein) measured in barred and walleye surfperch collected in 2015: naphthalene (NPH) and phenanthrene (PHN; Top) and benzo[a]pyrene (BaP; Bottom).*

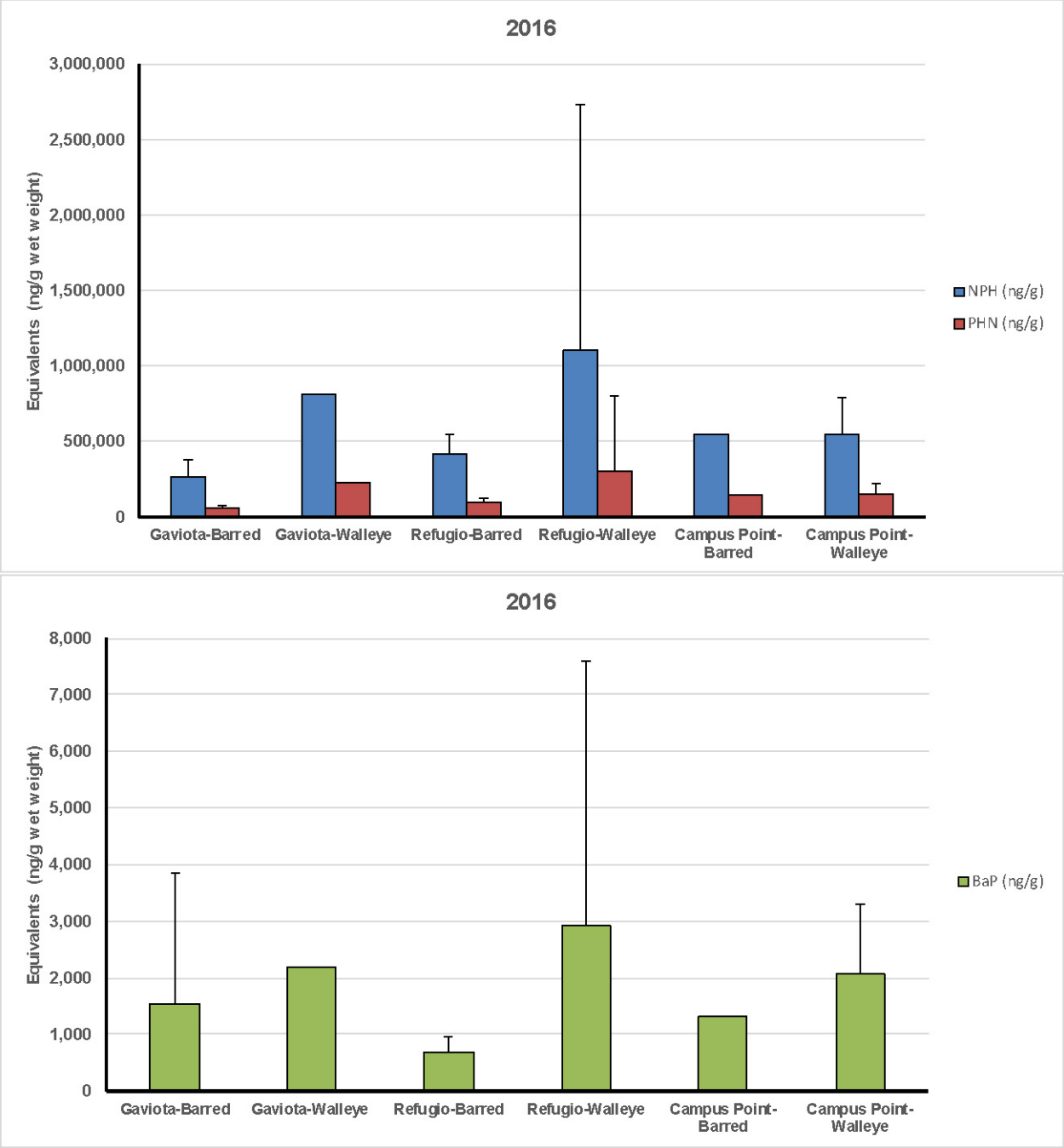

Figure 4. *Mean (±SD) concentrations of bile equivalents (ng/g bile wet weight) measured in barred and walleye surfperch collected in 2016: naphthalene (NPH) and phenanthrene (PHN; Top); and benzo[a]pyrene (BaP; Bottom).*

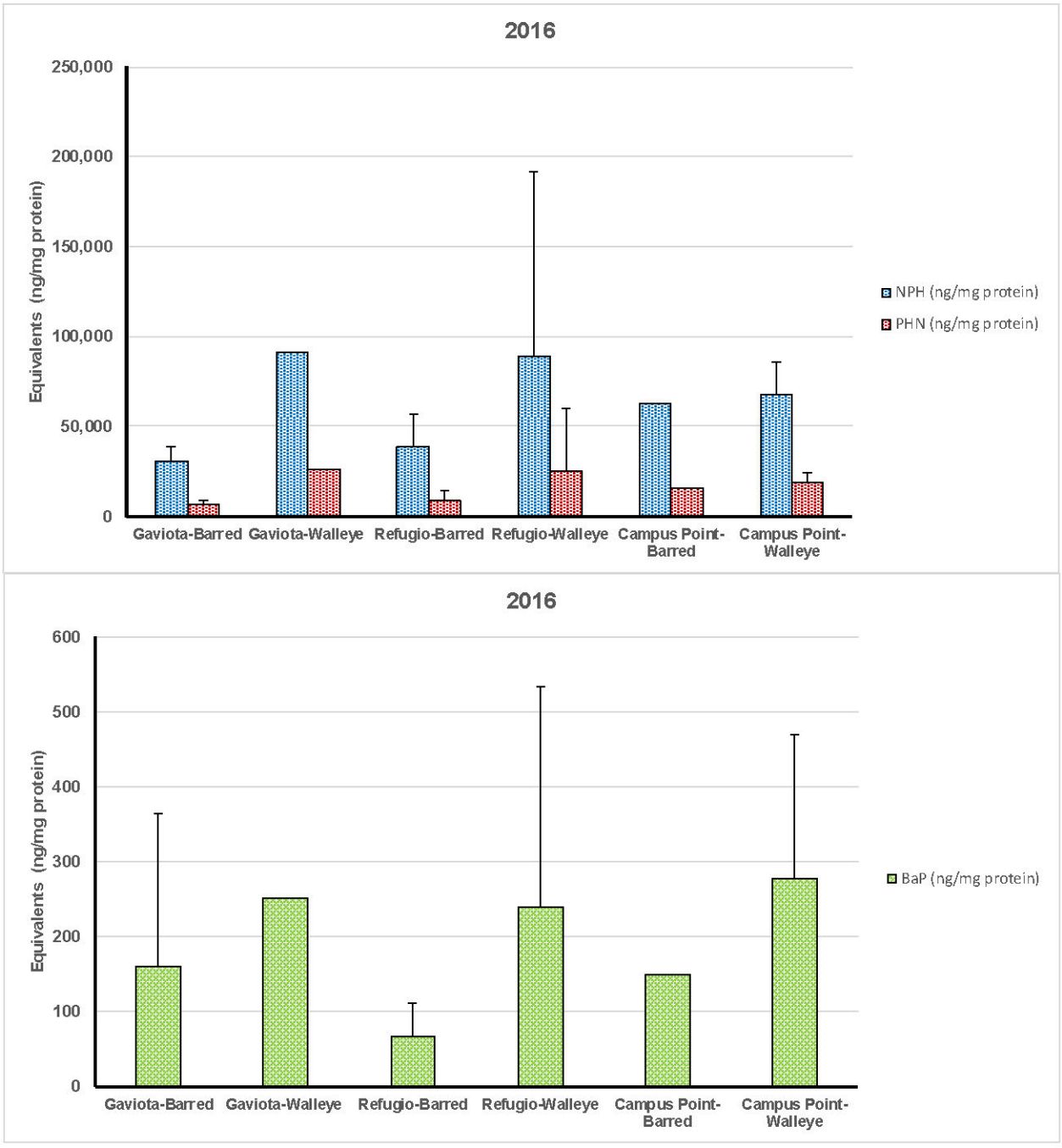

Figure 5. *Mean (±SD) concentrations of bile equivalents (ng/mg bile protein) measured in barred and walleye surfperch collected in 2016: naphthalene (NPH) and phenanthrene (PHN; Top); and benzo[a]pyrene (BaP; Bottom).*

At the Refugio State Beach site, mean concentrations of all PAH metabolites based on wet weight (Figure 4) in the 2016 collected fish were significantly higher in walleye surfperch compared to barred surfperch (ANOVA $p < 0.05$; t-test; log_{10} transformed data). Similarly, mean levels of protein-corrected PAH metabolites were significantly higher (ANOVA $p < 0.05$; t-test) in walleye compared to barred surfperch except protein-corrected NPH ($p = 0.0505$). Comparisons between species at the other two collection sites were not conducted due to inadequate numbers of bile samples.

Differences in mean concentrations of PAH metabolites based on sampling year for each species collected at the same site were examined. Barred surfperch collected at Refugio Beach in 2015 had significantly higher (ANOVA $p < 0.0001$; t-test; log_{10} transformed data) mean NPH, PHN and BaP concentrations (wet weight and protein-corrected) than those determined in the 2016 (Figure 6). In contrast, Gaviota barred surfperch collected in 2016 (Figure 6) had significantly higher mean concentrations (ANOVA $p < 0.05$; t-test; log_{10} transformed data) of NPH equivalents (wet weight and protein-corrected) and PHN equivalents (wet weight only) than the mean values of the 2015 fish. Walleye surfperch collected from Campus Point in 2016 had significantly higher mean concentrations of protein-corrected NPH, PHN and BaP equivalents, as well as NPH equivalents (wet weight only), compared to the 2015 fish. No other significant differences (ANOVA $p > 0.05$) in mean concentrations of PAH metabolites were found for walleye surfperch from this site.

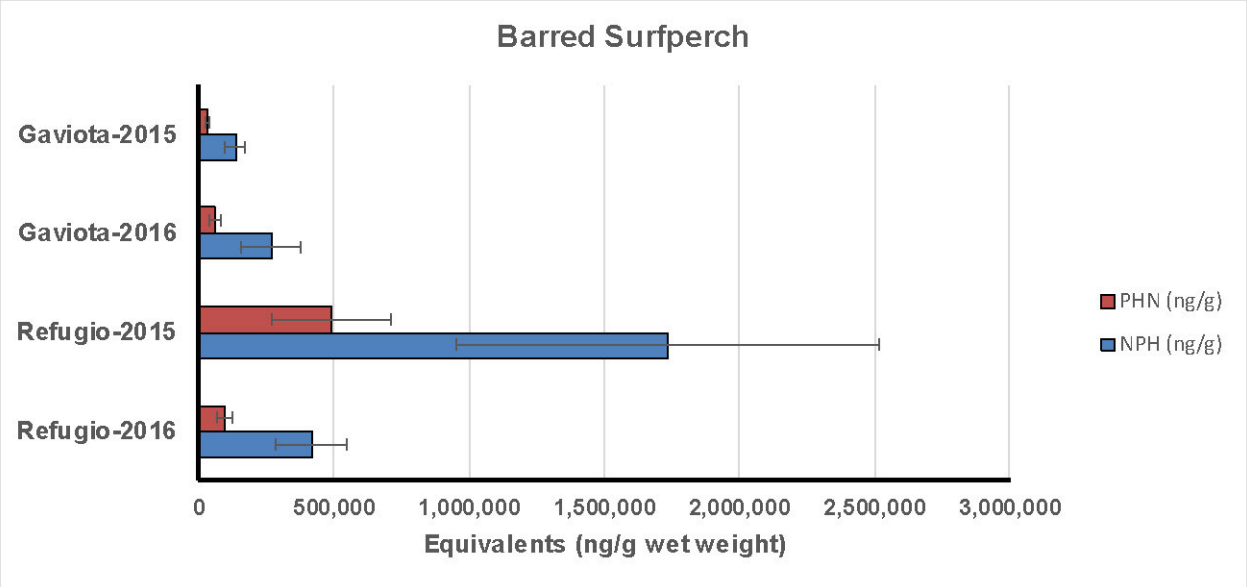

Figure 6. *Mean (±SD) concentrations of bile naphthalene (NPH) and phenanthrene (PHN) equivalents (ng/g bile wet weight) measured in barred surfperch collected in 2015 and 2016.*

Fish Muscle PAH Concentrations

The $TPAH_{45}$ concentration in skinless filets collected four days after the spill followed the pattern seen in bile, with highest concentrations observed at Refugio (88 ug/kg dw; Figure 7). Naphthalenes (parent and C1-C4 alkylated) were the primary PAHs detected at Gaviota (100%), Refugio (91%) and Campus Point (78%). Tricyclic PAHs were also

detected at Refugio (e.g., acenaphthene, dibenzothiophenes, and C1-phenanthrene/anthracene) and Campus Point (C1-phenanthrene/anthracene). Concentrations were almost three times lower at Refugio (30 ug/kg dw) 22 days post spill but were similar at Gaviota, consisting only of naphthalenes at both locations.

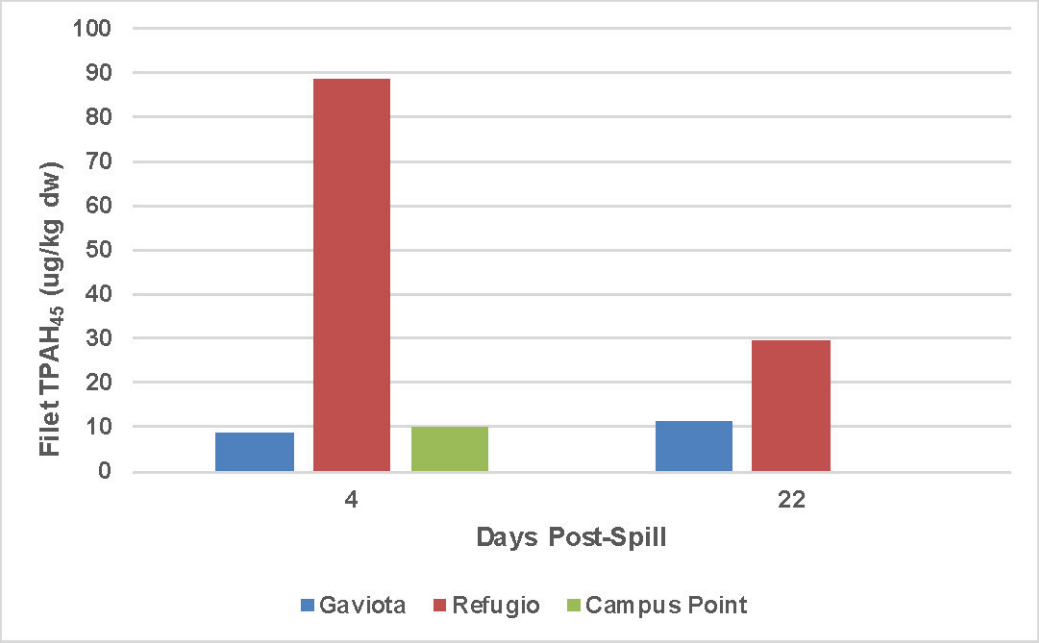

Figure 7. *$TPAH_{45}$ concentrations (ug/kg dw) in a composite sample of barred surfperch skinless filets 4 and 22 days after the spill from Gaviota (4; n=6: 22; n=9 fish)and Refugio (4; n=9: 22; n= 4 fish) and 4 days after the spill from Campus Point (4; n=6 fish).*

Surf Water PAH Concentrations

$TPAH_{45}$ concentrations in surf water 8 days after the spill were highest at Refugio (1.8 – 12.8 µg/l) when compared to Gaviota (0.06 – 0.18 µg/l) and Campus Point (0.12 – 0.30 µg/l; Figure 8). This is consistent with the 2015 spatial pattern observed in fish bile and muscle tissue. One year after the spill, $TPAH_{45}$ concentrations were lower at Refugio (0.16 – 0.53 µg/l) and Gaviota (0.0 – 0.04 µg/l), but variable at Campus Point (0.3 – 12.4 µg/l; Figure 8). Based on fingerprinting analysis (Stout, 2016), it was determined that the maximum concentrations at Refugio in 2015 and Campus Point in 2016 contained crude oil micro-droplets or emulsions, due to the presence of minimally soluble 4- to 6-ring PAHs. Further PAH composition analysis revealed that the Refugio 2015 sample was a probable match to the Line 901 oil, due to the high proportion of dibenzothiophenes, but the Campus Point 2016 PAH distribution was consistent with seep oil. The PAH composition in the maximum Campus Point 2015 sample was also consistent with seep oil.

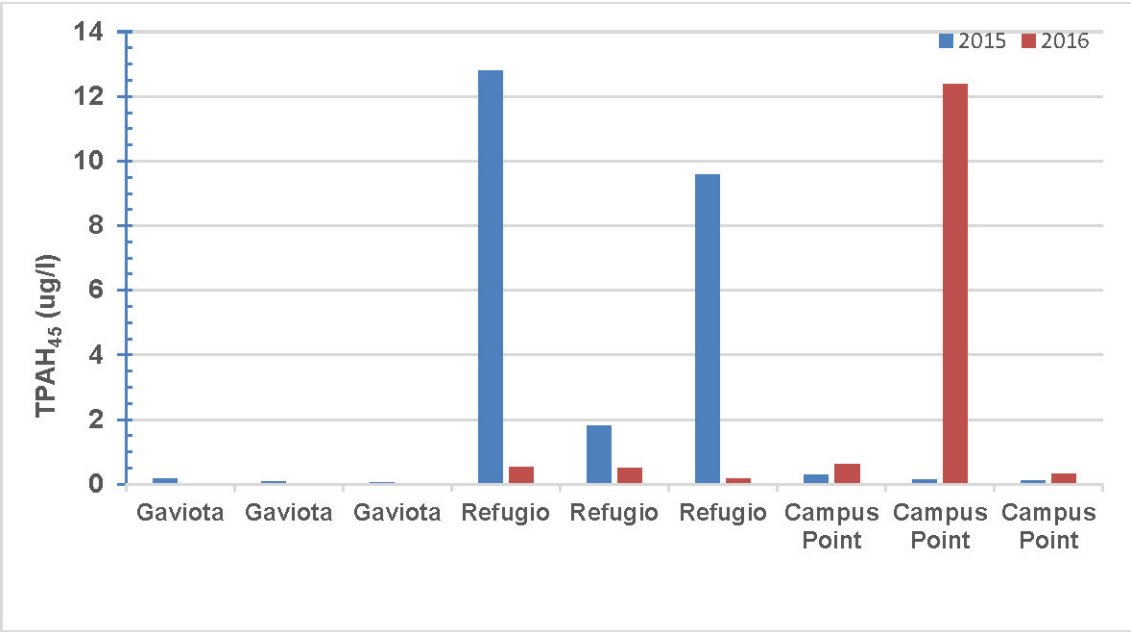

Figure 8. *$TPAH_{45}$ concentrations (µg/l) in triplicate surf water samples from Gaviota, Refugio and Campus Point collected 8 days after the spill (27 May 2015) and approximately one year after the spill (17 May 2016).*

DISCUSSION

Four days after the Line 901 oil release, surfperch biliary PAH metabolite concentrations were significantly higher at Refugio State Beach, compared to Campus Point and Gaviota State Beach. $TPAH_{45}$ concentrations in surfperch muscle and surf water reflected a similar spatial pattern in 2015. These results indicated surf zone fish exposures to PAHs were higher in the area adjacent to the oil release. One year after the oil spill, mean biliary PAH metabolite concentrations at Refugio declined, such that there was no longer a significant difference between the three sampling locations. Campus Point surfperch, continued to show elevated biliary PAH metabolite levels, compared to Gaviota State Beach, likely due to the presence of nearby natural oil seeps, consistent with elevated $TPAH_{45}$ levels in surf water at this location in 2016. Within site species differences between barred and walleye surfperch bile metabolite levels may have resulted from differences in food and habitat preferences (Carlisle et al, 1960; Feder et al, 1974; Hobson and Chess, 1986) but additional research would have to be conducted to further assess interspecies differences.

PAH metabolites in 2015 and 2016 bile samples were predominately naphthalene and phenanthrene derived metabolites, consistent with exposure to fresh crude oils, rather than higher molecular weight PAHs (e.g., BaP) that are associated with pyrogenic sources (Lee and Anderson, 2005). Exposure of fish to oil seep sediment has also resulted in bile PAH metabolites being dominated by NPH and PHN equivalents (Roy et al., 2003). However, levels measured in this study were somewhat higher than previously measured near Santa Barbara oil seeps. Spies et al (1996) sampled rainbow surfperch near the Isla Vista seeps at Coal Oil Point, at depths of 8-15m, and the Goleta

Pier in September 1990. Bile NPH and PHN equivalents were elevated near the seep site, compared to Goleta Pier (Figure 9). Liver cytochrome P-450 enzyme levels and mean gill and liver lesion scores were significantly higher in fish collected from the seep area, compared to Goleta.

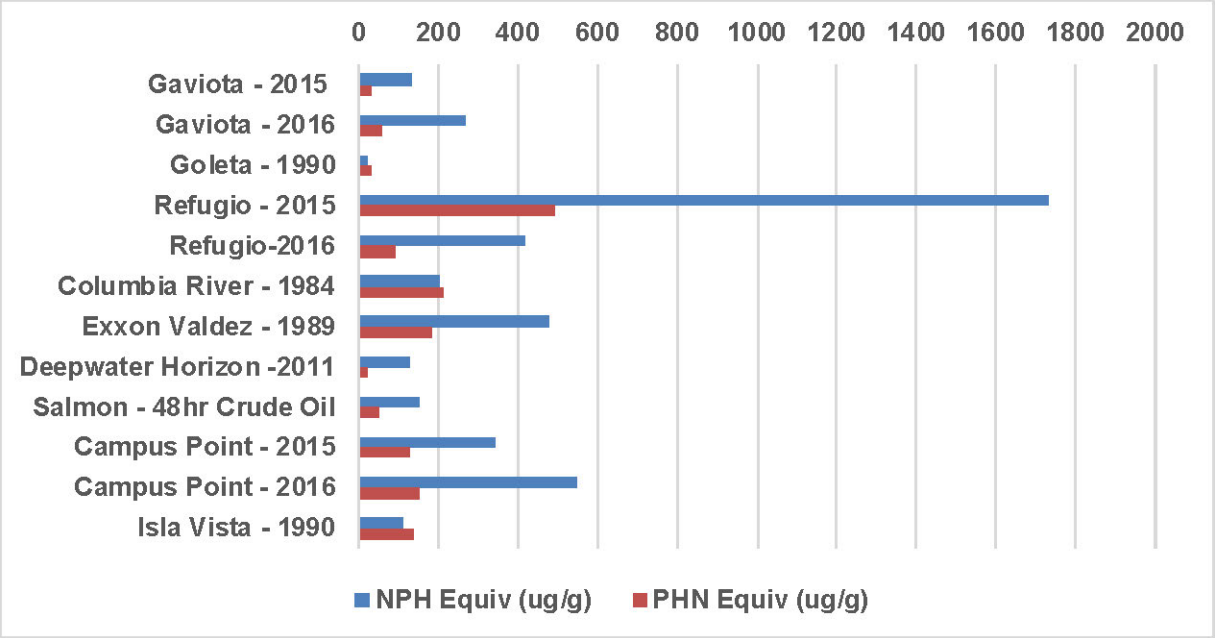

Figure 9. *Comparison of biliary naphthalene (NPH) and phenanthrene (PHN) equivalents (ug/g wet weight) mean concentrations from this study (2015, 2016 and salmon-48hr crude oil standard; barred surfperch at Gaviota and Refugio and walleye surfperch at Campus Point), previous studies in Santa Barbara (Goleta and Isla Vista, 1990; Spies et al., 1996) and following other oil spills (Krahn et al, 1986; Hom et al., 2008; Snyder et al., 2015).*

NPH equivalent mean concentrations measured in barred surfperch bile at Refugio State Beach in 2015 were higher than measured following other oil spills (Figures 9 and 10). In 1984, a tanker released more than 170,000 gallons of residual fuel oil into the Columbia River (Krahn et al., 1986). White sturgeon collected 5 days later and 57 miles downriver from the spill had significantly elevated mean concentrations of NPH (200 ug/g) and PHN (210 ug/g) equivalents, compared to the upriver reference site (32 and 9.7 ug/g respectively). Fish downriver of the spill were observed to have oil in their mouths and showed physical signs of stress (e.g., excess mucus secretion; Kennedy and Baca, 1984). In March 1989, 11 million gallons of Prudhoe Bay crude oil from the Exxon Valdez were released into Prince William Sound. Sol et al (2000) collected Dolly Varden (*Salvelinus malma*) 2-3 months after spill and found elevated levels of PAH metabolites in bile, associated with reduced plasma estradiol. Hom et al (1996; 2008) reported elevated PHN equivalents in pink salmon bile collected at an oiled site in 1989, compared to a reference location. Several studies documented that the Exxon Valdez spill adversely effected early life stages resulting in adverse effects on salmonid populations (Geiger et al., 1996; Incardona et al, 2013). One month following the North Cape oil spill of No. 2 fuel oil, winter flounder (*Pleuronectes americanus*) had elevated

levels of NPH and PHN equivalents in bile, compared to a reference site (Collier et al., 1997; Figure 10). It was concluded that exposure levels were sufficient to cause reproductive impairment, associated with reduced plasma estradiol levels. Effects to winter flounder early life stages were also reported (Hughes, 1999). Most recently, elevated levels of fish biliary PAH metabolites were reported following the 2010 Deepwater Horizon oil spill in the Gulf of Mexico (Snyder et al, 2015). An elevated incidence of skin lesions was observed in fish in 2011 and the incidence rate declined in 2012 (Murawski et al., 2014).

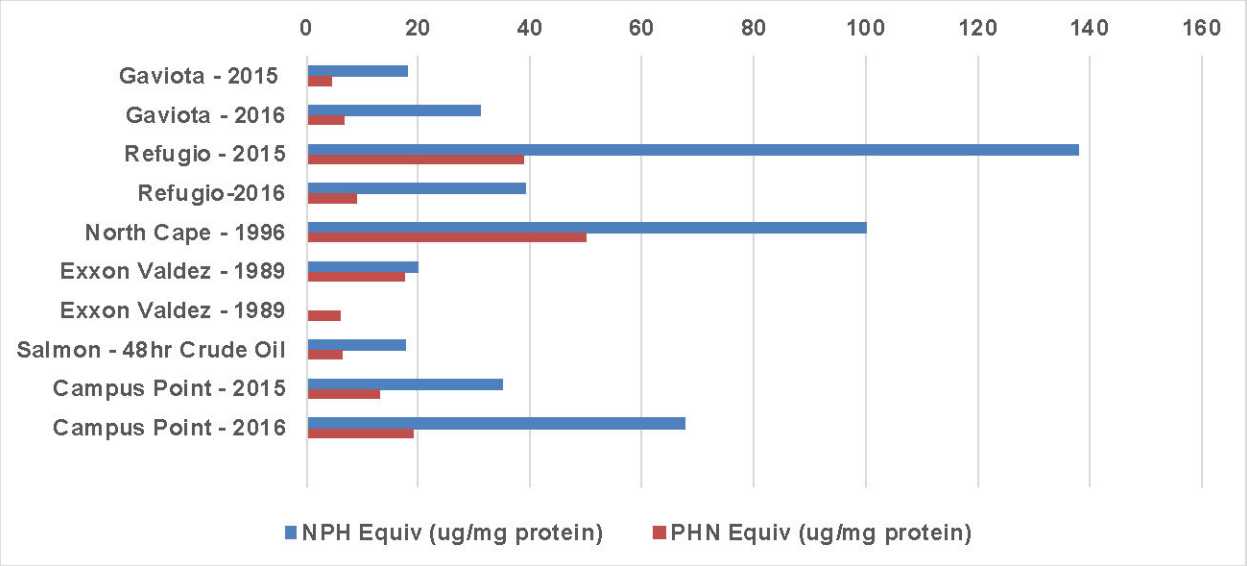

Figure 10. *Comparison of biliary naphthalene (NPH) and phenanthrene (PHN) equivalents (ug/mg bile protein) mean concentrations from this study (2015, 2016 and salmon-48hr crude oil standard; ; barred surfperch at Gaviota and Refugio and walleye surfperch at Campus Point) and following other oil spills (Collier et al., 1997; Hom et al., 1996; Sol et al., 2000).*

Biliary PAH metabolites have been shown to indicate uptake to fish from all exposure routes, providing an integrated estimate of recent PAH exposure (Meador et al., 2008). Many studies have used them as a biomarker of exposure for petroleum related contamination. In this assessment, a quantitative estimate of PAH exposure to surfperch was obtained, indicating that elevated exposure occurred in the surf zone at Refugio State Beach following the 2015 oil spill.

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Appendices:

Appendix 1: Analysis of Bile of Fish for Metabolites of Polycyclic Aromatic Compounds (PACs): Results from Samples Collected following the Refugio Beach Oil Spill, May 2015

Appendix 2: Analysis of Bile of Fish for Metabolites of Polycyclic Aromatic Compounds (PACs): Results from Samples Collected One Year after the 2015 Refugio Beach Oil Spill

Appendix 1

Analysis of Bile of Fish for Metabolites of Polycyclic Aromatic Compounds (PACs): Results from Samples Collected Following the Refugio Beach Oil Spill, May 2015

Bernadita Anulacion and Gina Ylitalo

Northwest Fisheries Science Center, NOAA Fisheries

Summary

Analyses that screen for metabolites of polycyclic aromatic compounds (PACs) were conducted for bile samples of fish collected from three sites in Santa Barbara, CA in the area of the Refugio Beach oil spill. Bile samples were collected from barred surfperch (*Amphistichus argenteus*) from the three sites that included the spill site, a natural oil seep site that was not reported as being oiled the day prior to the 23 May 2015 sampling and a reference site. Additionally, walleye surfperch (*Hyperprosopon argenteum*) were collected at the natural seep site. Concentrations of bile PAC metabolites showed site differences in the 3 types of metabolites measured, naphthalene (NPH), phenanthrene (PHN) and benzo[*a*]pyrene (BaP), based on wet weight or protein content. The levels in fish collected at both the spill and natural seep sites were higher than the reference site, and the concentrations in fish from the spill site were higher overall than the natural seep site. Barred surfperch from the Refugio Beach oil spill site had the highest levels of PAC metabolites, with concentrations being an order of magnitude higher than barred surfperch from Gaviota, the reference site, and approximately two times higher than barred surfperch from Campus Point, the natural seep site. Concentrations of PAC metabolites measured in bile of barred surfperch and walleye surfperch collected from Campus Point, the natural seep site, were 3 to 8 times higher than those in barred surfperch from Gaviota, the reference site. Bile PAC metabolites levels in barred surfperch from Refugio Beach were 2 times higher than those measured in barred surfperch from Campus Point and were 2 to 5 times higher compared to the walleye surfperch collected at this seep site.

Introduction

PACs are chemical contaminants that include polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, phenanthrene, pyrene, benzo[*a*]pyrene), as well as heterocyclic aromatic compounds (e.g., dibenzothiophene) that are primarily derived from petroleum or their combustion products. Concerns have been raised over the effects of exposure to PACs, alone or in combination with other toxic contaminants, on terrestrial and marine organisms because of the worldwide use of fossil fuels (Geraci and St. Aubin 1990; Peterson et al., 2003) and the occurrence of oil spills in regions that support populations of fish, birds, turtles and amphibians. In other vertebrates, such as fish, biological effects associated with exposure to PACs include developmental abnormalities, immunosuppression, hepatic lesions and altered growth (Myers et al. 1994; Heintz et al. 2000; Arkoosh et al. 2001; Meador et al. 2006; Reynaud and Deschaux

2006; Incardona et al. 2004; Incardona et al. 2012). Routes of PAC exposure in fish include consumption of contaminated food, inhalation, and dermal absorption.

Vertebrates (e.g., fish, marine mammals) rapidly take up PACs present in their food and the environment and quickly metabolize these compounds to more polar compounds. The more polar PAC metabolites are then secreted into fluids such as bile and urine for elimination via the gastroenteric tract or kidneys (Roubal et al., 1977; Varanasi et al., 1989; Krahn et al., 1984). Therefore, assessment of bile for PACs provides information on recent input and exposure to these compounds.

Methods

Bile samples were collected on May 23, 2015, 4 days after the spill occurred. Bile of barred surfperch was collected from Refugio Beach (n= 20), Campus Point (n = 5), and Gaviota (n = 5). Bile from walleye surfperch was collected from Campus Point (n= 7). Bile samples were collected from the gall bladder immediately after fish were sacrificed, placed into 4mL amber vials and kept on ice. Then, the samples were frozen and transported to the Northwest Fisheries Science Center and stored at -20°C until analyses.

Bile samples were analyzed using a high-performance liquid chromatography/fluorescence (HPLC-F) method described in Krahn et al., 1984. Briefly, bile was injected directly onto a Waters high-performance liquid chromatography/fluorescence system equipped with a C-18 reverse-phase column (Phenomenex Synergi Hydro). The fluorescent PAC metabolites were eluted with a linear gradient from 100% water (containing a trace amount of acetic acid) to 100% methanol at a flow of 1.0 mL/min. Chromatograms were recorded at the following wavelength pairs: 1) 292/335 nm where many 2-3 benzene ring aromatic compounds (e.g., naphthalene) fluoresce, 2) 260/380 nm where several 3-4 ring compounds (e.g., phenanthrene) fluoresce and 3) 380/430 nm where 4-5 ring compounds (e.g., benzo[a]pyrene) fluoresce. Peaks eluting after 9 minutes were integrated and the areas of these peaks were summed. The concentrations of fluorescent PACs in the bile samples of the fish were determined using naphthalene (NPH), phenanthrene (PHN) or benzo[a]pyrene (BaP) as external standards and converting the fluorescence response of bile to phenanthrene (ng PHN equivalents/g bile), naphthalene (ng NPH equivalents/g bile) or benzo[*a*]pyrene (ng BaP equivalents/g bile) equivalents. In addition, protein analysis as described in da Silva et al. (2006) was completed for all bile samples as previous laboratory contaminant exposure studies on fish have shown that normalization of biliary PAC metabolite concentrations to protein values can help account for variation in metabolite levels based on feeding status (Collier and Varanasi 1991).

To ensure that the HPLC/fluorescence system was in proper operating condition, a NPH/PHN/BaP calibration standard was analyzed numerous times ($n \geq 5$) until a relative standard deviation < 15% was obtained for each PAC. As part of our laboratory quality assurance (QA) plan (Sloan et al. 2006), a method blank and a fish bile control sample (bile of Atlantic salmon exposed to 25 µg/mL of Monterey crude oil for 48 hours) were analyzed with each batch of fish bile samples. In addition, an aliquot of a harbor seal bile sample (Bile_Ref_Mat) was also analyzed during the sample sequence as part of the QA plan.

Results

Concentrations of fluorescent PAC metabolites (based on wet weight or biliary protein) and levels of protein measured in the bile samples reported in Table 1. Two bile samples (RFB011BI and RFB001BI) were not analyzed for protein content due to inadequate bile volume (< 10 µL). For the barred surfperch from Refugio Beach, NPH equivalent concentrations ranged from 780,000 to 4,400,000 ng/g bile, wet weight and 67,000 to 270,000 ng/mg protein, PHN equivalents ranged from 200,000 to 1,200,000 ng/g bile, wet weight and 20,000 to 74,000 ng/mg protein and BaP equivalent values ranged from 1,100 to 6,700 ng/g bile, wet weight and 91 to 410 ng/mg protein. For the barred surfperch from Campus Point, NPH equivalent concentrations ranged from 420,000 to 1,800,000 ng/g bile, wet weight and 31,000 to 130,000 ng/mg protein, PHN equivalents ranged from 150,000 to 500,000 ng/g bile, wet weight and 10,000 to 35,000 ng/mg protein and BaP equivalent values ranged from 880 to 2,300 ng/g bile, wet weight and 55 to 160 ng/mg protein. For the walleye surfperch from Campus Point, NPH equivalent concentrations ranged from 150,000 to 610,000 ng/g bile, wet weight and 11,000 to 52,000 ng/mg protein, PHN equivalents ranged from 49,000 to 200,000 ng/g bile, wet weight and 3,700 to 19,000 ng/mg protein and BaP equivalent values ranged from 550 to 3,500 ng/g bile, wet weight and 48 to 340 ng/mg protein. For the barred surfperch from Gaviota, NPH equivalent concentrations ranged from 86,000 to 200,000 ng/g bile, wet weight and 14,000 to 26,000 ng/mg protein, PHN equivalents ranged from 22,000 to 43,000 ng/g bile, wet weight and 3,400 to 5,500 ng/mg protein and BaP equivalent values ranged from 240 to 510 ng/g bile, wet weight and 32 to 65 ng/mg protein. In addition, biliary protein concentrations ranged from 6.2 to 21.0 mg/mL in the barred surfperch bile samples and 4.2 to 15.2 mg/mL in the walleye surfperch.

The mean (± SD) biliary NPH, PHN and BaP equivalent concentrations (ng/g bile, wet weight) measured in barred surfperch collected at Refugio Beach, Campus Point and Gaviota are shown in Figure 1A–C. Significant differences (ANOVA $p < 0.05$; Tukey-Kramer HSD test) in mean NPH, PHN and BaP equivalent concentrations (based on wet weight or biliary protein) were found among collection sites. For each PAC metabolite, barred surfperch from the oiled site had a significantly higher mean level than those determined in fish from the seep site or from the reference site. Mean PAC metabolite concentrations measured in bile of barred surfperch from the seep site were significantly higher than the same metabolites measured in barred surfperch from the reference site. At the Campus Point site, mean levels of biliary NPH and PHN equivalents (based on wet weight only) were significantly higher (ANOVA $p < 0.05$; t-test) in barred surfperch than those measured in walleye surfperch; no other significant differences were found for mean PAC equivalent concentrations between species.

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Appendix 1

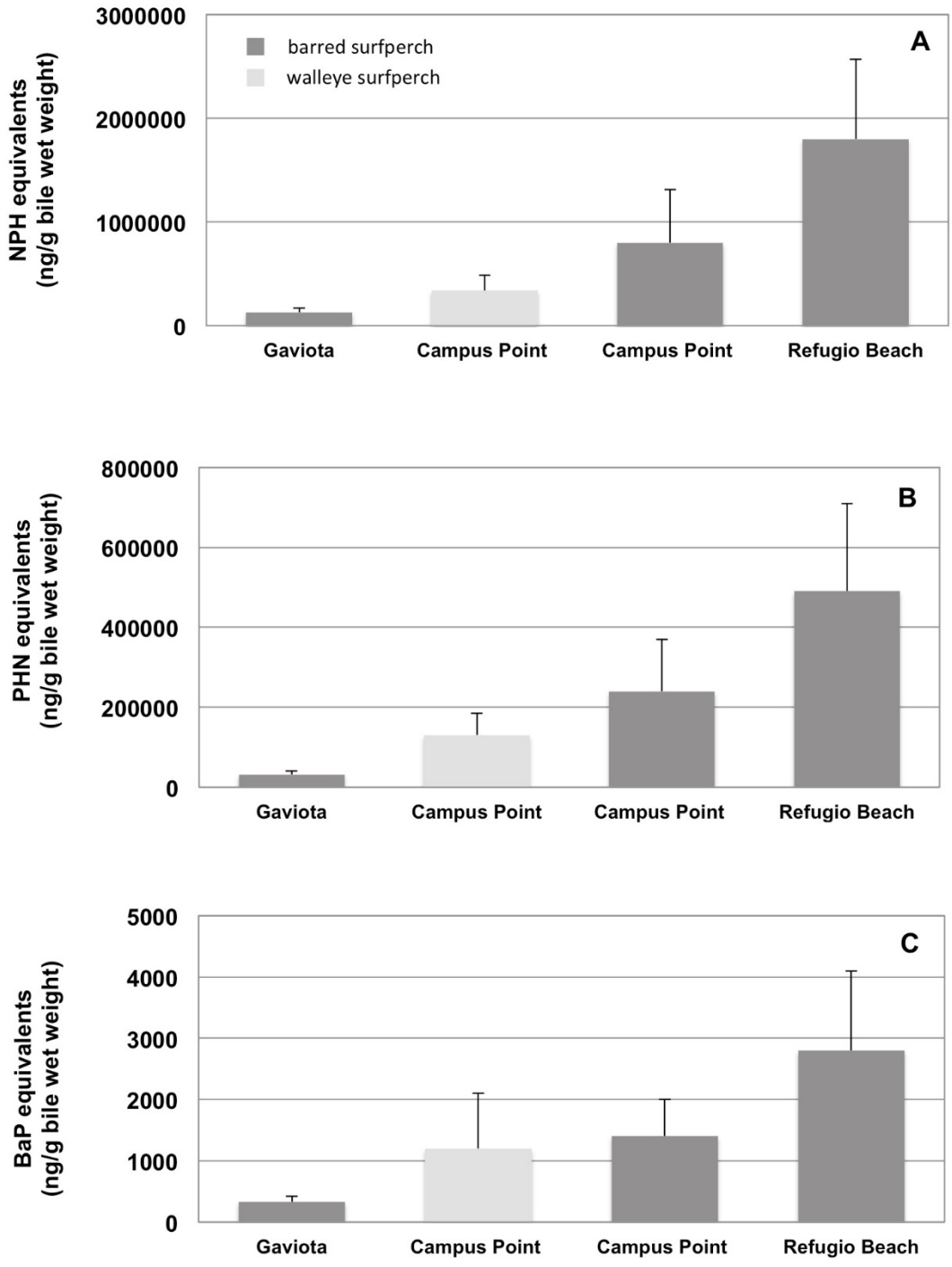

Figure 1. Mean (±SD) concentrations of bile equivalents of (A) naphthalene, NPH, (B) phenanthrene, PHN and (C) benzo[*a*]pyrene, BaP (ng/g bile wet weight) measured in two fish species collected from three sites in the area of the Refugio Beach oil spill.

Appendix 1

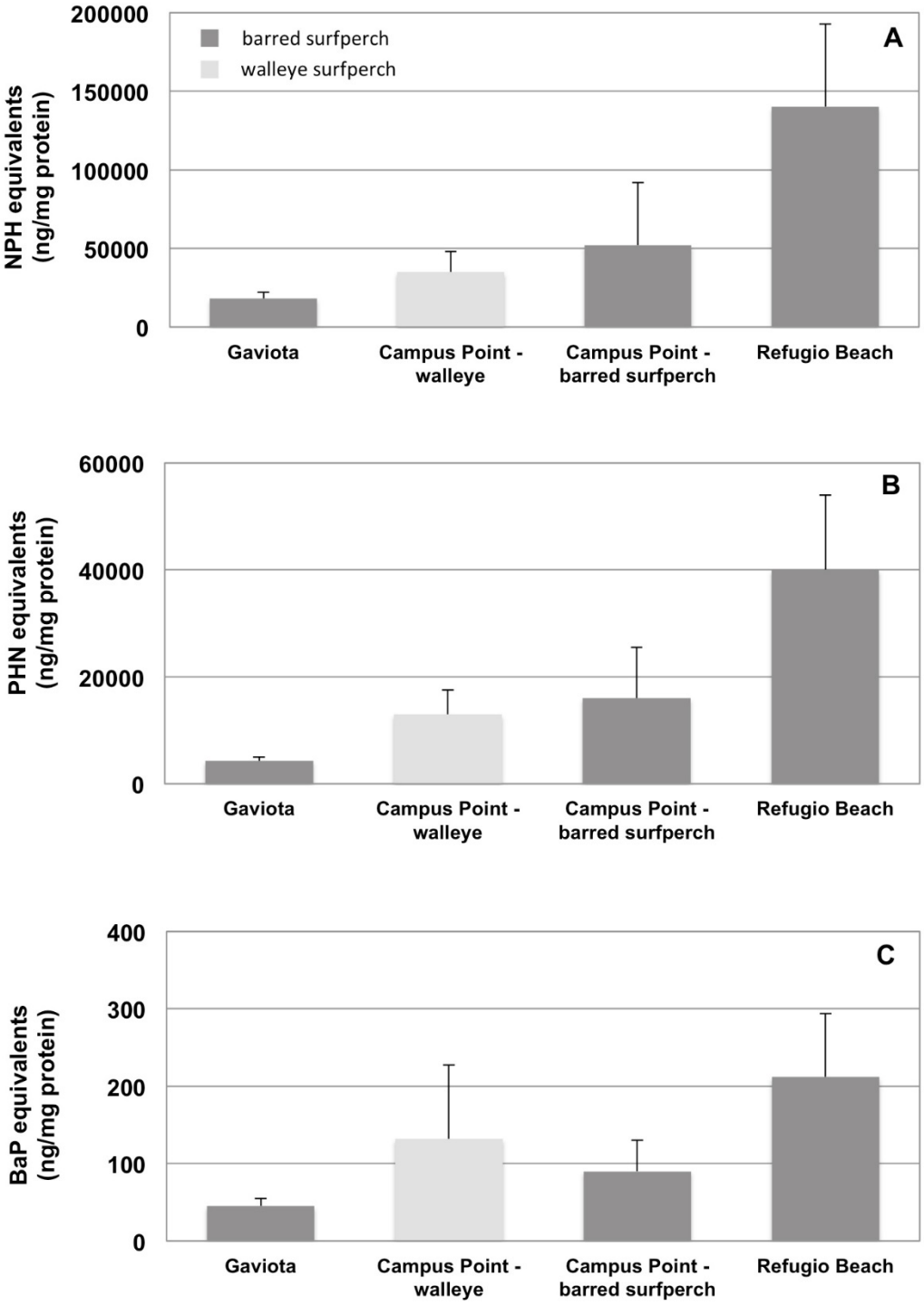

Figure 2. Mean (±SD) concentrations of bile equivalents (A) naphthalene, NPH, (B) phenanthrene, PHN and (C) benzo[*a*]pyrene, BaP (ng/mg protein) measured in two fish species collected from three sites in the area of the Refugio Beach oil spill.

Appendix 1

Table 1. Concentrations of metabolites in polycyclic aromatic compounds measured in bile of fish collected on May 23, 2015 in the area of the Refugio Beach oil spill, Santa Barbara, CA

				Equivalents of fluorescent aromatic compounds (ng/g bile, wet weight)			Equivalents of fluorescent aromatic compounds (ng/mg protein)		
Site	**FIELD NUMBER**	**SPECIES**	**Protein mg/mL**	**NPH Equivalents[1]**	**PHN Equivalents[2]**	**BaP Equivalents[3]**	**NPH Equivalents[1]**	**PHN Equivalents[2]**	**BaP Equivalents[3]**
Refugio Beach	RFB009BI	barred surfperch	12.6	1,000,000	280,000	1,500	79,000	22,000	120
Refugio Beach	RFB007BI	barred surfperch	15.1	3,100,000	940,000	5,100	210,000	62,000	340
Refugio Beach	RFB015BI	barred surfperch	9.0	1,500,000	400,000	2,300	170,000	44,000	260
Refugio Beach	RFB019BI	barred surfperch	9.9	2,100,000	560,000	3,100	210,000	57,000	310
Refugio Beach	RFB012BI	barred surfperch	8.7	1,100,000	320,000	2,000	130,000	37,000	230
Refugio Beach	RFB018BI	barred surfperch	16.3	4,400,000	1,200,000	6,700	270,000	74,000	410
Refugio Beach	RFB002BI	barred surfperch	17.6	1,400,000	370,000	1,600	80,000	21,000	91
Refugio Beach	RFB020BI	barred surfperch	9.3	1,700,000	440,000	2,500	180,000	47,000	270
Refugio Beach	RFB017BI	barred surfperch	15.5	1,500,000	560,000	2,800	97,000	36,000	180
Refugio Beach	RFB014BI	barred surfperch	19.7	1,900,000	530,000	3,000	96,000	27,000	150
Refugio Beach	RFB004BI	barred surfperch	8.8	780,000	200,000	1,100	89,000	23,000	130
Refugio Beach	RFB011BI	barred surfperch	*ND*	2,200,000	560,000	5,300			
Refugio Beach	RFB008BI	barred surfperch	7.2	1,100,000	280,000	1,300	150,000	39,000	180

Refugio Beach	RFB006BI	barred surfperch	16.1	2,200,000	620,000	3,300	140,000	39,000	200
Refugio Beach	RFB010BI	barred surfperch	8.6	1,500,000	430,000	2,400	170,000	50,000	280
Refugio Beach	RFB001BI	barred surfperch	*ND*	1,900,000	560,000	3,500			
Refugio Beach	RFB016BI	barred surfperch	13.0	1,400,000	380,000	2,200	110,000	29,000	170
Refugio Beach	RFB003BI	barred surfperch	12.0	1,400,000	380,000	2,100	120,000	32,000	180
Refugio Beach	RFB005BI	barred surfperch	10.6	1,700,000	470,000	2,700	160,000	44,000	250
Refugio Beach	RFB013BI	barred surfperch	16.5	1,100,000	330,000	1,800	67,000	20,000	110
Campus Point	CMP002,003BI	barred surfperch	12.6	420,000	150,000	920	33,000	12,000	73
Campus Point	CMP004,005BI	barred surfperch	14.9	480,000	180,000	1,000	32,000	12,000	67
Campus Point	CMP006,007BI	barred surfperch	16.1	570,000	180,000	880	35,000	11,000	55
Campus Point	CMP008,009BI	barred surfperch	14.4	1,800,000	500,000	2,300	130,000	35,000	160
Campus Point	CMP010,011BI	barred surfperch	21.0	660,000	210,000	1,700	31,000	10,000	81
Campus Point	CMP017BI	walleye surfperch	4.2	220,000	79,000	550	52,000	19,000	130
Campus Point	CMP012,013BI	walleye surfperch	7.3	380,000	140,000	1,900	52,000	19,000	260
Campus Point	CMP025,026BI	walleye surfperch	11.7	610,000	190,000	1,200	52,000	16,000	100
Campus Point	CMP018,019BI	walleye surfperch	10.3	240,000	97,000	3,500	23,000	9,400	340
Campus Point	CMP020,021BI	walleye surfperch	13.1	150,000	49,000	630	11,000	3,700	48

Appendix 1

Campus Point	CMP014,015BI	walleye surfperch	15.2	500,000	200,000	990	33,000	13,000	65
Campus Point	CMP023,024BI	walleye surfperch	8.0	250,000	92,000	740	31,000	12,000	93
Gaviota	GAV002BI	barred surfperch	6.2	96,000	26,000	270	15,000	4,200	44
Gaviota	GAV004,005BI	barred surfperch	7.9	110,000	27,000	250	14,000	3,400	32
Gaviota	GAV006,007BI	barred surfperch	7.4	140,000	35,000	340	19,000	4,700	46
Gaviota	GAV008BI	barred surfperch	7.8	200,000	43,000	510	26,000	5,500	65
Gaviota	GAV009BI	barred surfperch	8.7	170,000	40,000	390	20,000	4,600	45

[1]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of naphthalene standard at 292/335 nm wavelengths.

[2]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of phenanthrene standard at 260/380 nm wavelengths.

[3]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of benzo[*a*]pyrene standard at 380/430 nm wavelengths.

ND – no data due to inadequate amount of sample to conduct protein analyses.

Appendix 2: Analysis of Bile of Fish for Metabolites of Polycyclic Aromatic Compounds (PACs):

Results from Samples Collected One Year after the 2015 Refugio Beach Oil Spill

Appendix 2

Analysis of Bile of Fish for Metabolites of Polycyclic Aromatic Compounds (PACs): Results from Samples Collected One Year after the 2015 Refugio Beach Oil Spill

Bernadita Anulacion and Gina Ylitalo

Northwest Fisheries Science Center, NOAA Fisheries

Summary

Analyses that screen for metabolites of polycyclic aromatic compounds (PACs) have been completed for bile samples of fish collected from three sites in Santa Barbara, CA one year after the 2015 Refugio Beach oil spill. Bile samples were collected from barred surfperch (*Amphistichus argenteus*) and walleye surfperch (*Hyperprosopon argenteum*) from the three sites that included the spill site, a natural oil seep site that was not reported as being oiled the day prior to the 23 May 2015 sampling but may have had some oil exposure subsequently and a reference site. Mean biliary concentrations of PAC metabolites did not show significant site differences in the three types of metabolites measured, naphthalene (NPH), phenanthrene (PHN) and benzo[*a*]pyrene (BaP), based on wet weight or protein content except the mean PHN equivalent concentrations in barred surfperch from the oiled Refugio Beach site and the reference site (Gaviota). At the Refugio Beach oiled site, mean concentrations of all PAC metabolites in the 2016 collected fish were significantly higher in walleye surfperch compared to barred surfperch except protein-corrected NPH. Barred surfperch collected at Refugio Beach in 2015 had significantly higher mean NPH, PHN and BaP concentrations (wet weight and protein-corrected) than those determined in the 2016 barred surfperch collected one year later. In contrast, Gaviota barred surfperch collected in 2016 had significantly higher mean concentrations of NPH equivalents (wet weight and protein-corrected) and PHN equivalents (wet weight only) than the mean values of the 2015 Gaviota barred surfperch. Similarly, walleye surfperch collected from Campus Point in 2016 had significantly higher mean concentrations of protein-corrected NPH, PHN and BaP equivalents, as well as NPH equivalents (wet weight only) than walleye collected from this site in 2015.

Introduction

Polycyclic aromatic hydrocarbons (PACs) are chemical contaminants that include polycyclic aromatic hydrocarbons (PAHs) such as naphthalene, phenanthrene, pyrene, benzo[*a*]pyrene), as well as heterocyclic aromatic compounds (e.g., dibenzothiophene) that are primarily derived from petroleum or their combustion products. Concerns have been raised over the effects of exposure to PACs, alone or in combination with other toxic contaminants, on terrestrial and marine organisms because of the worldwide use of fossil fuels (Geraci and St. Aubin 1990; Peterson et al., 2003) and the occurrence of oil spills in regions that support populations of fish, birds, turtles and amphibians. In other vertebrates, such as fish, biological effects associated with exposure to PACs include developmental abnormalities, immunosuppression, hepatic

lesions and altered growth (Myers et al. 1994; Heintz et al. 2000; Arkoosh et al. 2001; Meador et al. 2006; Reynaud and Deschaux 2006; Incardona et al. 2004; Incardona et al. 2012). Routes of PAC exposure in fish include consumption of contaminated food or sediment, respiration and dermal absorption.

Vertebrates (e.g., fish, marine mammals) rapidly take up PACs present in their food and the environment and quickly metabolize these compounds to more polar compounds. The more polar PAC metabolites are then secreted into fluids such as bile and urine for elimination via the gastroenteric tract or kidneys (Roubal et al., 1977; Varanasi et al., 1989; Krahn et al., 1984). Therefore, assessment of bile for PACs provides information on recent input and exposure to these compounds.

Methods

Bile samples were collected on May 18, 2016, one year after the Refugio Beach oil spill occurred. Bile samples of barred surfperch were collected from Refugio Beach (n = 7), Campus Point (n = 1), and Gaviota (n = 5). Bile from walleye surfperch were collected from Refugio Beach (n = 13), Campus Point (n= 19), and Gaviota (n = 1). Bile samples were collected from the gall bladder immediately after fish were sacrificed, placed into 4mL amber vials and kept on ice. Then, the samples were frozen and transported to the Northwest Fisheries Science Center and stored at -20°C until analyses.

Bile samples were analyzed using a high-performance liquid chromatography/fluorescence (HPLC-F) method described in Krahn et al., 1984. Briefly, bile was injected directly onto a Waters high-performance liquid chromatography/fluorescence system equipped with a C-18 reverse-phase column (Phenomenex Synergi Hydro). The fluorescent PAC metabolites were eluted with a linear gradient from 100% water (containing a trace amount of acetic acid) to 100% methanol at a flow of 1.0 mL/min. Chromatograms were recorded at the following wavelength pairs: 1) 292/335 nm where many 2-3 benzene ring aromatic compounds (e.g., naphthalene) fluoresce, 2) 260/380 nm where several 3-4 ring compounds (e.g., phenanthrene) fluoresce and 3) 380/430 nm where 4-5 ring compounds (e.g., benzo[*a*]pyrene) fluoresce. Peaks eluting after 9 minutes were integrated and the areas of these peaks were summed. The concentrations of fluorescent PACs in the bile samples of the fish were determined using naphthalene (NPH), phenanthrene (PHN) or benzo[*a*]pyrene (BaP) as external standards and converting the fluorescence response of bile to phenanthrene (ng PHN equivalents/g bile), naphthalene (ng NPH equivalents/g bile) or benzo[*a*]pyrene (ng BaP equivalents/g bile) equivalents. In addition, protein analysis as described in da Silva et al. (2006) was completed for all bile samples as previous laboratory contaminant exposure studies on fish have shown that normalization of biliary PAC metabolite concentrations to protein values can help account for variation in metabolite levels based on feeding status (Collier and Varanasi 1991).

To ensure that the HPLC/fluorescence system was in proper operating condition, a NPH/PHN/BaP calibration standard was analyzed numerous times ($n \geq 5$) until a relative standard deviation < 15% was obtained for each PAC. As part of our laboratory quality assurance (QA) plan (Sloan et al. 2006), a method blank and a fish bile control sample (bile of

Atlantic salmon exposed to 25 µg/mL of Monterey crude oil for 48 hours) were analyzed with each batch of fish bile samples.

Results

Concentrations of fluorescent PAC metabolites (based on wet weight or biliary protein) and protein content measured in the bile samples collected in 2016 are reported in Table 1. One bile sample from a walleye surfperch from Refugio Beach (RSBFI1051816BI7) was not analyzed for protein content due to inadequate bile volume (< 10 µL). All sample batches met our laboratory quality assurance criteria (Tables 2 and 3).

A wide range of fluorescent PAC metabolite concentrations were measured in the bile of the fish collected in 2016 (Table 1). For the barred surfperch from Refugio Beach, the NPH equivalent concentrations ranged from 230,000 to 590,000 ng/g bile, wet weight and 23,000 to 78,000 ng/mg protein, PHN equivalents ranged from 51,000 to 140,000 ng/g bile, wet weight and 4,900 to 22,000 ng/mg protein and BaP equivalent values ranged from 430 to 1,200 ng/g bile, wet weight and 33 to 180 ng/mg protein. For the barred surfperch from Campus Point, NPH equivalent concentrations were 550,000 ng/g bile, wet weight and 63,000 ng/mg protein, PHN equivalents were 140,000 ng/g bile, wet weight and 16,000 ng/mg protein and BaP equivalent values were 1,300 ng/g bile, wet weight and 150 ng/mg protein. For the barred surfperch from Gaviota, the NPH equivalent concentrations ranged from 190,000 to 530,000 ng/g bile, wet weight and 22,000 to 44,000 ng/mg protein, PHN equivalents ranged from 44,000 to 100,000 ng/g bile, wet weight and 4,400 to 9,800 ng/mg protein and BaP equivalent values ranged from 540 to 7,200 ng/g bile, wet weight and 29 to 650 ng/mg protein. For the walleye surfperch from Refugio Beach, the NPH equivalent concentrations ranged from 410,000 to 6,700,000 ng/g bile, wet weight and 29,000 to 420,000 ng/mg protein, PHN equivalents ranged from 93,000 to 2,000,000 ng/g bile, wet weight and 7,300 to 130,000 ng/mg protein and BaP equivalent values ranged from 550 to 19,000 ng/g bile, wet weight and 74 to 1,200 ng/mg protein. For the walleye surfperch from Campus Point, the NPH equivalent concentrations ranged from 150,000 to 1,100,000 ng/g bile, wet weight and 19,000 to 94,000 ng/mg protein, PHN equivalents ranged from 41,000 to 290,000 ng/g bile, wet weight and 5,100 to 27,000 ng/mg protein and BaP equivalent values ranged from 550 to 5,900 ng/g bile, wet weight and 68 to 840 ng/mg protein. For the walleye surfperch from Gaviota, the NPH equivalent concentrations were 810,000 ng/g bile, wet weight and 91,000 ng/mg protein, PHN equivalents were 230,000 ng/g bile, wet weight and 26,000 ng/mg protein and BaP equivalent values were 2,200 ng/g bile, wet weight and 250 ng/mg protein. Biliary protein concentrations in barred surfperch and walleye surfperch ranged from 4.5 to 22.5 ng/mg and 4.3 to 19.9 ng/mg, respectively.

Mean NPH, PHN and BaP equivalent concentrations based on wet weight (Figure 1) or biliary protein (data not shown) (log_{10} transformed data) for each species collected at the oiled (Refugio Beach), natural seep (Campus Point) and reference (Gaviota) sites were not significantly different (ANOVA $p > 0.05$) in fish collected one year after the spill except PHN

equivalent levels (wet weight only) in barred surfperch from the oiled Refugio Beach site and Gaviota (reference site) ($p = 0.0487$). This is in contrast to our findings for barred surfperch collected at the same three sampling sites approximately one week (May 2015) after the spill occurred (Anulacion and Ylitalo 2015), in which the mean PAC metabolite levels in fish collected at both the spill and natural seep sites were significantly higher than those at the reference site, and the concentrations in barred surfperch from the spill site were higher overall than the natural seep site.

At the Refugio Beach site, mean concentrations of all PAC metabolites based on wet weight (Figure 1) ($\log_{10}$ transformed data) in the 2016 collected fish were significantly higher in walleye surfperch compared to barred surfperch (ANOVA $p < 0.05$; t-test). Similarly, mean levels of protein-corrected PAC metabolites were significantly higher (ANOVA $p < 0.05$; t-test) in walleye compared to barred surfperch except protein-corrected NPH ($p = 0.0505$). Comparisons between species at the other two collection sites were not conducted due to inadequate numbers of bile samples ($n = 1$ for barred surfperch from Campus Point and $n = 1$ for surfperch from Gaviota).

We examined differences in mean concentrations of PAC metabolites based on sampling year for each species collected at the same site (Figure 2). Barred surfperch collected at Refugio Beach in 2015 had significantly higher (ANOVA $p < 0.0001$; t-test) mean NPH, PHN and BaP concentrations (wet weight and protein-corrected) ($\log_{10}$ transformed data) than those determined in the 2016 collected barred surfperch. In contrast, Gaviota barred surfperch collected in 2016 had significantly higher mean concentrations ($\log_{10}$ transformed data) (ANOVA $p < 0.05$; t-test) of NPH equivalents (wet weight and protein-corrected) and PHN equivalents (wet weight only) than the mean values of the 2015 fish; no other significant differences were found for barred surfperch from this site. Walleye surfperch collected from Campus Point in 2016 had significantly higher mean concentrations of protein-corrected NPH, PHN and BaP equivalents, as well as NPH equivalents (wet weight only). No other significant differences (ANOVA $p > 0.05$) in mean concentrations of PAC metabolites were found for walleye surfperch from this site.

References

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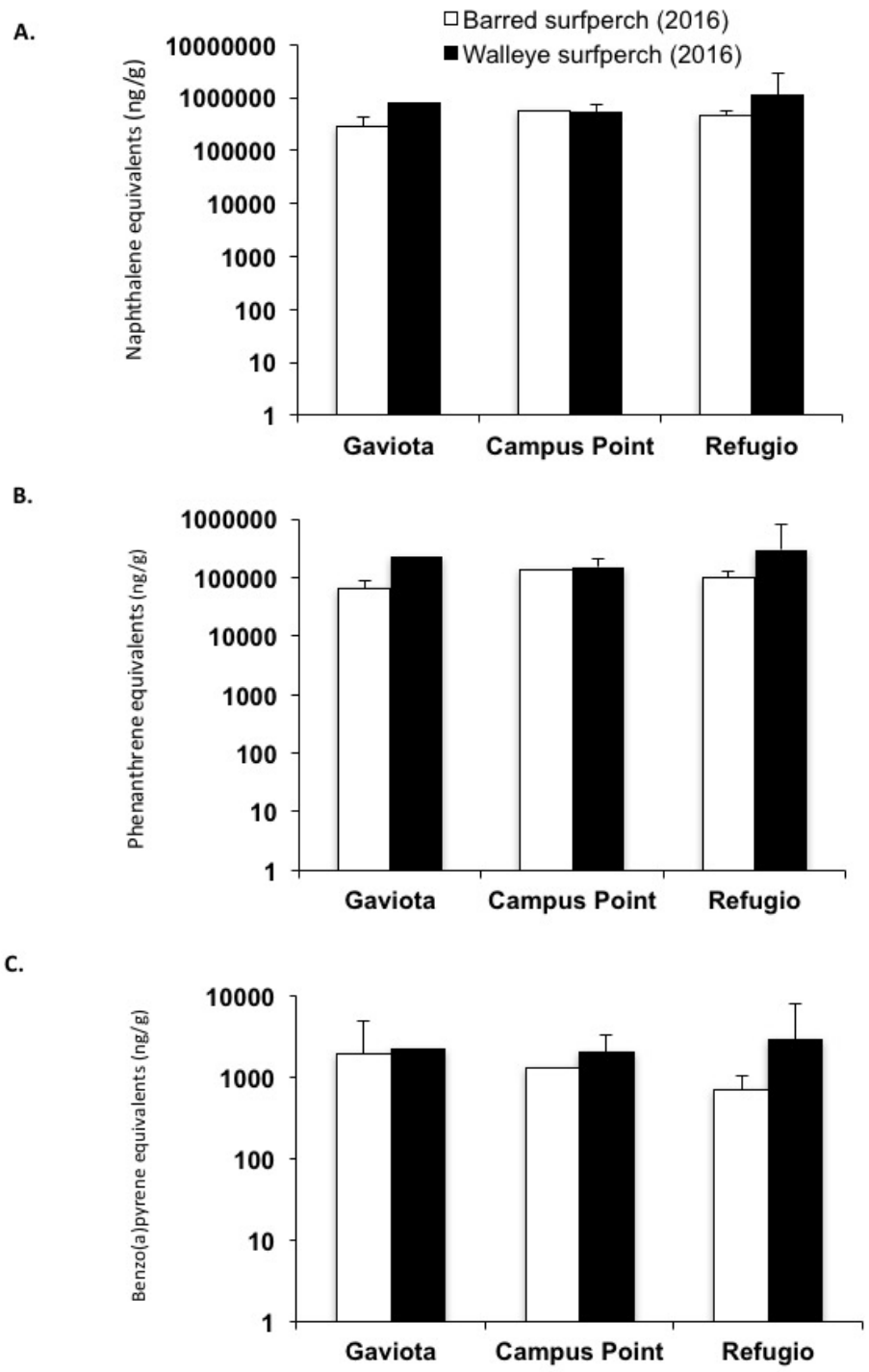
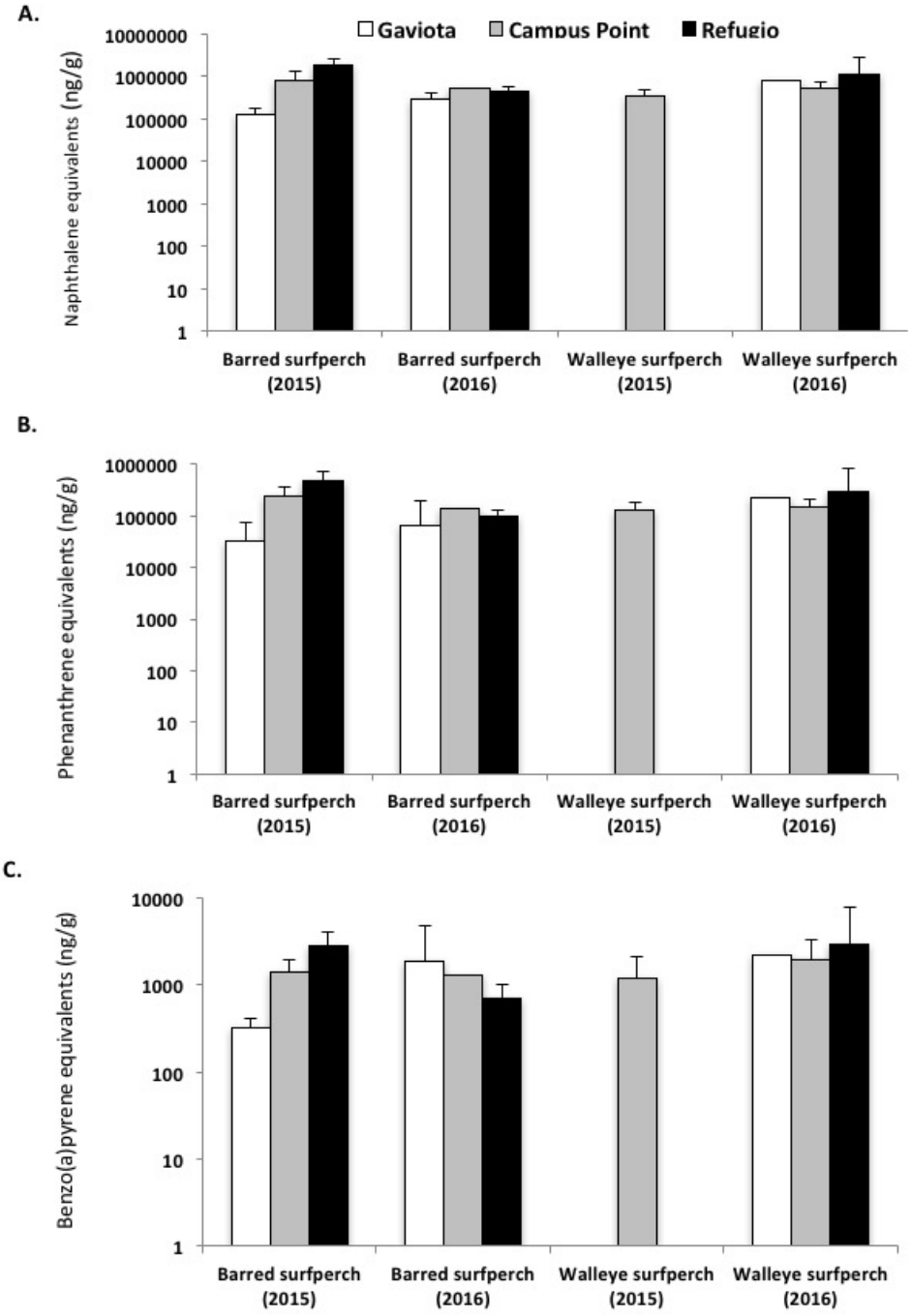
Appendix 2

Figure 1. Mean (±SD) concentrations of bile equivalents of (A) naphthalene, NPH, (B) phenanthrene, PHN and (C) benzo[*a*]pyrene, BaP (ng/g bile wet weight) measured in two fish species collected one year following the 2015 Refugio Beach oil spill.



Appendix 2

Figure 2. Mean (±SD) concentrations of bile equivalents (A) naphthalene, NPH, (B) phenanthrene, PHN and (C) benzo[*a*]pyrene, BaP (ng/g bile wet weight) measured in two fish species collected in 2015 and 2016 showing the differences in mean concentrations of PAC metabolites based on sampling year for each species collected at the same site



Appendix 2

Table 1. Concentrations of metabolites of polycyclic aromatic compounds measured in bile of barred surfperch and walleye surfperch collected in 2016, one year after the 2015 Refugio Beach Oil Spill.

Species	Laboratory ID number	Collection site	Field ID number	Protein, mg/mL	Equivalents of fluorescent aromatic compounds (ng/g bile, wet weight)			Protein-corrected equivalents of fluorescent aromatic compounds (ng/mg biliary protein)		
					NPH[1]	PHN[2]	BaP[3]	NPH[1]	PHN[2]	BaP[3]
Barred surfperch	136-0436	Campus Point	CMPFI1051816BI20	8.8	550,000	140,000	1,300	63,000	16,000	150
	136-0444	Gaviota	GAVFI1051816BI5	4.5	200,000	44,000	540	44,000	9,800	120
	136-0393	Gaviota	GAVFI1051816BI4	7.7	220,000	47,000	560	29,000	6,100	73
	136-0422	Gaviota	GAVFI1051816BI2	11.1	240,000	63,000	7,200	22,000	5,700	650
	136-0434	Gaviota	GAVFI1051816BI3	10.4	280,000	64,000	630	27,000	6,200	61
	136-0404	Gaviota	GAVFI1051816BI1	22.5	530,000	100,000	650	24,000	4,400	29
	136-0406	Refugio Beach	RSBFI1051816BI4	7.1	240,000	52,000	460	34,000	7,300	65
	136-0400	Refugio Beach	RSBFI1051816BI5	15.4	360,000	75,000	510	23,000	4,900	33
	136-0414	Refugio Beach	RSBFI1051816BI6	14.6	360,000	80,000	500	25,000	5,500	34
	136-0412	Refugio Beach	RSBFI1051816BI1	14.4	500,000	110,000	950	35,000	7,600	66
	136-0402	Refugio Beach	RSBFI1051816BI17	6.5	510,000	140,000	1,200	78,000	22,000	180
	136-0431	Refugio Beach	RSBFI1051816BI2	20.2	560,000	120,000	910	28,000	5,900	45
	136-0432	Refugio Beach	RSBFI1051816BI3	10.3	590,000	120,000	430	57,000	12,000	42
Walleye surfperch	136-0413	Campus Point	CMPFI1051816BI10	8.1	150,000	41,000	550	19,000	5,100	68
	136-0405	Campus Point	CMPFI1051816BI17	5.5	290,000	81,000	4,600	53,000	15,000	840
	136-0430	Campus Point	CMPFI1051816BI8	4.3	290,000	81,000	1,000	67,000	19,000	230
	136-0440	Campus Point	CMPFI1051816BI11	4.5	330,000	98,000	1,100	73,000	22,000	240
	136-0398	Campus Point	CMPFI1051816BI1	4.5	360,000	110,000	1,100	80,000	24,000	240
	136-0443	Campus Point	CMPFI1051816BI3	5.7	370,000	110,000	1,100	65,000	19,000	190
	136-0401	Campus Point	CMPFI1051816BI19	8.8	390,000	98,000	5,900	44,000	11,000	670
	136-0437	Campus Point	CMPFI1051816BI2	10.9	410,000	130,000	1,000	38,000	12,000	92
	136-0397	Campus Point	CMPFI1051816BI18	4.9	440,000	120,000	2,900	90,000	24,000	590
	136-0396	Campus Point	CMPFI1051816BI4	6.1	480,000	120,000	1,700	79,000	20,000	280
	136-0421	Campus Point	CMPFI1051816BI14	7.4	510,000	160,000	1,500	69,000	22,000	200
	136-0435	Campus Point	CMPFI1051816BI16	6.8	540,000	170,000	1,700	79,000	25,000	250
	136-0419	Campus Point	CMPFI1051816BI12	9.1	550,000	150,000	1,800	60,000	16,000	200
	136-0442	Campus Point	CMPFI1051816BI13	8.4	670,000	200,000	1,900	80,000	24,000	230
	136-0418	Campus Point	CMPFI1051816BI15	8.6	730,000	230,000	2,200	85,000	27,000	260
	136-0420	Campus Point	CMPFI1051816BI6	7.8	730,000	210,000	1,800	94,000	27,000	230
	136-0415	Campus Point	CMPFI1051816BI5	15.1	740,000	170,000	2,000	49,000	11,000	130
	136-0394	Campus Point	CMPFI1051816BI9	9.1	760,000	200,000	2,700	84,000	22,000	300
	136-0425	Campus Point	CMPFI1051816BI7	15.3	1,100,000	290,000	2,400	72,000	19,000	160
	136-0408	Gaviota	GAVFI1051816BI6	8.9	810,000	230,000	2,200	91,000	26,000	250
	136-0399	Refugio Beach	RSBFI1051816BI15	9.4	410,000	93,000	1,300	44,000	9,900	140
	136-0426	Refugio Beach	RSBFI1051816BI14	6.8	420,000	120,000	1,000	62,000	18,000	150
	136-0407	Refugio Beach	RSBFI1051816BI7	IS[5]	470,000	96,000	550	ND	ND	ND
	136-0433	Refugio Beach	RSBFI1051816BI16	13.3	520,000	120,000	1,300	39,000	9,000	98
	136-0441	Refugio Beach	RSBFI1051816BI18	17.8	520,000	130,000	1,400	29,000	7,300	79
	136-0438	Refugio Beach	RSBFI1051816BI12	14.8	540,000	110,000	1,100	36,000	7,400	74
	136-0439	Refugio Beach	RSBFI1051816BI20	8.1	550,000	160,000	1,600	68,000	20,000	200
	136-0416	Refugio Beach	RSBFI1051816BI13	11.1	600,000	170,000	1,900	54,000	15,000	170
	136-0417	Refugio Beach	RSBFI1051816BI10	5.6	740,000	200,000	1,600	130,000	36,000	290
	136-0424	Refugio Beach	RSBFI1051816BI9	14.5	780,000	210,000	2,100	54,000	14,000	140
	136-0395	Refugio Beach	RSBFI1051816BI11	12.3	870,000	240,000	2,300	71,000	20,000	190
	136-0423	Refugio Beach	RSBFI1051816BI8	19.9	1,200,000	320,000	2,800	60,000	16,000	140
	136-0403	Refugio Beach	RSBFI1051816BI19	15.9	6,700,000	2,000,000	19,000	420,000	130,000	1,200

[1]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of naphthalene standard at 292/335 nm wavelengths.

[2]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of phenanthrene standard at 260/380 nm wavelengths.

[3]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of benzo[a]pyrene standard at 380/430 nm wavelengths.

[4]IS - insufficient sample available for protein analysis

ND = protein-corrected PAC metabolite concentrations not determined due to insufficient sample available for protein analysis

Appendix 2

Table 2. Concentrations of metabolites of polycyclic aromatic compounds measured in bile reference material and method blanks analyzed one year after the 2015 Refugio Beach Oil Spill.

Quality assurance sample type	Quality assurance sample information	Analysis date	Equivalents of fluorescent aromatic compounds (ng/g bile, wet weight) NPH[1]	PHN[2]	BaP[3]
ASMBC2[4]	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/22/16	160,000	53,000	1,500
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/22/16	180,000	58,000	1,700
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/22/16	180,000	56,000	1,700
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/24/16	160,000	52,000	1,500
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/24/16	160,000	52,000	1,500
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/24/16	160,000	52,000	1,600
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/25/16	160,000	52,000	1,600
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/25/16	160,000	50,000	1,400
	Atlantic salmon exposed to Monterey Bay crude oil for 48 hours.	06/25/16	160,000	51,000	1,500
Method blank[5]	Methanol blank A	06/22/16	610	83	21
	Methanol blank C	06/22/16	400	54	26
	Methanol blank F	06/22/16	640	94	26
	Methanol blank A	06/24/16	600	100	29
	Methanol blank C	06/24/16	890	100	27
	Methanol blank F	06/24/16	810	90	26
	Methanol blank A	06/25/16	640	130	27
	Methanol blank C	06/25/16	780	91	27
	Methanol blank F	06/25/16	590	99	23

Bile Reference Material ASMBC2	Equivalents of fluorescent aromatic compounds (ng/g bile, wet weight) NPH[1]	PHN[2]	BaP[3]
Mean	150,000	50,000	1,200
SD	14,000	4,900	230
Upper Control Limit	180,000	60,000	1,700
Lower Control Limit	120,000	40,000	740

[1]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of naphthalene standard at 292/335 nm wavelengths.

[2]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of phenanthrene standard at 260/380 nm wavelengths.

[3]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of benzo[a]pyrene standard at 380/430 nm wavelengths.

[4]NWFSC Quality Assurance Criterion (from Sloan et al. 2006): Reference material (3/set): analyte concentrations will be ≤ 2 SD of historic values

[5]NWFSC Quality Assurance Criterion (from Sloan et al. 2006): Method blank (3/set): analyte concentrations in samples will be ≥ 10 times the maximum blank value.

Appendix 2

Table 3. Results of duplicate analyses[1] for metabolites of polycyclic aromatic compounds of selected bile samples of field captured fish analyzed one year after the 2015 Refugio Beach Oil Spill.

Species	Laboratory ID number	Collection site	Field ID number	Protein mg/mL	Equivalents of fluorescent aromatic compounds (ng/g bile, wet weight)			Protein-corrected equivalents of fluorescent aromatic compounds (ng/mg biliary protein)		
					NPH[2]	PHN[3]	BaP[4]	NPH[2]	PHN[3]	BaP[4]
Barred surfperch	136-0444	Gaviota	GAVFI1051816BI5	4.5	200,000	44,000	540	44,000	9,800	120
	136-0444R	Gaviota	GAVFI1051816BI5	4.5	190,000	42,000	560	42,000	9,300	120
Barred surfperch	136-0406	Refugio Beach	RSBFI1051816BI4	7.1	240,000	52,000	460	73,000	34,000	65
	136-0406R	Refugio Beach	RSBFI1051816BI4	7.1	230,000	51,000	480	72,000	32,000	68
Walleye surfperch	136-0425	Campus Point	CMPFI1051816BI7	15.3	1,100,000	290,000	2,400	72,000	19,000	160
	136-0425R	Campus Point	CMPFI1051816BI7	15.3	1,100,000	280,000	2,400	72,000	18,000	160

[1]NWFSC Quality Assurance Criterion (from Sloan et al. 2006): Sample duplicates (at least 1 for every 20 field samples analyzed): relative percent difference for each analyte ≤ 60% for duplicates.

[2]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of naphthalene standard at 292/335 nm wavelengths.

[3]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of phenanthrene standard at 260/380 nm wavelengths.

[4]Concentrations in part per billion (ng/g) based on total area compared to the fluorescence of benzo[a]pyrene standard at 380/430 nm wavelengths.