Chapter 4
Analysis of PAH body burdens in blue mussels in winter 2008

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Introduction

More than 100 kilometers of coastline on Unalaska Island were oiled by the *Selendang Ayu* grounding, creating both short- and long-term biological consequences, including biochemical evidence of continued oil exposure through 2008 in harlequin ducks. The objective of this chapter is to evaluate potential biological effects to other biota within the spill-impact zone (Objective 3). To accomplish this, indigenous mussels were collected from intertidal areas within the *Selendang Ayu* oil spill area, a reference area, and a human-impacted area – and analyzed for PAHs. Mussels were collected at two different time periods in 2008: February (during the harlequin duck surveys; this chapter) and July/August (during the lingering oil surveys; Chapter 3). This chapter focuses on the winter mussel sampling, which did not include samples from the reference site. Therefore, mussels from the least impacted areas were used to provide a relative measure of impacts to mussels from the most heavily impacted areas.

Only a handful of studies have reported the relationship between tissue body burdens and the adverse toxicological effects of total polycyclic aromatic hydrocarbon (TPAH) in mussels[1-4]. These studies were used to assess the effect of TPAH in blue mussels via two effect endpoints: scope for growth and lysosomal destabilization. Scope for growth (SFC) is a measure of an individual organism's available energy for growth, as it balances energy gains and expenditure losses[5]. SFG has been used as a sensitive indicator of contaminant-induced stress under field condition scenarios[5, 6]. Lysosomes are intracellular organelles involved in essential cellular functions (i.e., membrane turnover, nutrition, and cellular defense), and they can also sequester a variety of contaminants[3, 7]. Lysosomal destabilization assays have been used to indirectly quantify the effects of contaminant body burdens on cellular functions, damage, and apoptosis. Stable lysosomes of cells incubated in neutral red dyes retain and accumulate the pigment, while damaged lysosomes released the pigment into the cytosol. Dye release reflects membrane destabilization, thus this effects endpoint has been widely used as an indicator of cellular injury and as a good early warning indicator of further effects[1-4].

As discussed in Chapter 3, the July/August 2008 TPAH concentrations in mussel tissues in the heavily oiled areas were very low. Chapter 3 included analysis of the potential for sublethal effects from low levels of PAH in mussel tissues. Because the February 2008 samples contained higher PAH concentrations, further analysis of the potential sublethal effects was conducted for these samples using two effect endpoints: scope for growth and lysosomal destabilization. Effect endpoints (scope for growth and lysosomal destabilization) were calculated using TPAH (ng/g, dw) as the sum of individual PAH analytes (naphthalene to benzo[g,h,i]perylene; 52 PAHs).

Methods

The February 2008 mussel samples consisted of replicates, as composites of $\geq$ 20 mussels that were within a 2-3 cm size class (representing the typical larger size range), collected at six locations within a 50 m radius at each collection site within a bay[8]. Samples were collected from three heavily oiled sites (Skan Bay North and South, Makushin Bay, and Humpback Bay; SKN11, SKS04, HMP11 and MKS11, respectively), a moderately oiled site (Cannery Bay; CNB17), a lightly oiled site (Anderson Bay; AND2/3), and a (not oiled) human-impact site in Chernofski Harbor (CFEG; Fig. 4.1). Although the Anderson Bay sampled location was classified by SCAT as unoiled, most of the adjacent shorelines were classified as light oiling.

Mussel tissue samples were analyzed for PAHs according to Alpha Analytical's Standard Operating Procedures, and following guidelines from the National Oceanic and Atmospheric Administration (NOAA) National Status and Trends Mussel Watch Program[9]. Briefly, samples were homogenized, and 10 -15 g of sample placed into glass extraction jars for chemistry analysis. Samples and laboratory controls were spiked with surrogate recovery standards, dried with sodium sulfate, and extracted three times with methylene chloride. Samples were filtered through sodium sulfate and glass wool plug, or filter paper into kuderna-danish (KD) vessels, and concentrated under a gentle stream of nitrogen. Extracts were run through an alumina column, and further concentrated via KD and nitrogen to a 5 mL volume. Two mL per sample were analyzed on a high performance liquid chromatograph (HPLC) equipped with a gel permeation chromatography size exclusion column, and 2 mL were archived. Post-HPLC extracts were spiked with PAH internal standards and submitted at a 250 µL volume.

Sample extracts were analyzed by gas chromatography/mass spectrometry (GC/MS) operated in the selected ion monitoring (SIM) mode for PAH and heteroatomic compound target analytes. Reference oils and an independent calibration check standard were analyzed with each initial calibration. Analyte final concentration was quantified versus internal standards spiked into the sample extract prior to analysis. The target PAH concentrations were quantified using average response factors (RF) generated from a five-point calibration curve. Alkyl homologues were quantified using the RF of the parent compound.

Results

The February 2008 results were reported only as wet weight. Therefore, TPAH concentrations were converted from a wet to dry weight basis by multiplying the tissue PAH concentration by the sample's wet weight divided by its estimated dry weight. Dry weights were calculated by multiplying the wet weight tissue sample by a dry-to-wet weight conversion factor of 0.122. This conversion factor was obtained from the mean dry-to-wet ratio (0.122± 0.028) for the 112 blue mussel samples collected in July/August 2008. In February 2008, total PAH ranged from 150 to 1,457 ng/g dw in (Table 4.1). The large variation in PAH tissue concentration from oiled sites was due to a sample from North Scan Bay (SKN11-C) which contained relatively high PAH concentrations (1,457 ng/g dw), particularly chrysenes (Table 4.1). The laboratory obviously thought that this measurement was suspect, because the sample was re-run and the duplicate analysis of this sample (SKN11-C2) was 377 ng/g dw, more in line with PAH concentration in

samples from this area. Because of the uncertainty associated with SKN11-C, analyses were conducted with and without this sample (and including the duplicate analysis for SKN-11C2). Mean PAH tissue concentrations from heavily oiled sites were not significantly different from lightly to moderately oiled sites (pooled mean 219 ± 38 ng/g) only when SKN11-C was included in the analysis (pooled mean 397 ± 316 ng/g with SKN11-C, p= 0.08; pooled mean 285 ± 81 ng/g without SKN11-C, p=0.01).

Scope for Growth

Based on Scope for Growth (SFC)[5, 6], we determined that low/no stress to mussels would be predicted at a critical TPAH (2- and 3- ring PAHs) body residue of ca. ≤ 2,250 dw (SFG >15 J/g/h), moderate stress between 2,500 and 25,000 ng/g dw (SFG = 5-15 J/g/h), and high stress above 25,000 ng/g dw (SFG < 5 J/g/h). The February 2008 mussels had TPAH (2- and 3- ring PAHs) tissue concentrations nearly one order of magnitude lower than the predicted 2,250 ng/g dw critical body residue, suggesting that in terms of SFG, these individuals may not be suffering from contaminant-induced stress. It is important to note that the quantification method for PAH from which SFG-TPAH relationships were derived[6] (i.e., ultraviolet fluorescence data from HPLC analysis) differs from more standard PAH quantification techniques such as GC/MS. This suggests that the current body residues are not directly comparable to those of Widdows and Donkin[5] and Widdows et al.[6, 10] (Widdows pers. comm., 27 January 2009). Therefore, comparisons were made relative to lightly to moderately oiled sites in terms of order of magnitude changes. Widdows et al.[6] stated that SFG is reduced by a ca. 10 J/g/h for every order of magnitude increase in toxic hydrocarbons (regardless of units). Widdows et al.[10] also calculated a SFG of 29 J/g/h in clean reference sites as the optimum SFG in animals that exhibited maximum growth potential (see Figure 9 in Widdows et al.[10]). SFG reduction (J/g/h) in samples from heavily oiled sites and the human-impacted area were, therefore, calculated as the differences between TPAH concentration (ng/g, dw; Log) from heavily oiled sites and the mean TPAH concentration (ng/g, dw; Log) in samples from lightly to moderately oiled sites multiplied by the SFG 10 J/g/h factor. TPAH concentrations included mean values from each lightly to moderately oiled site (Cannery Bay [CNB17] and Anderson Bay [AND2/3]) and the pooled mean from all samples (CNB17 and AND2/3 combined). Percent SFG reduction was calculated by dividing the SFG reduction (J/g/h) by the maximum SFG potential of 29 J/g/h (see above).

Theoretical percent SFG reduction (i.e., SFG reduction > 0; Figure 4.2) in heavily oiled sites ranged from 1.5% (HMP11-B/AND2/3) to 30% (SKN11-C/CBN17) (equivalent to a SFG of 1.45 and 8.9 J/g/h, respectively), with a mean SFG reduction of 8.4% (equivalent to a SFG of 2.4 J/g/h). Percent SFG reduction was slightly elevated in the human-impacted site (range 10.58% to 18.64%) compared to most oiled sites (except for SKN11-C). Two of the oiled sites (MKS11-A and MKS11-C) exhibited a positive percent SFG change relative to background. Minor sublethal effects may have been observed in SKN11-A, and in all human-impacted sites (SFG reduction > 10%), while mild sublethal effects may have been observed in SKN11-C (SFG reduction > 25%). However, since SFG was estimated using previously published data, further studies would be necessary to determine if these TPAH concentrations would induce changes in SFG of blue mussels in the area affected by the *Selendang Ayu* oil spill.

Lysosomal Destabilization

Lysosomal destabilization (LD) assays have been used as biomarkers of exposures to a variety of contaminants in bivalves. Studies often report LD as the percent of the total incubated cells collected from stressed animals that fail to retain the dye.

Two models were used to calculate the percent of LD in blue mussels collected in February 2008. In the first model, LD vs. TPAH relationships were fitted using data from field-collected oysters exposed to a variety of contaminants (including TPAH mixtures; data from Hwang et al.[1], Figure 1 a&b; Hwang et al.[2]). In these samples, 37 PAHs were analyzed. The second model consisted of the LD vs. TPAH relationship described in Hwang et al.[2] for oysters exposed under laboratory conditions to a known mixture of 24 PAHs. Both of these models and respective equations are shown in Figure 4.3. Large circles represent the TPAH concentration at which LD reaches 100% (Field Model = 6,000 µg/kg; Exposure Model = 9,100 µg/kg). As stated by Hwang et al.[2] , field-collected oysters exhibited higher LD rates at lower TPAH concentrations, because of the additional stress posed by contaminant mixtures and extra PAHs; therefore, this model can serve as an upper bound LD rate at most TPAH concentrations.

The predicted rate of LD for all samples was calculated using both models and using the sum of the 52 PAHs analyzed as TPAH. Because Hwang et al.[1, 2] calculated TPAH from <52 PAHs, we assumed additive PAH effects on LD from the additional PAHs. This assumption is scientifically substantiated by extensive experimental evidence showing that PAHs in mixtures act additively to produce toxic effects[11-13] but at lower concentrations, where multiple mechanisms of action prevail, comparison of the two analyses are ambiguous because additive conditions are not expected[14]. LD results from the human-impacted and heavily oiled sites are discussed relative to mean LD values from samples within each lightly to moderately oiled site (CNB17 and AND2/3) and the pooled mean from all samples (CNB17 and AND2/3 combined).

The calculated rate of LD ranged from < 0.5% (HMP11-B/AND2/3) to 9% (SKN11-C/CNB17) with the Experimental Model, and from 2% (HMP-02/AND2/3) to 51% (SKN11-C/CNB17) with the Field Model (Figure 4.4). Across heavily oiled sites and relative to the mean from lightly to moderately oiled samples, LD was 2% with the Experimental Model and 12% with the Field Model. The calculated LD values were slightly elevated in the human-impacted site compared to most oiled sites (except for SKN11-C). Two of the oiled sites (MKS11-A and MKS11-C) exhibited a lower rate of LD relative to lightly to moderately oiled samples (i.e., LD < 0), suggesting that environmental quality at these sites are slightly better than conditions in lightly to moderately oiled sites.

Ringwood et al.[7] established a LD value of < 30% in reference/control oysters and suggested that LD values of > 40% would indicate serious cell damage that can be translated into adverse effects at the organism level. LD values > 40% have been correlated with poor gamete viability and linked to adverse effects on reproductive success[7]. Only one of the samples collected in North Skan Bay (SKN11-C) had a calculated rate of change in the LD of > 40% (Field Model) relative to low to moderately impacted areas. In summary, the levels of TPAH in most tissue samples detected in February 2008, except for one sample (SKN11-C), are not likely to have significant sublethal effects on mussels as indicated by increases in lysosomal destabilization over low impacted site levels.

Discussion

Despite the use of mussels in large-scale environmental monitoring, our knowledge and understanding of the cause and effect relationships between TPAH tissue residues and meaningful biological responses is still limited. Early work by Widows and colleagues[1,2,9] followed by more recent studies[4-6] has facilitated the use of effect endpoints to elucidate the potential effects of TPAHs based on tissue residue data. The two effect endpoints used in the current analysis suggested little to no adverse effects of TPAHs in blue mussels. In only one tissue sample (SKN11-C) mild sublethal effects (SFG reduction > 25%) and increased lysosomal destabilization (experimental model = 9% and Field Model = 51%) were found relative to tissue samples from lightly to moderately oiled sites. However, only one sample indicating potential adverse effects does not allow generalization of effects to the entire blue mussel population or to other invertebrates. Duplicate chemical analysis of this sample suggests potential sample contamination of the chrysene series (Table 4.1), though this observation has not been definitively confirmed. Lack of adverse effects in mussels is uncertain based on two considerations: 1) "true" background samples were not collected and, therefore, effects relative to samples from Cannery Bay and Anderson Bay may have added uncertainty to estimates of adverse effects via the two effects endpoints; and 2) mussels are relatively tolerant to contaminants, thus lack of effects in mussels do not indicate lack of potential toxicity to other invertebrates. For example, acute toxicity data (i.e., 24-, 48- and 96h- LC_{50}) for naphthalene extracted from NOAA's Chemical Aquatic Fate and Effects[15] (CAFÉ) tool ranks marine bivalves between the 80th to 90th percentile across three multi-species sensitivity distribution curves (13 to 23 species per curve; Fig. 4.5). In this example, bivalves were less sensitive to naphthalene than several crustacean and fish species.

The February 2008 tissue concentrations were reported as wet weight and therefore a dry-to-wet weight conversion factor was used to report dry weight concentrations. This conversion factor likely introduced uncertainty into tissue concentration estimates. Estimated variation based on the standard deviation of the conversion factor indicated that tissue concentrations may have been overestimated or underestimated by 19% and 30%, respectively, relative to the reported value. However, scope for growth and lysosomal destabilization comparisons were made relative to estimated TPAH concentrations from mussels collected from lightly to moderately oiled sites and, therefore, the conclusions of the analysis are not influenced by potential uncertainties introduced with the use of the conversion factor.

Although TPAH concentration in mussel tissues from the most heavily oiled sites were relatively low (range 150-486 ng/g, dw), except for SKN11-C (1,457 ng/g, dw), studies with bivalves have documented sublethal effects at concentrations within the ranges documented here (Table 4.2). Despite obvious differences across studies in chemical analysis (GC/MS vs. HPLC) and reporting (number of PAHs analyzed, tissue concentrations in dry or wet weight) most of such research reported cellular stress linked to PAH exposure[16-17, 18,19]. These studies attribute increase in cellular stress to a series of cellular alterations including the activation of catalyzing multifunctional enzymes (i.e., gluthatione-*S*-transferase) involved in the metabolism of xenobiotics, and increased oxidative stress (i.e., formation of DNA adducts and production of lipid peroxidation). Not surprisingly, increased lysosomal destabilization has shown a strong correlation with decreased total antioxidant capacity in cells[20], indicative of compromised

efficiency in neutralizing strong oxidizing chemical species, and increased micronuclei frequency, indicative of severe chromosomal damage[21].

In conclusion, based on a relatively small sample size of blue mussels, the two effect endpoints did not suggest severe adverse effects to mussels in winter 2008 following exposure to oil residues from the *Selendang Ayu* grounding. This conclusion is consistent with results from summer mussels (Chap 3). Generalizations beyond these findings may not be appropriate given limitations inherent to the design in the sampling of mussel tissue.

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Table 4.1. TPAH (naphthalene to benzo[g,h,i]perylene) concentration (ng/g, dw) in blue mussel tissues collected in February 2008, and percent contribution of major PAH groups Naph= napthalenes, Fluor=fluorenes, Dibenz=dibenzothiophenes, Phen/Anth= phenanthrenes/anthracenes, and Chrys=chrysenes.

Sample Name	TPAH (ng/g, dw)	% Naph	% Fluor	% Dibenz	% Phen/Anth	% Chrys
Lightly to moderately oiled sites						
AND2/3-A	277	29	1	12	33	1
AND2/3-B	211	35	2	10	32	1
AND2/3-C	245	26	1	4	32	2
CNB17-A	222	42	6	11	13	2
CNB17-B	152	29	11	10	25	3
CNB17-C	209	43	4	10	20	1
Heavily oiled sites						
SKN11-A	468	20	5	11	20	6
SKN11-B	349	18	7	12	19	7
SKN11-C	1,457	5	2	19	14	38
SKN11-C2	377	14	4	14	21	7
SKS04-A	308	24	10	11	20	2
SKS04-B	326	36	9	12	21	2
SKS04-C	286	15	3	12	19	7
MKS11-A	180	26	14	8	26	2
MKS11-B	343	40	10	8	10	2
MKS11-C	150	27	14	11	24	2
HMP11-A	295	18	4	12	30	5
HMP11-B	270	17	1	12	31	7
HMP11-C	352	18	0.4	14	30	6
Human-impacted site						
CFEG-A	544	16	6	1	30	5
CFEG-B	495	24	4	0	28	6
CFEG-C	675	10	3	5	32	5

Table 4.2. Mussel studies showing chronic effects linked to PAH critical body burdens reported as wet weight (ww) or dry weight (dw).

Species	Exposure Conditions	Response	Critical Body Residue
Blue mussel (*Mytilus edulis*)[16]	Samples from areas impacted by the *Sea Empress* oil spill. Exposure duration: 132 days post spill	ca. 110 to 125 mean lysosomal neutral red retention (min)	105,000 to 150,000 ng/g ww (PAH mixture)
Clams (*Ruditapes decussates*)[22]	Transplanted clams to a PAH contaminated site. Exposure duration: 28 days	Significant increase in glutathione S-transferase and lipid peroxidation	304 ng/g ww (PAH mixture)
Blue mussel (*Mytilus edulis*)[23]	Samples across a contamination gradient	25th percentile and 50% below reference-lysosomal labilization	ca. 170 ng/g dw and 970 ng/g dw (16 PAHs)
Blue mussel (*Mytilus edulis*)[17]	Exposure to microencapsulated phenanthrene, fluoranthene benzo[a]pyrene mixture Exposure duration: 6 days	Adverse cytochemical and cytologcal responses in digestive tissue	Phenanthrene: 430 ng/g dw, fluoranthene: 650 ng/g dw benzo[a]pyrene: 85 ng/g dw
Mussels (*M. galloprovincialis*)[24]	Control laboratory exposure to the *Prestige* oil. Exposure duration: 12 days	Significant DNA damage	17,033 ng/g dw (sum of 36 PAHs)
Giant mussel (*Choromytilus chorus*)[18]	Field collected samples	Scope for growth below 0 J/g/h indicative of severe stress	ca. 1,000 ng/g dw PAH mixture
Green-lipped mussel (*Perna viridis*)[25]	Exposure to 100 µg/L B[a]P concentration. Exposure duration: 42 days	51% change in neutral red retention (mean) between days 6 and 42	ca. 105,000 ng/g dw on day 6 to 617,000 ng/g dw on day 42

Table 4.2. Cont.

Species	Exposure Conditions	Response	Critical Body Residue
Perna perna[19]	Transplanted mussels from a clean area to a site housing an oil terminal and refinery Exposure duration: 15 days	Reduced lysosomal neutral red retention (23-30 min) relative to controls (60 min)	64 ng/g (sum of 16 priority PAHs) and 260 to 307 ng/g (sum of 37 PAHs)
Eastern oyster (*Crassostrea virginica*)[1]	Field collected oysters from a contaminated site	74% and 81% lysosomal destabilization based on total incubated cells	1,780 and 2,390 ng/g dw (sum of 37 PAHs)
Eastern oyster (*Crassostrea virginica*)[26]	Field collected oysters from a contaminated site	81% lysosomal destabilization based on total incubated cells	650 ng/g dw (sum of 19 PAHs)
Eastern oyster (*Crassostrea virginica*)[2]	Oysters fed PAH mixture under controlled conditions Exposure duration: 25 days	50% lysosomal destabilization based on total incubated cells	2,100 ng/g dw or 9.3 nmol/g dw

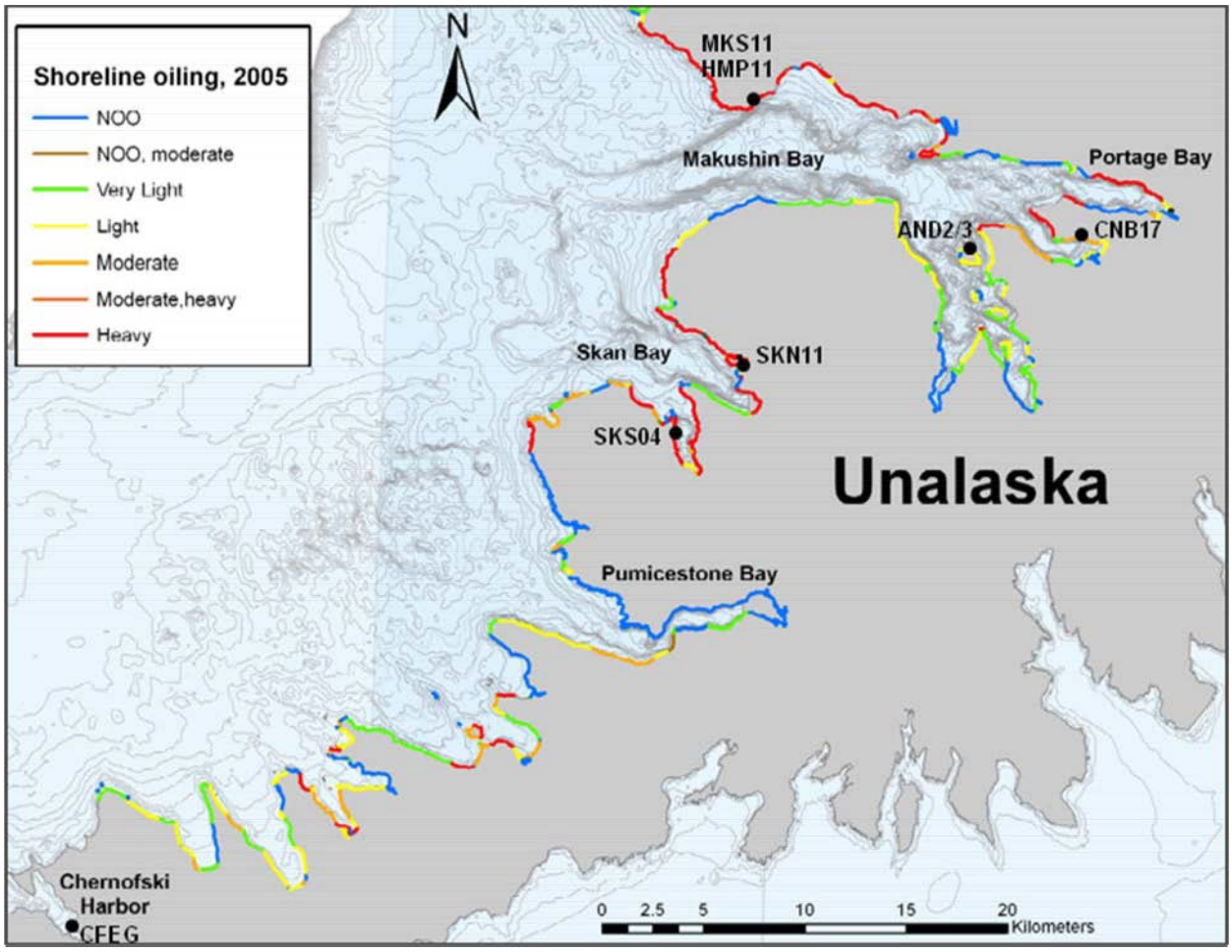

Figure 4.1. Map depicting the approximate location of mussel sampling (●; February 2008) sites within the oiled segments. Heavily oiled sites: Skan Bay North (SKN11) and South [SKS04], Makushin Bay (MKS11) and Humpback Bay (HMP11), and lightly to moderately oiled sites: Cannery Bay (CNB17) and Anderson Bay (AND2/3).

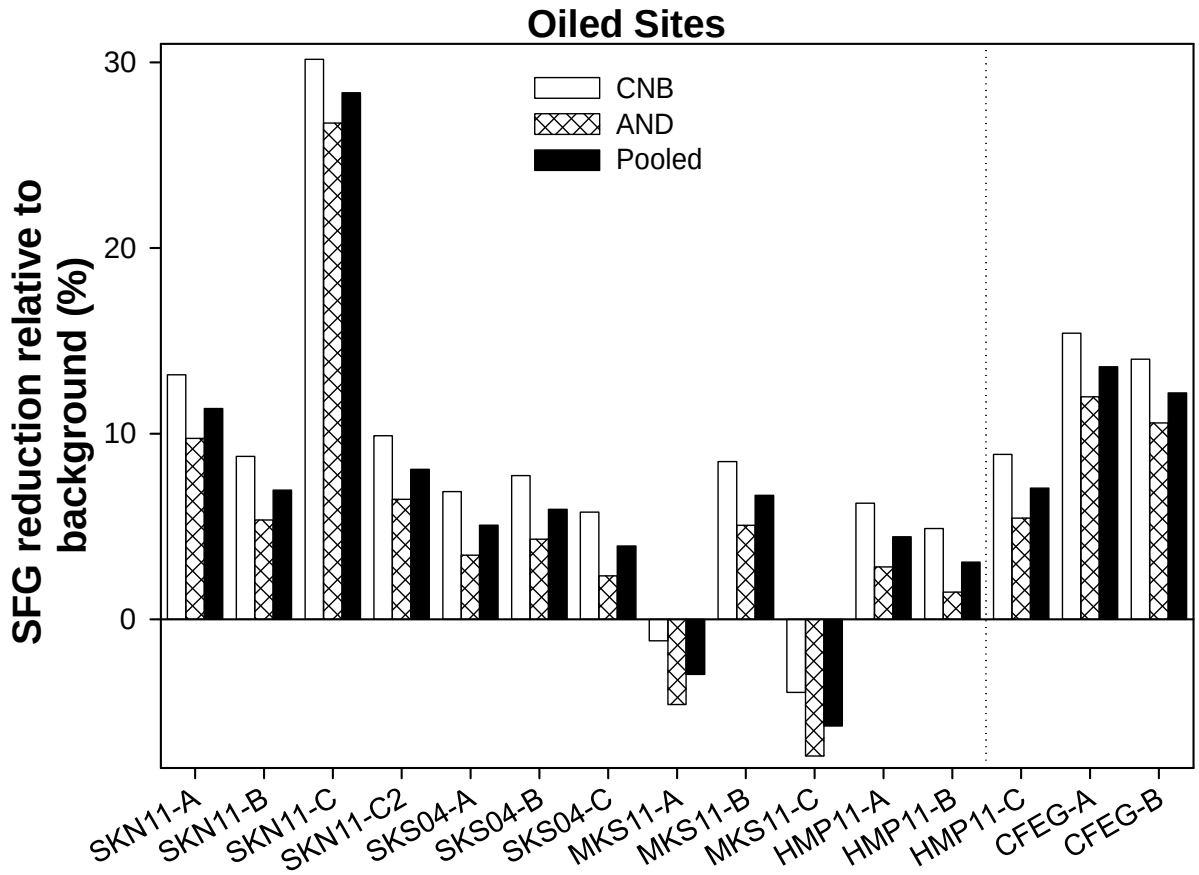

Figure 4.2. Theoretical scope for growth (SFG) changes relative to lightly to moderately oiled mussel tissue samples (Cannery Bay [CNB17] and Anderson Bay [AND2/3], and CNB17 and AND2/3 pooled) in samples collected from oiled sites and human-impacted sites, for February 2008 samples.

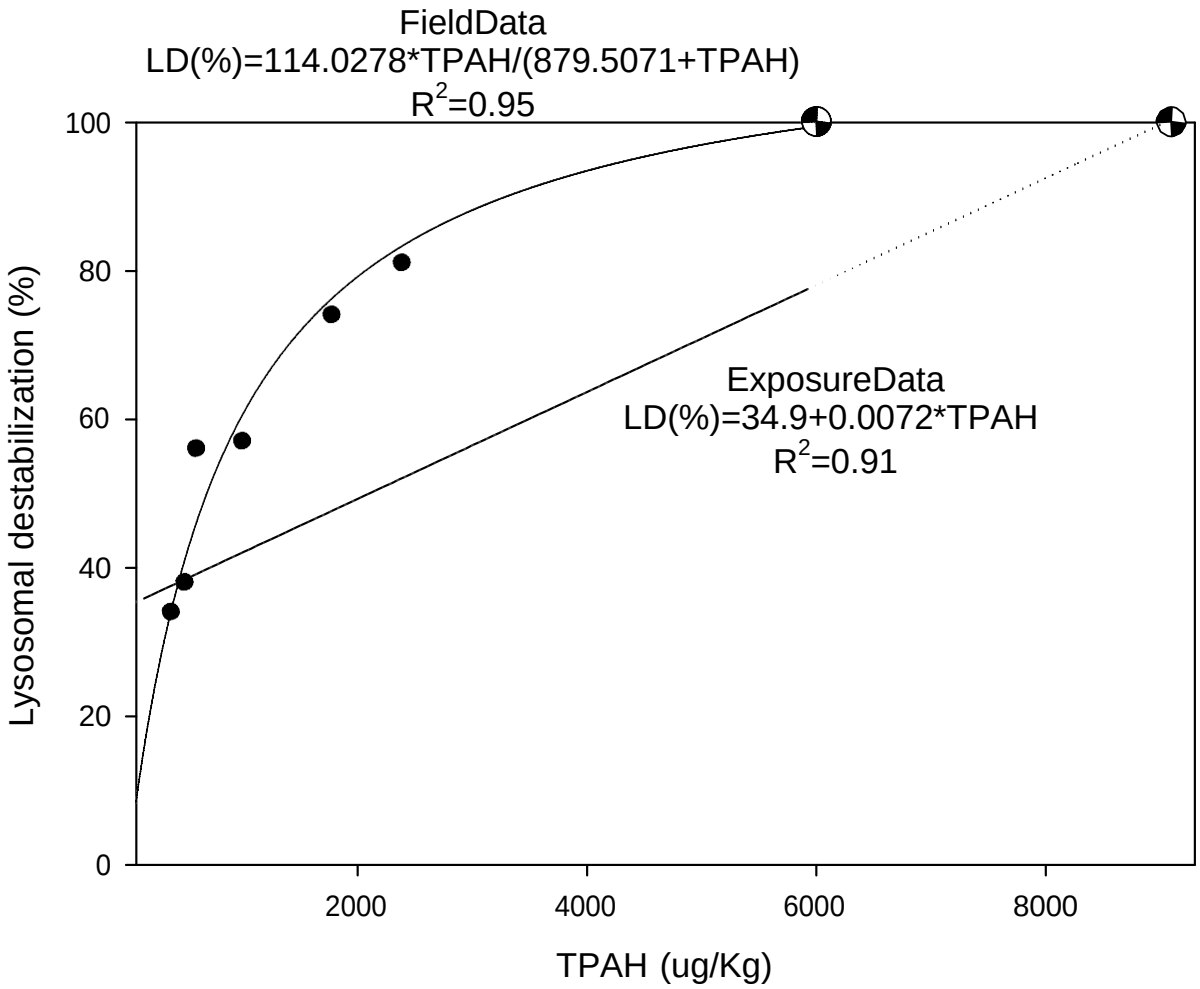

Figure 4.3. Lysosomal destabilization (LD%) models from field-collected oysters exposed to contaminant mixtures (Field Data) and from oysters exposed to a known PAH mixture (Exposure Data). Partially filled symbols indicate the TPAH concentration at which 100% LD is expected to occur. These models use data from Hwang et al.[1] and a LD vs. TPAH equation from Hwang et al.[2].

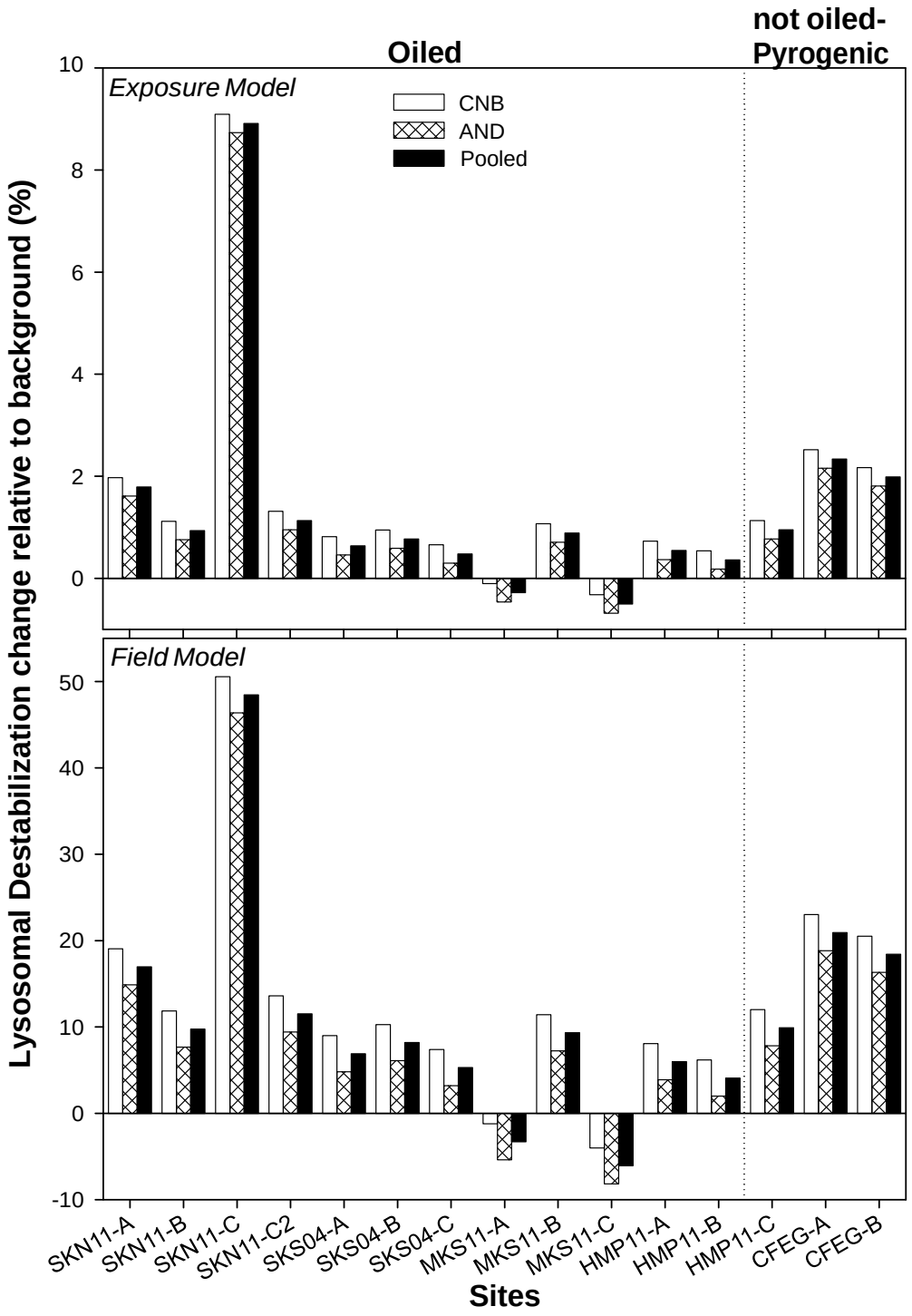

Figure 4.4. Predicted lysosomal destabilization (LD%) changes relative to lightly to moderately oiled samples (Cannery Bay [CNB17] and Anderson Bay [AND2/3], and CNB17 and AND2/3 pooled) in samples collected from oiled sites and human-impacted sites collected in February 2008. The Exposure Mode (top) uses data from oysters exposed to a known PAH mixture, while the Field Model (bottom) uses data from field collected oysters exposed to contaminant mixtures.

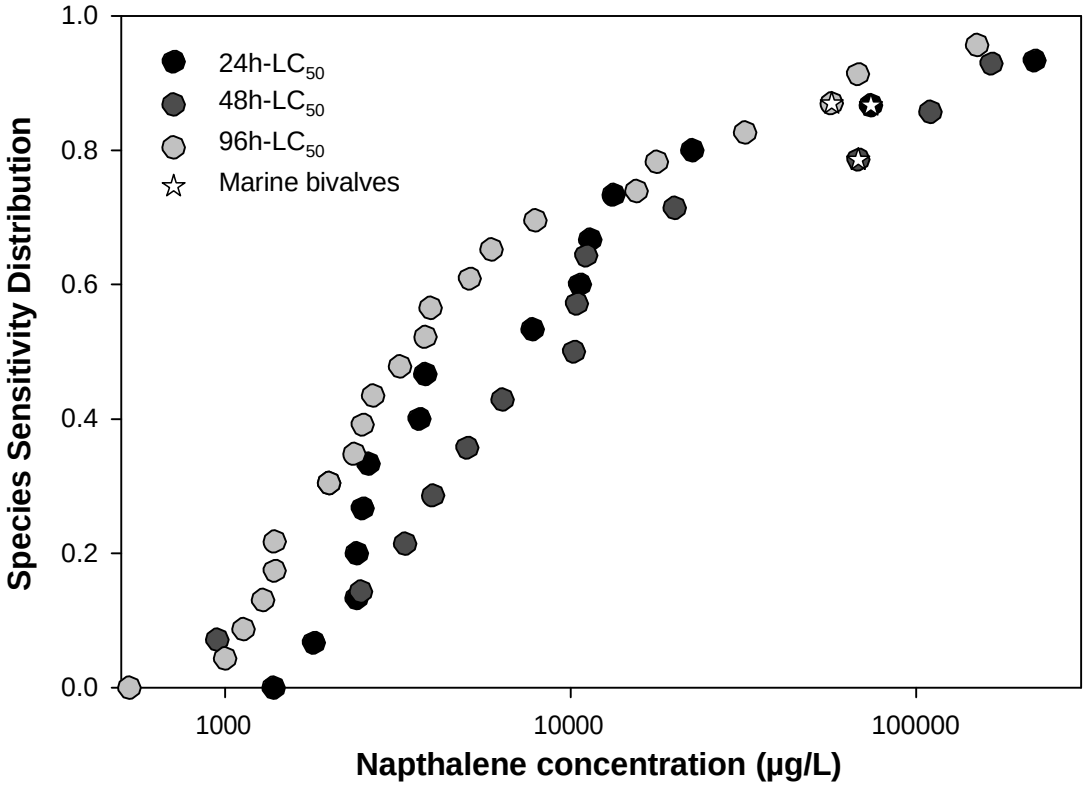

Figure 4.5. Species sensitivity distribution for naphthalene using acute toxicity data (i.e., 24-, 48- and 96h- LC_{50}) highlighting the position of marine bivalves within the curve. Data from the NOAA Chemical Aquatic Fate and Effects (CAFÉ) tool.