Exxon Valdez Oil Spill
Restoration Project Final Report

Comparison of Cytochrome P450 1A Induction in Blood and Liver Cells of Sea Otters

Restoration Project 01534
Final Report

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Study History: This project began in 2001 with the approval of a 1 year plan by the *Exxon Valdez* Oil Spill (EVOS) Trustee Council to further investigate expression of the cytochrome P450 1A (CYP1A) biomarker in sea otters, including a comparison of (1) the induction of the biomarker in blood and liver tissues, and (2) liver CYP1A values from 2001 and 1989. The project was integrated with sea otter capture and biosampling conducted as part of Restoration Project 01423, and was based on techniques developed as part of the Nearshore Vertebrate Predator Project, 95025-99025, designed to assess recovery of the nearshore ecosystem affected by the *Exxon Valdez* spill. In this report we present blood and liver CYP1A values of sea otters caught in 2001, liver CYP1A values from sea otters dying in 1989 post-spill, and preliminary histology information on liver tissues collected in 2001. Additional liver samples have been collected from sea otters captured in 2002 and 2003, and further analyses and interpretation of the liver CYP1A and histopathology findings will be included with the final report for Project //423, scheduled for completion in late 2003.

Abstract: Sea otters (*Enhydra lutris)* in the most heavily oiled areas of western Prince William Sound have not recovered from the EVOS, based on estimates of otter abundance and survival rates. Exposure of sea otters to lingering oil appears to be a likely mechanism explaining lack of full recovery. This study was initiated to obtain further information on oil exposure, using the cytochrome P450 1A (CYP1A) biomarker. Previous work (Project //025) found that sea otters in oiled areas of western PWS had elevated levels of CYP1A, in blood samples collected in 1996-98. In summer 2001, as part of Project 01423, we resampled sea otters in oiled and unoiled areas of PWS, to monitor health and blood CYP1A values. Liver was also sampled from these otters for assays of CYP1A and for examination of histopathological changes. In both blood and liver tissues, CYP1A levels in the oiled area were higher than those in the unoiled area. Histopathological lesions of the liver were observed in both the oiled and unoiled areas, but the most severe lesions were observed in the oiled area. We also assayed CYP1A in archived frozen liver samples from sea otters that were oiled and died in 1989, to enable comparison of current levels of CYP1A induction with levels in sea otters that had a known high degree of oil exposure. Levels of CYP1A induction in 1989 samples were higher than in livers collected from sea otters in an unoiled area in 2001, but lower than levels in livers of sea otters residing in oiled areas in 2001.

Key words: CYP1A, *Enhydra lutris*, *Exxon Valdez*, oil spill, sea otters.

Project Data: Data will be kept in digital format (MS Excel) at the USGS Alaska Science Center in Anchorage.

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TABLE OF CONTENTS

HISTORY AND ABSTRACT..... 3

LIST OF TABLES..... 5

LIST OF FIGURES..... 5

INTRODUCTION..... 6

PROJECT OBJECTIVES..... 6

METHODS..... 7

RESULTS..... 8

DISCUSSION..... 10

ACKNOWLEDGEMENTS..... 11

LITERATURE CITED..... 12

LIST OF TABLES

Table 1. Cytochrome P450 1A values[a] (mean, standard error, range, and number of samples) in blood and liver samples from sea otters captured in western PWS in 2001, and in liver samples from sea otters dying in 1989 post-spill.

Table 2. Data on sea otters dying in 1989 and sampled for liver cytochrome P450 1A (CYP1A) in 2001.

LIST OF FIGURES

Figure 1. CYP1A values in blood and liver samples collected from sea otters in oiled and unoiled areas of western PWS, 2001.

Figure 2. CYP1A values in liver samples collected from sea otters in oiled and unoiled areas of western PWS in 2001 and from sea otters dying post-spill in 1989.

INTRODUCTION

Sea otters in western Prince William Sound (PWS), Alaska, were exposed to large quantities of oil following the 1989 *Exxon Valdez* spill and mortality was high, particularly in areas with heavy shoreline oiling. In the western Sound overall, sea otter numbers have increased during the 1990's and the population is considered to be recovering. However, studies conducted in 1996-1998 as part of the NVP program (Restoration Project //025) and continued as part of Restoration Project //423, have provided evidence that sea otters in the area of northern Knight Island, where much of the shoreline was heavily oiled in 1989, had not fully recovered from oil spill injury (Bodkin et al. 2002). Sea otter abundance at northern Knight Island remains at about 50% of the estimated pre-spill abundance (Bodkin et al. 2002, Dean et al. 2000). Analysis of ages at death of beach-cast sea otters, found before and after the spill, identify elevated mortality rates of sea otters that survived the spill, as well as those born after 1989, as a factor delaying recovery (Monson et al. 2000). Cytochrome P450 1A (CYP1A), a biomarker of aromatic hydrocarbon exposure, was elevated in blood samples collected from sea otters in oiled areas (primarily around northern Knight Island) during 1996-98. Further, serum enzymes, particularly gamma glutamyl transferase (GGT), are elevated in sea otters from the oiled area, suggesting liver damage in those animals. Overall, these observations implicate exposure to oil as a factor limiting recovery of sea otters (Ballachey et al. 2002, Bodkin et al. 2002).

Additional monitoring of CYP1A in sea otters in western PWS, in the summer of 2001, was conducted as part of Project 01423. This study, Project 01534, was conducted as an adjunct to Project 01423, to supplement measurement of CYP1A in blood with assays of CYP1A in liver biopsies from the same individuals, to investigate the relation between CYP1A in the two tissue types. Further, we assayed archived liver samples, collected from sea otters that died in the summer of 1989, to obtain a comparison of CYP1A liver levels from 1989 and 2001. Finally, we examined liver biopsies from 2001 for histopathological alterations, comparing samples from the oiled and unoiled areas. Primary findings of the study include (1) elevated CYP1A levels in the oiled area (blood and liver) compared to those in the unoiled area, although there is some indication that blood CYP1A levels have declined since 1996-98, (2) histopathological lesions of the liver in both the oiled and unoiled areas, but with the most severe lesions seen only in the oiled area, and (3) CYP1A in 1989 liver samples at levels less than those found in livers of sea otters from oiled areas in 2001, but higher than levels in sea otters from unoiled areas in 2001.

OBJECTIVES

1. Measure and compare CYP1A in blood and liver samples from sea otters captured in summer 2001.

2. Measure CYP1A in archived liver samples of oiled sea otters from 1989; compare liver CYP1A values from 2001 to 1989 samples.

3. Conduct histopathological examination of liver biopsies from 2001, to assess relation between CYP1A levels and histological change in the liver.

4. Relate CYP1A levels in 1989 liver samples with hydrocarbon concentrations measured previously, and histopathology collected previously on those samples.

METHODS

In the NVP study, the RT-PCR assay (quantitative reverse transcriptase PCR assay; Vanden Heuvel et al. 1993, 1994; Snyder et al. 2001) was adapted to measure CYP1A levels in sea otters. This assay quantifies the messenger RNA (mRNA) that codes for the CYP1A protein, with results reported as molecules of mRNA per 100 ng of RNA. Previously, for sea otters, the assay had been applied only to peripheral blood mononuclear cells. In this study, the assay was applied to both blood and liver samples.

Study areas and animal capture. In summer 2001, sea otters were captured in western PWS, at northern Knight (oiled area) and Montague (unoiled area, in the vicinity of Stockdale Harbor and Port Chalmers) islands. Capture and handling methods were similar to those employed previously (Bodkin et al. 2002). Sea otters were sedated, body measurements taken, a tooth collected for age determination, and a blood sample taken by jugular venipuncture. Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood by density gradient centrifugation and isolated PBMC were cryopreserved in liquid nitrogen. In addition, three liver biopsies (weighing approximately 0.5 gm total) were surgically collected, using endoscopic procedures, from 15 otters per area. Two biopsies were frozen immediately in liquid nitrogen and a third biopsy fixed in neutral buffered formalin. Following reversal, sea otters were released in the same vicinity as captured.

Liver in formalin and cryopreserved PBMC and liver samples, as well as frozen archived liver samples from 1989, were shipped to Purdue University for analysis in the laboratory of Dr. Paul Snyder.

Isolation and Analysis of RNA. Total RNA was isolated from liver and PBMC using a rapid guanidinium-phenol extraction method originally adapted from Chomczynski and Sacchi (1987). To examine the quality of RNA extracted, two micrograms of each RNA sample were analyzed by electrophoresis in a 1% MOPS-EDTA-formaldehyde agarose gel and visualized by staining for 5 minutes with 100 μg/ml ethidium bromide in deionized water. Samples with evidence of degraded RNA (observed in a proportionof the 1989 liver samples) were not further utilized in the study. Aliquots of 20 μg native RNA were maintained at -70°C.

Preparation of Internal Standard. The use of an internal standard that contains target (i.e. sea otter CYP1A1) primer sequences negates tube-to-tube variability in PCR amplification and is essential to quantifying mRNA expression by RT-PCR. We

generated recombinant RNA (rcRNA) internal standards as described by Vanden Heuvel et al. (1993, 1994). Using this method, a rcRNA was generated that upon amplification with sea otter CYP1A1 primers results in a product (221 bp) that is easily resolved from target product (180 bp) following agarose gel electrophoresis.

Quantitative Competitive RT-PCR. Competitive PCR was performed essentially as described by Gilliland et al. (1990) as modified by Vanden Heuvel et al. (1993). For each sample, 8-10 aliquots of total RNA (0.1 mg) were prepared, and a dilution series of the rcRNA internal standard was spiked into these aliquots. Reverse transcription of RNA was performed in a final volume of 20 μl containing 25 mM Tris-HCl (pH 8.3 at 25°C), 50 mM $(NH_4)_2SO_4$, β-mercaptoethanol, 0.1 mg/ml bovine serum albumin, 5 mM $MgCl_2$, 1 mM of each deoxynucleotide triphosphate, 1 unit RNase inhibitor, 2.5 units M-MLV Reverse Transcriptase (Life Technologies, Inc.), 2.5 μM oligo$(dT)_{16}$, 0.1 μg total RNA, and varying amounts of rcRNA internal standard. The samples were incubated at 45°C for 15 min., and reverse transcriptase was inactivated by heating to 99°C for 5 min. PCR reaction mixture contained 3 mM $MgCl_2$, 2.5 units Taq polymerase, and 6 pmol of forward and reverse primers. The reactions were heated to 94°C for 3 min. and cycled 30 times through a 30-s denaturing step at 94°C, a 30-s annealing step at 54°C, and a 30-s elongation step at 72°C. Following the final cycle, a 5-min elongation step at 72°C was included.

Aliquots of the PCR reaction were electrophoresed on 2.5% NuSieve® 3:1 agarose (FMC Bio Products, Rockland, ME) gels, and PCR fragments were visualized with ethidium bromide staining. A photographic negative was prepared and densitometry was performed using a LKB Gel Scan II laser densitometer (LKB, Piscataway, NJ). Quantification of the amount of target mRNA present was determined as described by Gilliland et al. (1990). Initially a large internal standard concentration range (i.e., 10^2-10^{10} molecules/tube) was examined in order to estimate the concentration of target mRNA in each sample. Once the concentration of the CYP1A1 was determined, a more narrow range of internal standards was used to more precisely determine the levels of CYP1A1 mRNA. The actual numbers of molecules of CYP1A1 were determined by comparing the ratio of the volume of the internal standard to CYP1A1 mRNA. PCR products were plotted against the amount of internal standard added to individual tubes as previously described (Gilliland et al. 1990). Linear regression analysis was used to define the equation for the line through the data points. The amount of CYP1A1 mRNA present for individual animals was defined as the amount of rcRNA present where the volume ratio was equal to 1.

Histopathology. Liver samples in formalin were processed for histology and sections were examined microscopically.

RESULTS

CYP1A in blood and liver, 2001 samples: Blood samples were collected from 18 sea otters in the unoiled area and 16 sea otters from the oiled area, and liver from 15 sea

otters in each area. Both blood and liver samples from 2001 showed a similar pattern to that observed in blood samples from 1996-98 (Table 1, Figure 1), with elevated values and greater variablility observed in samples from the oiled area. CYP1A was significantly higher in blood cells from sea otters in the oiled area, relative to those from the unoiled area (t-test, $t = 3.19$, $P < 0.005$) and also in liver tissues from the oiled area relative to those from the unoiled area (t-test, $t = 6.04$, $P < 0.0001$). Liver and blood CYP1A values were positively correlated (0.55).

CYP1A in liver, 1989 samples: Archived liver samples from 9 sea otters that died at the rehabilitation centers in 1989, after being captured in oiled areas and with varying amounts of external oil, were judged to have adequate RNA quality and subsequently assayed for CYP1A (Table 2). Generally, CYP1A levels were at the lower end of the range of values observed for liver samples collected in the oiled area in 2001 (Table 1, Figure 2). CYP1A levels in 1989 samples were significantly lower than levels in 2001 samples from the oiled area (t-test, $t = 3.45$, $P < 0.002$), but significantly higher than in 2001 samples from the unoiled area (t-test, $t = 3.71$, $P < 0.001$).

For the 1989 sea otters, no obvious relation was noted between liver CYP1A levels and either (1) external oiling, (2) total concentration of aromatic hydrocarbons in the liver, or (3) pathological/histopathological lesions observed (Table 2).

Liver Examinations and Histology, 2001 samples: Endoscopy procedures allowed visualization of the livers sampled in 2001, as the endoscope was connected to a television monitor. Consequently, gross abnormalities, if present, could be readily observed. Upon visual examination, three sea otters from the oiled area (of 15 examined) showed marked liver changes, including swollen margins, irregular surfaces, and abnormally dark, mottled color. Two of these individuals (SO-01-21, a 3 year old female, and SO-01-28, a 5 year old male) also had the highest CYP1A blood values (25.1×10^6 and 17.83×10^6 respectively), and highest serum GGT levels (53 IU and 25 IU, respectively) of the group. These two sea otters also exhibited the most severe of the liver lesions observed (see below).

Microscopically, the livers had a spectrum of lesions that included regenerative, regenerative and inflammatory changes. Those lesions included: 1) telangiectasis; 2) lympohistiocytic inflammation; 3) vacuolar and fatty degeneration; 4) necrosis; 5) foci of regeneration; and 6) apoptosis. The inflammatory, degenerative, necrotic, and regenerative lesions were present in both groups (oiled and unoiled area animals) to varying degrees of severity. There was no discernable difference between oiled and non-oiled area animals with regard to inflammatory lesions. In general, the degenerative, regenerative and necrosis lesions were more severe in the oiled area animals. The telangiectasis (observed in two otters, SO-01-21 and SO-98-29) and apoptosis (observed in one otter, SO-01-28) lesions were limited to specific animals captured in the oiled area.

DISCUSSION

Generally, the CYP1A data from 2001 are similar to those collected from 1996-98, in that there is a marked elevation as well as high variability in CYP1A values of sea otters from the oiled area. This result further supports previous conclusions (Bodkin et al. 2002) that lingering oil is constraining recovery. There is some indication that overall, oil exposure at northern Knight Island is declining, as the mean value for CYP1A in blood samples from 2001 (7.91×10^6, n = 16) is lower than measured in previous years (Ballachey et al. 2002), including 1998 (13.07×10^6, n = 23). Nevertheless, the difference between oiled and unoiled areas is still highly significant, and the majority of the animals from the unoiled area continue to have CYP1A values below the minimum value noted in the oiled area, for both blood and liver samples. For example, 13 of 18 sea otters from the unoiled area have blood CYP1A values below 2×10^6, whereas none of the 16 sea otters sampled in the oiled area have blood CYP1A values below that level.

At 0.55, the correlation between blood and liver CYP1A values is, as expected, positive, but clearly there are different sources of variation influencing the two measures of hydrocarbon exposure. Further paired blood and liver samples have been collected in 2002 and 2003, and will be examined with the 2001 data as part of the finalization of Project //423.

The generally lower values among CYP1A values measured in the 1989 liver samples relative to those from otters in oiled areas in 2001 were not expected. We had anticipated that in 1989, the sea otters would have been exposed to much greater quantities of oil (all but one of the 1989 otters had some degree of external oiling at capture, and died shortly thereafter, presumably because of causes related to oil toxicity) and that this would be reflected in greatly elevated CYP1A values, relative to samples collected over a decade later.

Instability and time/storage-related loss of the CYP1A mRNA transcripts should not have been a factor in our results, as the 1989 liver samples were screened prior to CYP1A analyses and samples with evidence of degraded RNA were not used in the study. Nevertheless we cannot preclude the possibility that a difference in handling (cryopreservation of 2001 liver samples immediately after collection from a live sea otter, with analysis within months of collection, vs. sampling at necropsy some hours after death, and frozen storage at –20C for a 12 year period prior to analysis) may have affected our findings.

As stated, there were no apparent relations between CYP1A and external oiling, days in captivity prior to death, or pathological lesions (as reported by Lipscomb et al. 1993, 1996). Aromatic hydrocarbon concentrations in the liver tissue were available for 7 of the 9 otters sampled from 1989, and if anything, a weak, non-statistically significant negative relation (r = -0.39) existed between CYP1A and hydrocarbon concentration. Although the sea

otters came in with external oiling, if they were in reasonable condition during the time they survived in the rescue/rehab centers (ranging from 1 to 23 days), they may have been able to mount a CYP1A response to metabolize hydrocarbons in the liver thus contributing to a negative correlation between the two measures, and relatively low liver hydrocarbon concentrations (see below). Highest CYP1A levels actually may have occurred well prior to capture and death of the otters, and declined by the time of death.

Alternatively, the negative correlation may not be real, and the lack of relation between the CYP1A and liver hydrocarbon concentrations may be due in part to the relatively low hydrocarbon concentrations. The range of concentrations (31.90 – 122.88 ng/g) was, in fact, similar to or only slightly higher than concentrations observed in sea otters from clean areas of south-east Alaska (range 34.13 – 57.89 ng/g; Ballachey and Kloecker, 1997b), and far lower than some liver concentrations (range 87.94 – 973.46 ng/g) measured in sea otters recovered dead in 1989, with heavy external oiling. Furthermore, it seems likely that the otters which were brought in to the rescue centers and subsequently died, were severely compromised prior to death and consequently CYP1A induction in these individuals may not be representative of what would occur in a less compromised animal. We do not have a good understanding of the relative contributions of hydrocarbon toxicity, organ pathologies (secondary to hydrocarbon toxicity), and capture/holding stress to the death of these otters, nor of the ability of compromised individuals to generate a CYP1A response to hydrocarbons or other xenobiotics. Further research, examining CYP1A responses of healthy animals to known quantities of hydrocarbons under controlled conditions, would be of great benefit in interpreting CYP1A data obtained from early post-spill samples as well as samples collected a decade or more later.

Additional findings and conclusions from this study, particularly in relation to (1) the relation between blood and liver CYP1A levels, and (2) liver pathologies and CYP1A levels, will be incorporated into the final report for Project 03423, which will also cover samples of liver collected in 2002 and 2003. This report is scheduled for completion in November 2003.

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Table 1. Cytochrome P450 1A values[a] (mean, standard error, range, and number of samples) in blood and liver samples from sea otters captured in western PWS in 2001, and in liver samples from sea otters dying in 1989 post-spill.

Tissue	Unoiled area, 2001	Oiled area, 2001	Oiled area, 1989
Blood[b]	2.44 0.69 0.44 – 9.70 (18)	7.91 1.57 2.18 – 25.1 (16)	--
Liver	1.03 0.11 0.38 – 2.10 (15)	6.00 0.82 2.03 – 12.62 (15)	2.21 0.37 1.0 – 3.83 (9)

[a] CYP1A expressed as molecules of CYP1A mRNA x 10^6 per 100 ng RNA; measured by reverse-transcriptase polymerase chain reaction.
[b] CYP1A measured in peripheral blood mononuclear cells.

Table 2. Data on sea otters dying in 1989 and sampled for liver cytochrome P450 1A (CYP1A) in 2001.

Otter #	Sex	External Oiling[a]	Date of Arrival[b]	Days in Center[c]	Pathological Lesions[d]	Total Aromatics[e]	CYP1A[f]
VZ-013	F	Heavy	1 Apr	9	None found	38.41	3.83
VZ-035	M	Heavy	2 Apr	7	CLHN	122.88	1.6
VZ-135	M	Heavy	19 Apr	4	HL, RL	No data	1.04
VZ-100	F	Moderate	8 Apr	1	EMP, GE, HL, RL	79.22	1.0
VZ-109	M	Moderate	9 Apr	4	GE	52.45	3.49
VZ-074	M	Moderate	6 Apr	23	None found	66.64	1.18
VZ-099	M	Light	8 Apr	23	None found	79.60	3.34
SW-170	M	None	17 July	18	HL	31.90	1.86
VD-110[g]	M	Moderate	9 Apr	--	None found	No data	2.53

[a] External oiling, assessed at arrival at Rescue/Rehab Center.
[b] Date of arrival in 1989 at Rehab/Rescue Center.
[c] # of days in captivity prior to death.
[d] Pathological and histopathological lesions, as reported by Lipscomb et al., 1996. CLHN: centrilobular hepatic necrosis; HL: hepatic lipidosis; RL: renal lipidosis; EMP: emphysema; GE: gastric erosion.
[e] Total aromatics, in ng/g of wet weight of liver tissue, computed as the sum of all aromatic hydrocarbons measured and reported for that sample. Hydrocarbon analyses were done by GERG, Texas A&M University. See Ballachey and Kloecker 1997a,b for further information on hydrocarbon analyses in sea otters.
[f] CYP1A expressed as molecules of CYP1A mRNA x 10^6 per 100 ng RNA; measured by reverse-transcriptase polymerase chain reaction.
[g] Sea otter recovered dead on 9 April 1989.

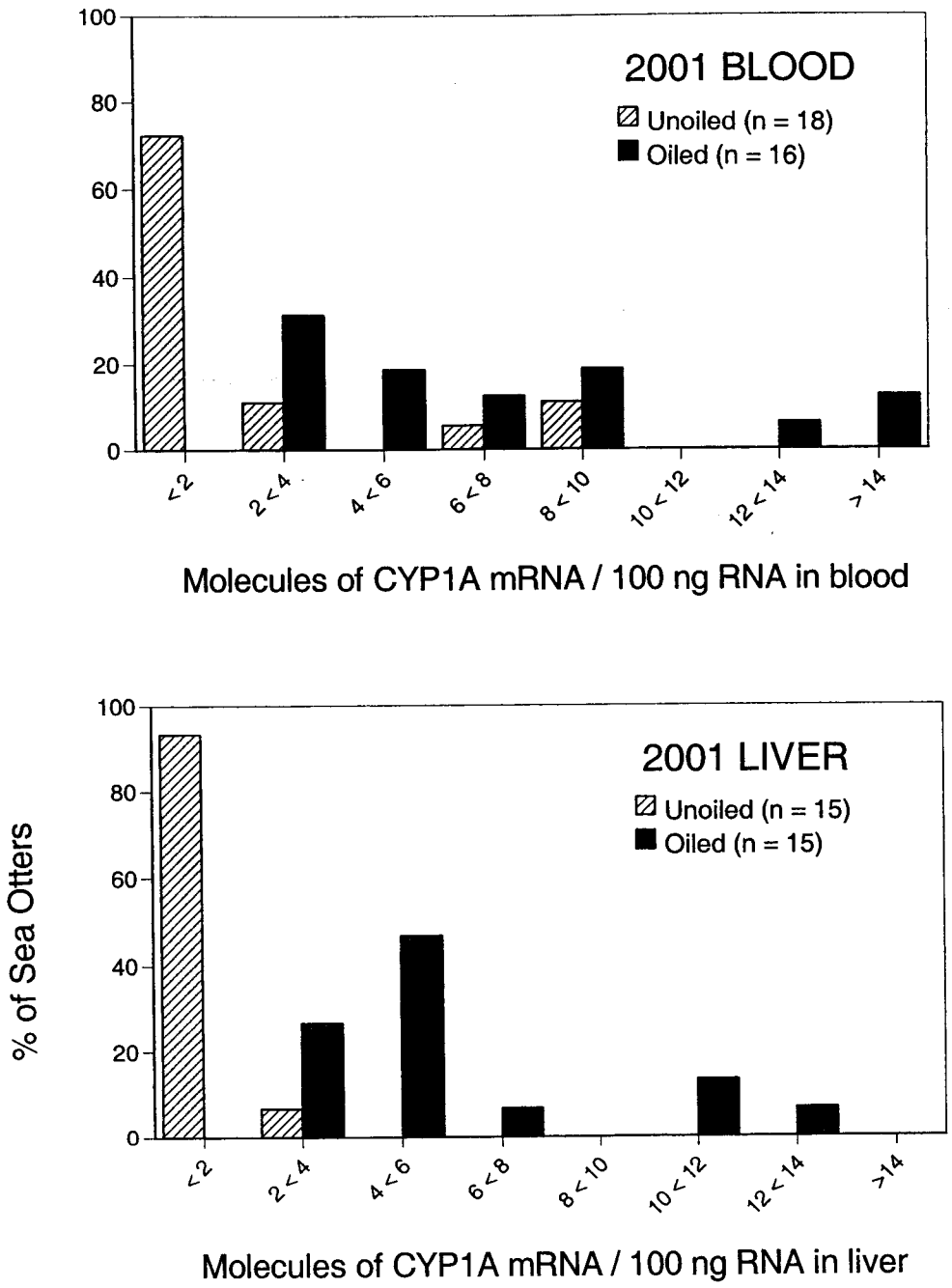

Figure 1. CYP1A values in blood and liver samples collected from sea otters in oiled and unoiled areas of western PWS, 2001.

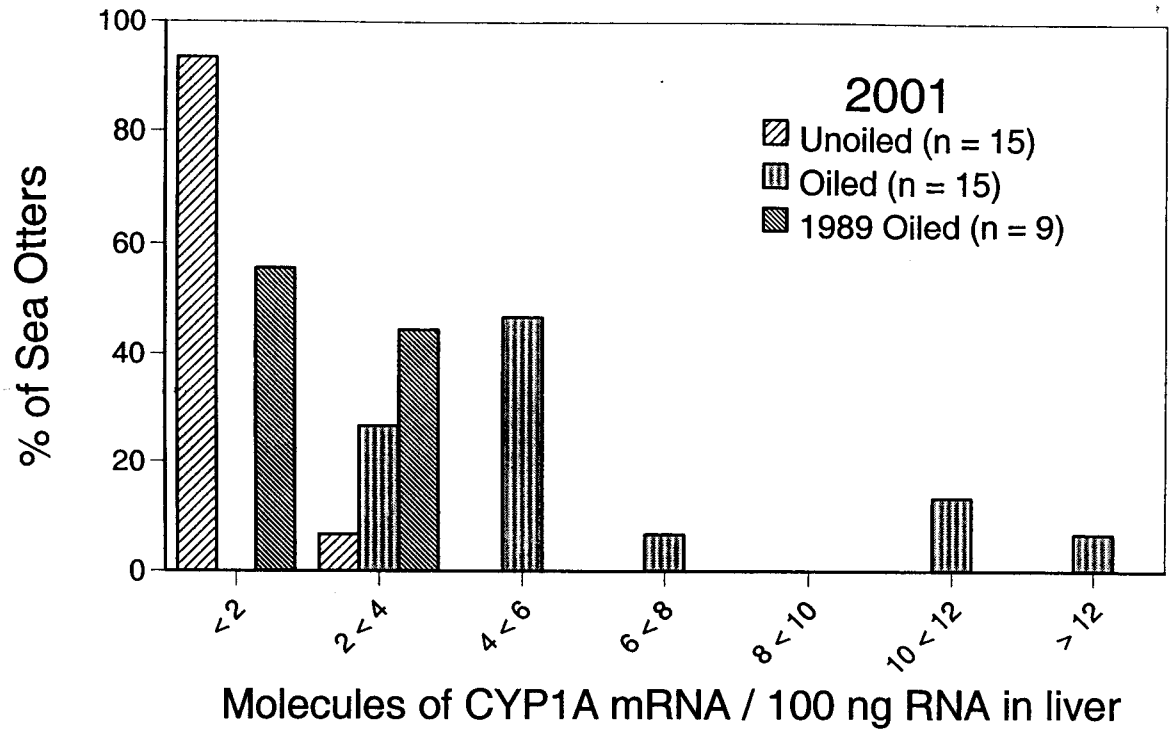

Figure 2. CYP1A values in liver samples collected from sea otters in oiled and unoiled areas of western PWS in 2001 and from sea otters dying post-spill in 1989.