Validation of a cell culture bioassay for detection of petroleum exposure in mink (*Mustela vison*) as a model for detection in sea otters (*Enhydra lutris*)

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Objective—To validate a luciferase bioassay, which is based on a recombinant mouse hepatoma cell line, for the detection of exposure to petroleum in mustelid species.

Animals—122 American mink (*Mustela vison)* and 15 sea otters (*Enhydra lutris*).

Procedures—Mink were exposed to Bunker C fuel oil or Alaska North Slope crude oil externally as a single exposure or internally via low dose concentrations in their ration for 6 months. Serum samples were analyzed for cytochrome P450 1A1 induction by quantification of luciferase activity in the bioassay. Mink liver specimens were also evaluated for cytochrome P450 1A1 induction by quantification of ethoxyresorufin-o-deethylase activity. Serum collected from exposed and unexposed sea otters was also analyzed using the luciferase bioassay.

Results—Serum samples from mink externally exposed to petroleum had significantly increased luciferase activities at 1 week after exposure. Serum samples taken at later time points or from mink exposed to either product in the ration did not cause significant luciferase induction. Samples from otters exposed to petroleum had significantly higher luciferase induction as compared with samples from otters not exposed to petroleum at 2 and 8 years after the spill. Cytochrome P450 1A1 activity in liver specimens collected from mink that were internally exposed through diet was significantly increased at the conclusion of our study.

Conclusion and Clinical Relevance—The luciferase bioassay is a sensitive and specific method for determining recent exposure to petroleum in mink. The lack of luciferase activity in serum samples collected from mink greater than 1 week after experimental exposure was likely attributable to lower overall petroleum exposure in our trial, compared with natural exposures. (*Am J Vet Res* 2002;63:963–968)

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Crude and refined petroleum products can contain thousands of organic and inorganic compounds; however, they are predominantly (50 to 98%) composed of various aromatic and nonaromatic hydrocarbons.[1] The release of such products into the marine environment can cause substantial morbidity and mortality in exposed organisms.[2] The most dramatic recent example of this was the **Exxon Valdez oil spill (EVOS)**, which was ultimately responsible for the deaths of hundreds of thousands of animals, including several thousand sea otters. Otters are at high risk, because the insulating capacity of their coat is destroyed when exposed to oil, leading to acute mortality from hypothermia.[3,4] In addition, grooming of oiled fur by exposed otters results in substantial internal exposure to petroleum products and their associated **polycyclic aromatic hydrocarbon (PAH)** components by ingestion. Exposure to these aromatic compounds can cause many health effects in otters, including CNS depression, respiratory distress and emphysema, organopathies, anemia, and death.[5-7]

Exposure to oil induces many metabolic enzymes including **cytochrome P450 1A1 (CYP1A1)**, a microsomal-bound hemoprotein with wide species and tissue distributions. Induction of CYP1A1 is mediated by the **Ah receptor (AhR)**, a soluble intracellular receptor protein that binds PAH and related chemicals with high affinity.[8-10] After binding of the inducing chemical, AhR accumulates within the nucleus, and its interaction with a specific DNA-binding sequence immediately adjacent to the CYP1A1 promoter results in increased CYP1A1 gene transcription and an increase in CYP1A1-derived protein (P4501A1).[9,10] Oxidative metabolism of the inducing PAH and related compounds by P4501A1 results in PAH detoxification and enhanced elimination,[11] thus playing a key role in an organism's defense against potentially damaging xenobiotics.

Although external exposure to oil may be easy to assess by visual observation, quantification of internal exposure by ingestion has been difficult. Determination of CYP1A1 activity (usually through measurement of **ethoxyresorufin-o-deethylase [EROD]** activity) has been used as a biomarker of contaminant exposure in marine mammals.[12-14] However, the utility of EROD analyses in determining the extent of petroleum exposure is somewhat limited because of differing intra- and interspecies responses,[9,15,16] the variety of structurally dissimilar chemicals that can activate the AhR signal transduction pathway and inhibit EROD

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activity,[9,15-17] and the assay's requirement for tissues rich in CYP1A1 activity (ie, liver specimens). Chemical methods, such as gas and liquid chromatography, have been used to assess petroleum exposure in otters.[18] However, these techniques are often prohibitively expensive, require extensive time-consuming extraction methods, and rely on tissues acquired at necropsy or samples difficult to acquire from debilitated animals (ie, bile or large volumes of blood).

A cell-based bioassay, originally developed to screen natural and manufactured products for the presence of dioxin-like chemicals, was recently modified for the direct analysis of serum.[19] This assay uses a recombinant luciferase reporter gene to detect AhR-dependent activation, thereby allowing the quantification of dioxin-like chemicals present in samples and sample extracts. Induction of luciferase activity occurs in a time-, dose-, AhR-, and chemical-specific manner.[20] Results of additional studies indicate that numerous PAH and petroleum products can be detected and semiquantitated through the evaluation of bioluminescence produced through coactivation of luciferase and CYP1A1 genes, and their induction potencies compared with the most potent inducer **2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)**.[21,22]

In this study, we examined the utility of the luciferase bioassay as a tool for epidemiologic assessments of sea otter populations, as well as its ability to determine exposure in individual otters during oil spills. We validated the luciferase bioassay through an experimental exposure of American mink (*Mustela vison*), a laboratory model previously used for sea otters,[23] to 2 commonly used and transported petroleum products, **Alaska North Slope crude oil (ANSCO)** and **bunker C fuel oil (BCFO)**. These data were compared with bioassay results from 3 sea otter populations in Alaska with a history of known oil exposure.

Materials and Methods

Experimental animals—This study was part of a large ,multi-faceted project to determine the effects of petroleum exposure in mustelids.[23,24] One hundred twenty-two yearling female American mink were selected from a commercial mink-breeding facility and randomly assigned to 1 of 6 oil exposure groups. These groups included mink externally exposed to ANSCO (n = 24), BCFO (24), or seawater (11), or internally exposed through diet to ANSCO (24), BCFO (24), or a standard mink ration (15). Sample sizes were chosen to account for possible mortalities associated with exposure, to minimize the total number of mink required, and to maximize the likelihood of detecting a true difference of 45% in bioassay results between exposed and nonexposed mink with 80% power and 95% confidence. A university animal care and use committee approved all procedures.

Petroleum **exposure—**Animal exposure was accomplished as previously described.[23,24] Briefly, external exposures were accomplished by placing mink in seawater containing a 1.5-cm-thick oil slick (achieved by adding 500 ml of the appropriate product to 4 L of water in a 5 L container), which approximates the quantity of oil present in a heavy oil slick. Mink were allowed to be in contact with this product for 1 minute, which allowed for complete dermal exposure down to the skin, as well as minimized acute morbidity associated with exposure. Internal exposures through diet were accomplished by feeding each mink a standard mink ration containing 500 ppm (vol/vol) of the appropriate product throughout the 6-month study period. External and internal dosages were extrapolated from previously conducted studies documenting oil content in the environment during a spill and in typical otter food items after a spill event.[25] Mink were identified with a coding number and personnel performing observations, feedings, and sample collection were blinded as to treatment status.

Serum samples from mink—Mink were bled prior to exposure, 1 week after exposure, and at 2-week intervals thereafter for 7 weeks (with the exception of week 3, where only externally exposed mink were bled, and week 5, where only internally exposed mink were bled). Mink were given atropine sulfate (0.02 mg/kg, IM) and ketamine hydrochloride (25 mg/kg, IM), after which approximately 2.5 ml of whole blood was drawn by jugular or cardiac venipuncture, with care being taken to collect blood aseptically to prevent potential contamination of samples from dermal petroleum exposure. Blood was placed in serum separation tubes,[a] allowed to clot for 15 to 30 minutes, centrifuged for 5 minutes, and aliquots of serum were aseptically placed into cryovials and stored at –80 C until analyzed. At the conclusion of our study (6 months after exposure), all mink were humanely euthanatized individually by exposure to carbon monoxide and necropsied. Liver specimens were stored frozen in glass containers at –80 C until analyzed.

Sea otters—Representative serum samples randomly selected from samples collected from free-ranging sea otters captured in the Prince William Sound area at various times after the EVOS were analyzed. Samples included those collected during the initial medical evaluation of otters that underwent rehabilitation at the Seward Otter Rehabilitation Center 1 to 2 months after the EVOS in 1989 (n = 5), and samples collected 2 years (1991; 5) and 8 years (1997; 5) after the spill during damage assessment and restoration projects in and around the spill area.

Luciferase bioassay—Recombinant mouse hepatoma (H1L1.1c2) cells, which contain the stably transfected PAH and halogenated aromatic hydrocarbons-inducible luciferase expression vector pGudLuc1.1, as well as the protocol for analysis of samples using a 96-well luciferase bioassay format, have been previously described.[20,26] Briefly, 100-mm-diameter plates of cells were trypsinized, resuspended in culture media,[b] diluted to 300,000 cells/ml, and 200 µl aliquots were added to wells in sterile 96-well culture plates.[c] Plates were incubated for 24 hours, washed with PBS solution, followed by the addition of 75 µl of control fetal bovine serum[b] (containing dimethyl sulfoxide or TCDD at a 1% final concentration) or mink serum (at a 50% dilution in minimum essential medium) in triplicate. Plates were incubated for 3 hours at 37 C, washed twice with PBS solution, followed by the addition of 25 µl of lysis buffer[d] to each well. Luciferase activity was measured using an automated microplate luminometer[e] in enhanced flash mode after the addition of 50 µl of luciferase reagent.[d] Correction for intraplate variation was accomplished through protein quantification using fluorescamine[f] (at 500 µg/ml) in acetonitrile,[g] and detection with a multiplate fluorometer[h] at 400 nm excitation and 460 nm emission wavelengths. Interplate variation was corrected by standardizing each plate to the luciferase activity induced by 1 n*M* TCDD.[19] Final results were expressed as relative light units per milligram protein.

Ethoxyresorufin-o-deethylase bioassay—Liver microsomes were prepared using standard procedures. Briefly, liver specimens were homogenized, centrifuged at 10,000 × g for 20 minutes, and the supernatant further centrifuged at 105,000 × g for 65 minutes. The resulting microsomal pellet

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was washed, recentrifuged at 105,000 × g for an additional 65 minutes, and resuspended in buffer and stored frozen at –80 C until used. Final EROD activity was measured as previously described[27] with minor modification. Final incubation mixtures contained 7-ethoxyresorufin[g] (3.5 µ*M*) and microsomal proteins (0.05 to 0.1 mg protein/ well), and each sample was assayed in triplicate. Reactions were initiated by addition of NADPH[g] (0.6 n*M*) and production of resorufin was measured kinetically for 15 minutes using a microplate fluorometer with excitation and emission wavelengths of 550 and 585 nm, respectively. The reaction was terminated by the addition of 50 µl of fluorescamine (500 µg/ml in acetonitrile), and results were standardized to protein concentration determined by fluorescence.[28]

Data analysis—Differences in luciferase bioassay results and EROD activities among mink groups and otter populations were analyzed by the nonparametric Kruskal-Wallis ANOVA technique.[i] When significant differences were detected, pairwise analyses were done using Mann-Whitney U tests. A value of $P < 0.05$ was used to indicate significance. Correlations between luciferase induction and EROD results were assessed using the Spearman rank order method.

The optimal cut-off value for classification of luciferase bioassay results as positive or negative was determined by 2 methods. First, cut-off values were assigned on the basis of the mean plus 2 SD for all samples collected at week 0, and from all control samples from week 1. Sensitivity and specificity values, and 95% **confidence intervals (CI)** were determined for each product at the selected cut-off value using previously described methods.[29] A **two-graph receiver operator characteristic (TG-ROC)** method[30] was also used to determine optimal cut-off values. This method plots sensitivity and specificity against various threshold values and determines the optimal cut-off where sensitivity and specificity are approximately equal (thereby minimizing the number of misclassified samples), as well as calculating an intermediate range where samples are deemed suspect at a preselected accuracy value.

Results

Large increases in luciferase activity were observed for serum samples from all mink exposure groups at week 1, with significant increases in luciferase activity in mink externally exposed to BCFO and ANSCO (**Fig 1**). Similarly, sea otters exposed to oil during the EVOS had serum samples with significantly higher luciferase induction results than serum samples from otters tested 2 and 8 years after the spill (**Table 1**).

The EROD activity of liver specimens collected from mink surviving to the end of the trial revealed significant increases for groups exposed to either petroleum product in the ration (**Fig 2**). Additionally, externally exposed mink that died between weeks 3 and 9 of the trial had greater EROD responses than those that died immediately following exposure or those that survived to the conclusion of our study (**Fig 3**). No significant correlations between induction of luciferase activity and EROD activities were detected.

Cut-off values using the mean plus 2 SD resulted in high specificity estimates (range, 89.5 to 96.1%; 95% CI, 75.7 to 100%) for both products using week-0 and week-1 controls. In contrast, sensitivity estimates using the week-0 controls resulted in high sensitivity estimates (range, 90.9 to 100%; 95% CI, 73.9 to 100%); use of week-1 control groups resulted in low estimates (range, 8.3 to 27.2%; 95% CI, 0 to 53.6%). Using the TG-ROC method, sensitivity values improved for both products using the week 1 controls (76.9 and 81.8% for ANSCO and BCFO, respec-

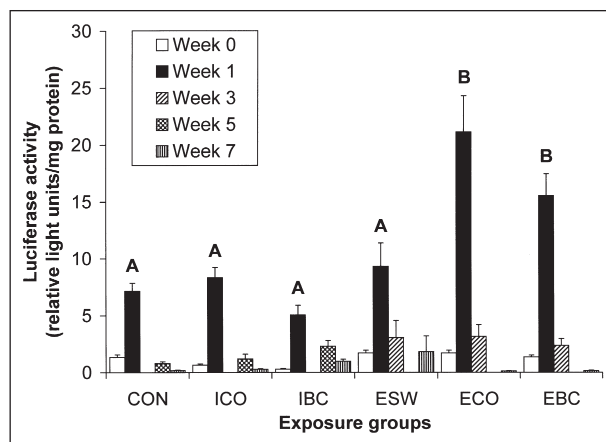

Figure 1—Induction of luciferase activity in serum samples from mink exposed to petroleum products internally through ingestion of control diet (CON; ie, no exposure to petroleum products), Alaska North Slope crude oil (ICO), or bunker C fuel oil (IBC), or externally by placement in salt water (ESW; ie, no exposure to petroleum products), Alaska North Slope crude oil (ECO), or bunker C fuel oil (EBC). Values represent the means ± SD from triplicate determinations. Bars that do not share a common letter differ significantly ($P < 0.05$).

Table 1—Median (and range) values of luciferase activity of serum samples from 3 sea otter populations

Location	Year	Time after spill*	No. of sea otters	Luciferase activity (relative light units/mg protein)
Seward Otter				
Rehabilitation center	1989	30–45 days	5	11.653 (7.140–13782)[a]
Prince William Sound	1991	2 years	5	0.172 (0.018–0.337)[b]
Prince William Sound	1997	8 years	5	0.169 (0.154–0.273)[b]

*Exxon Valdez oil spill, March 24, 1989.
[a,b]Values with different superscript letters are significantly ($P < 0.05$) different.

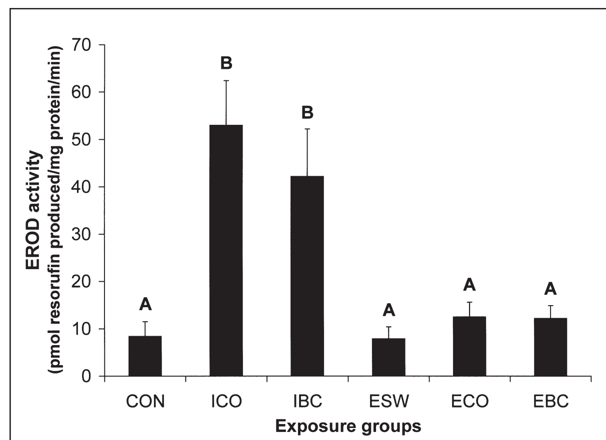

Figure 2—Induction of liver ethoxyresorufin-o-deethylase (EROD) activity in serum samples from mink exposed to petroleum products internally through ingestion of CON, ICO or IBC, or externally by placement in ESW, ECO, or EBC. Values represent the means ± SD from triplicate determinations. Bars that do not share a common letter differ significantly ($P < 0.05$).

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tively; 95% CI, 54.0 to 100%), with only a slight decrease in specificity (71.4 and 73.7% for ANSCO and BCFO, respectively; 95% CI, 52.1 to 93.5%). Sensitivity and specificity values for both products on the basis of week-0 controls remained high (range, 90.9 to 97.1%; 95% CI; 73.9 to 100%) with the TG-ROC method (**Fig 4**).

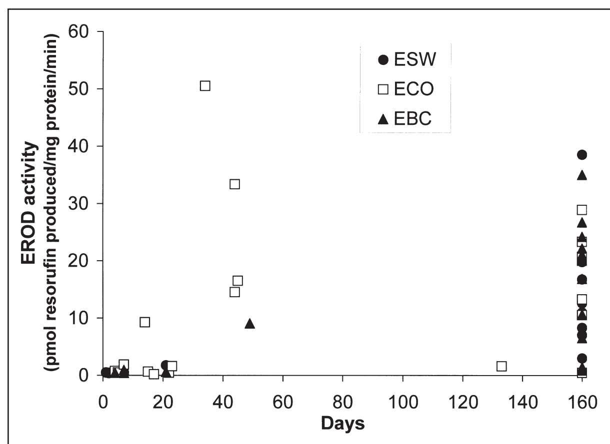
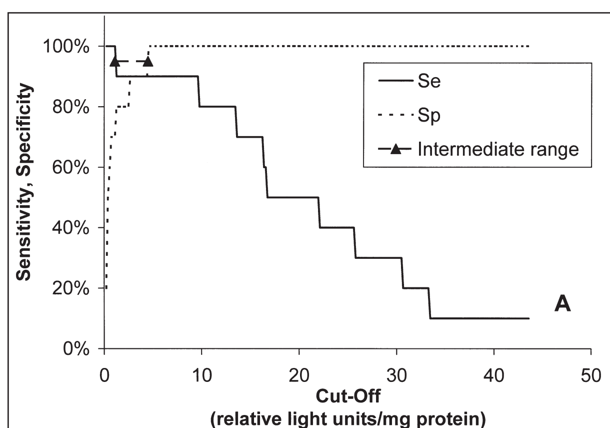


Figure 3—Induction of liver EROD activity in mink externally exposed to ESW, ECO, or EBC. Values represent the means from triplicate determinations.



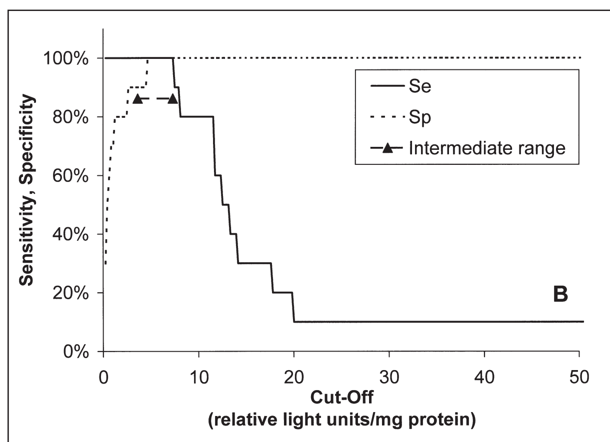


Figure 4—Two-graph receiver operator characteristic (TG-ROC) plot of luciferase activity in serum samples from mink externally exposed to Alaska North Slope crude oil (panel A) or bunker C fuel oil (panel B). Serum samples from week 0 were used as negative controls.

Discussion

Induction of luciferase activity in serum samples from mink exposed to an acute high dose of ANSCO and BCFO was expected, because mink groomed and ingested petroleum that was then taken up by the blood. However, the concomitant increase in luciferase activity in serum samples of control groups, although significantly lower than that of externally exposed mink, was unexpected. It is possible that all mink in the first week of the experiment were exposed to compounds with substantial AhR-activity resulting in induction of luciferase activity. However, as mink are exquisitely sensitive to dioxin-like compounds[31,32] and no lesions were observed in tissues on necropsy, such an exposure would most likely be to weaker, less potent AhR agonists (ie, less active halogenated aromatic hydrocarbons or other natural or synthetic compounds such as indoles, flavones, or carotenoids).[9,33,34] As all mink were housed in a single building and shared similar management methods and caging (other than diet), it is also possible that environmental conditions during this period, such as an air pollutant or a compound in bedding material with AhR activity, caused a facility-wide increase in blood contaminant concentrations. The recent finding that a range of structurally dissimilar chemicals can activate AhR supports this explanation.[9] Whatever the cause, however, it did not mask the significant increase in luciferase activity that was detected in serum samples from externally exposed mink.

Between 1 and 3 weeks after exposure, luciferase activity in serum samples from all groups declined to values comparable to those evident prior to the trial. Although this finding might suggest that the bioassay has limited application in spill situations, analysis of serum samples from otters exposed to oil during the EVOS revealed that serum from otters captured 30 to 45 days after the onset of the spill event still induced luciferase activity (Table 1). Therefore, the substantial decline in values detected in serum samples from mink is most likely attributable to differences between the experimental exposure and natural exposure. These differences may be attributable simply to a lower dose exposure in mink as compared with natural exposure of otters, or may be attributable to repeated exposures of otters during spill events. In otters affected during oil spills, ongoing grooming-exposure activities are likely to occur as a result of the natural behavior of otters, persistent oil in the environment, and oil contamination of prey. Continued spilling of product from the source would also result in increased total duration of exposure to AhR-active ligands. This reexposure cycle is likely to continue until otters are captured for rehabilitation, emigrate from the spill site, or succumb to hypothermia or other oil-related physiologic problems. The externally exposed mink in our trial had large single-dose exposures. Therefore, on the basis of our preliminary analysis of oil-exposed otters, we believe that the bioassay can be used in an analytically sensitive and clinically relevant manner to detect oil exposure in mustelid species.

Significantly increased EROD values were observed in mink exposed to ANSCO and BCFO in their ration

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but not in the control mink or those externally exposed 6 months earlier (Fig 2). For those mink that died during our study, higher EROD activity was found for those that died between weeks 4 and 7, compared with those that died within the first 3 weeks (Fig 3). These data, in combination with the serum bioassay results, indicate that the increase in concentrations of AhR-active chemicals detected in the serum samples during the first 3 weeks after exposure appeared to be ineffective at inducing hepatic CYP1A1-dependent EROD activity in mink. However, interestingly, when increases in hepatic EROD activity were observed at weeks 4 to 7 (Fig 3), the serum from mink contained little or no detectable AhR-active chemicals (Fig 1). These results would suggest that an AhR-active inducer accumulated or formed within the liver tissue to an amount that resulted in induction of EROD activity. The fact that little AhR-active activity was observed in serum samples from mink during this period would suggest the latter possibility. Alternatively, it is possible that an AhR active chemical was present in the blood of the exposed mink, yet not detected by the luciferase cell bioassay. The existence of a species difference in the ligand binding specificity of the AhR between the mink and the mouse AhR (species in which the cell for the bioassay system is derived) would be consistent with our results. Our previous identification of significant differences in the ability of single-hydroxylated metabolites of benzo[a] pyrene to bind to the AhR from various species,[15] coupled with published reports documenting other species differences in AhR ligand binding specificity, are in support of this hypothesis.[16,20,35] The chemicals responsible for the EROD induction in mink 4 to 7 weeks after external exposure remains to be identified and is an interesting direction for future studies. Finally, the temporal decrease in bioassay response observed with serum from mink 4 to 7 weeks after external exposure likely results from a decrease in serum PAH concentration resulting from continued metabolism and elimination of these products in mink. Therefore, joint interpretation of luciferase and EROD results might not only allow for more appropriate interpretation of the degree and duration of petroleum exposure, but it may provide an avenue in which to identify other AhR-active chemicals which may be formed in vivo.

To assess the validity of the luciferase bioassay, 2 methods were used to assign cut-off values for experimentally exposed mink. The first, assigning the value of the mean of the control plus 2 SD, is a commonly used technique in immunologic and other serologic diagnostic assays,[36] however, it assumes that data are normally distributed and does not take into account known positive results (thereby optimizing specificity at the expense of sensitivity). The second method, the TG-ROC technique, reduces these effects and can provide an intermediate range where upper and lower cut-off values are established to classify samples from suspect animals (ie, animals with nonoil-related exposure). Because of the unexplained increase in baseline induction in the controls at week 1, 2 separate comparison groups were evaluated for the TG-ROC method: results for the week-1 internal and external control samples, and data from all mink prior to the start of the exposures (week 0). Using the control data from week 1, sensitivity values were 76.9 and 81.8% for ANSCO and BCFO, respectively. When control data from week 0 were used, all sensitivity and specificity estimates increased to more than 90% (Fig 4). Use of an intermediate range for ANSCO using the week-0 controls reclassified a previously identified false-negative and 5 of 9 previously identified false-positive results as suspect, thereby increasing sensitivity to 100% and specificity to 95.1%. This range, however, classified 17 of the 94 nonexposed test-negative samples as suspect. For BCFO, the use of an intermediate range caused the single previously false-negative sample to become suspect (increasing sensitivity from 91.7 to 100%), however it did not correctly reclassify any of the 3 false-positive results. In fact, the use of this technique reduced overall specificity from 96.5 to 95.1%. This reduction was caused by the overall test accuracy for BCFO previously being 96.5%; by ascribing 95% accuracy to the range, a poorer discrimination of test results occurred. Therefore, it appears that the TG-ROC technique with an intermediate range, in many instances, can be a valuable method to reduce misclassification of potentially exposed animals. However, cut-off values should be flexible depending on the cost of a misclassified animal and the uncertainty of baseline pollutant content in the sample population.

In conclusion, on the basis of these data and results of previous optimization studies,[19,21,22] the luciferase bioassay is a time-, cost- and sample-conserving diagnostic method to accurately characterize mink petroleum exposure immediately following oil exposure. Results of prior work[22] have established a minimum detection limit of this bioassay system of 6.67 ng/g of serum for these products. Because results from tissue analyses on otters that died during the EVOS revealed concentrations of aromatic hydrocarbons of 700 to 2,000 ng/g,[18] and otters tested 1 year after the spill had median concentrations of 22.06 ng/g total aromatics in whole blood,[37] this assay has the potential to be a valuable diagnostic tool for otters following a spill. Because the rehabilitation process may cause morbidity and mortality as a result of stress- and captivity-related problems, the rapid assessment and release of unaffected animals can minimize stress and reduce costs associated with spill response. The luciferase bioassay may also be valuable if used on serially collected blood samples to determine appropriate treatments and release times. Although this assay is not designed to be the sole diagnostic method to determine exposure, its use as a screening tool for internal exposure by ingestion may aid veterinarians and rehabilitators in the treatment of affected animals, as well as assist in subsequent litigation and mitigation processes.

[a]Vacutainer, Becton Dickinson, Rutherford, NJ.
[b]Life Technologies, Gaithersburg, Md.
[c]Packard Instruments, Meriden, Conn.
[d]Promega, Madison, Wis.
[e]ML3000, Dynatech, Chantilly, Va.
[f]Molecular Probes, Eugene, Ore.
[g]Sigma Chemicals, St Louis, Mo.
[h]Fluostar, SLT, Salzburg, Austria.
[i]SigmaPlot, SPSS, Chicago, Ill.

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