Bull. Environ. Contam. Toxicol. (1997) 59:513-519

Bulletin of Environmental Contamination and Toxicology

Field Assay for the Detection of Petroleum Products on Wildlife

J. A. K. Mazet,[1,2] I. A. Gardner,[1] D. A. Jessup,[2] J. H. Rittenburg[3]

[1]Wildlife Health Center, VM:Medicine and Epidemiology, University of California, Davis, California 95616, USA
[2]Office of Oil Spill Prevention and Response, California Department of Fish and Game, 1701 Nimbus Road, Suite D, Rancho Cordova, California 95670, USA
[3]Biocode, Inc., 3599 Farm School Road, Ottsville, Pennsylvania 18942, USA

Received: 1 March 1996/Accepted: 11 July 1997

Marine wildlife are at substantial risk of petroleum product exposure both from catastrophic oil spill incidents and low-level intermittent petroleum discharges. Because many marine mammals and birds have dark pelts or plumage with a natural sheen, it is often difficult to determine whether an animal in the vicinity of environmental oil has been exposed. Additionally, capture, restraint, and rehabilitation of potentially exposed wildlife are stressful to the animal and costly to undertake (Estes 1991).

Concerns regarding the capture and subsequent rehabilitation of sea otters which may not have been exposed to oil from the *Exxon Valdez* incident in 1989 prompted the development of a field diagnostic test for the detection of petroleum products on wildlife. Prior to the development of such a test, standard laboratory methods, including gas chromatography (GC), were found to be time consuming and expensive. Because of the potential for low level exposures, some animals were rehabilitated without being definitively diagnosed as petroleum product exposed. Determination of oiling via laboratory methods in these cases would have been impossible in a clinically relevant period of time. The sea otters in question most likely would have groomed away and subsequently ingested the crude oil from their coats, increasing their potential toxic insult, if processing had been delayed until laboratory results could have been obtained. While processing of these animals through the normal oil removal and rehabilitation protocols at that time was the only viable option, it subjected those sea otters to handling stress and captivity which may have been avoided if a rapid sensitive and specific diagnostic test for petroleum product exposure had been available. In fact, histopathologic studies of sea otters which died at rehabilitation centers and were determined to be uncontaminated or lightly oil contaminated by visual inspection showed the presence of gastric erosions which have been attributed to stress (Lipscomb et al. 1993). In addition, almost half (46.7%) of otters which were rehabilitated, implanted with radio transmitters, and released were either dead or missing within eight months of release (Monnett et al. 1990) indicating that post-release survival for animals subjected to the stress of rehabilitation may be low.

Correspondence to: J. A. K. Mazet

For a field assay which detects hydrocarbons on wildlife to have practical application during an oil spill event, it should be cost effective, rapid, and relatively simple to perform so that multiple field personnel could be immediately trained in its use. All of these traits are characteristic of immunoassays, and these techniques have been applied to detect residues of environmental pollutants, such as pesticides and gasoline, in soil and water (Goh et al. 1992, Stocker et al. 1993). If possible, a field assay should also be able to detect a variety of petroleum products and be sufficiently quantitative to allow clinical decisions to be made based on the extent and severity of oiling. In order to meet these goals, we evaluated a direct competitive immunoassay developed by Quantix Systems (Cinnaminson, New Jersey) for the rapid analysis of polynuclear aromatic hydrocarbon (PAH) contamination in soil for adaptation to analysis of PAH exposure on wildlife (the soil assay is now available from Idetek, Inc., Sunnyvale, California). The Quantix system was selected because it enables the user to obtain on-site quantitation of PAH in a cost effective manner. The immunoassay system has a detection limit of 0.7 ppm for a 16 component PAH standard with sensitivities for individual components even lower. The reproducibility obtained using the immunoassay is comparable to that obtained using the U.S. Environmental Protection Agency methods 8015 and 8270 over the linear range of the assay (Kikani et al. 1994).

MATERIALS AND METHODS

The Quantix™ Portable Workstation system for the detection of PAH in soil utilizes a direct competitive immunoassay performed on the microporous surface of a disposable plastic analyte detector (Rittenburg et al. 1993). Antibody production and the preparation of conjugates followed Kikani et al. (1994). Briefly, immunogens and conjugates were prepared by using PAH derivatives bound to either keyhole limpet hemocyanin (Pierce, Rockford, Illinois), bovine serum albumin, chicken albumin (Sigma Chemical Company, Saint Louis, Missouri), or alkaline phosphatase (Boehringer Manheim, Indianapolis, Indiana). Hapten to protein conjugation and removal of unreacted hapten were confirmed using high performance liquid chromatography (HPLC). Antisera were produced in rabbits immunized with immunogens diluted with Freund's complete adjuvant. The resulting antibody and hapten-alkaline phosphatase conjugate were selected based upon sensitivity to a 16 component PAH mixture containing 0.2 mg/mL of acenaphthene, acenaphthylene, anthracene, benz[a]anthracene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[g,h,i]perylene, benzo[k]fluoranthene, chrysene, dibenz[a,h]anthracene, fluoranthene, fluorene, indeno[1,2,3-cd]pyrene, naphthalene, phenanthrene, and pyrene as described by United States Environmental Protection Agency method 610 (Accustandard Inc., New Haven, Connecticut).

Because we were not able to initially assess the validity of the assay using either experimentally or naturally-exposed sea otters, we used a laboratory animal model to determine the appropriateness of the assay's application to the field situation. This live-animal model was required to assess the natural weathering of oil on the exterior

surfaces of wildlife over time which, in conjunction with normal grooming behavior, is expected to result in a gradual decrease in external oil contamination. Additionally, the model was necessary for quantitative evaluation of assay performance through calculation of the assay's sensitivity and specificity. The American mink (*Mustela vison*) was selected as a model for sea otters because, like the sea otter, it is a mustelid and has similar fur quality; it is domesticated; and the mink has been used as toxicological model for a variety of environmental contaminants (Wren 1991). Because they are commonly transported or used as fuel along the Pacific coastline and thus have the highest probability of being spilled, Alaskan North Slope crude oil (ANS crude oil) and Bunker C fuel oil were chosen as test products.

To provide some consistency among quantitative data, a protocol for the use of the commercially available Quantix system was developed. Eleven mink were exposed to ANS crude oil in sea water, 11 were exposed to the fuel oil in sea water, and 11 controls were exposed to sea water alone. All animals moved freely for 1 minute in the oil/sea water (or sea water alone) mixture (500 mL oil to 4 L sea water which resulted in an oil slick of approximately 1.5 cm in depth). The immunoassay procedure was carried out 48 hours, 1 week, and 3 weeks after the mink were externally exposed. Each mink was manually restrained, and a fixed 44 cm^2 area (circular area defined by a template with a 7.5 cm diameter) was swabbed for 15 seconds. Swabs consisted of 1 ply - 10.2 cm by 10.2 cm rayon/polyester general use gauze saturated with 4 mL of technical grade isopropanol. Mink were swabbed on the dorsum just anterior to the tail head.

After the fin of the mink had been swabbed, the cotton gauze was placed into an extraction tube containing 4 mL isopropanol. Isopropanol was selected for extraction because in a comparison of isopropanol to methylene chloride/acetone (90:10), the solvent of choice in the GC method, an average PAH recovery from soil containing varying concentrations of the 16 component PAH standard of 77.0% was achieved with isopropanol, while that obtained using the methylene chloride/acetone mixture was 70.3% (Kikani et al. 1994). The two sets of extracts were analyzed by method 8015. The extraction tube containing the guaze swab was shaken for 1 minute to extract the PAH components. The extract was filtered using a piston filter and then diluted 10-fold. This first dilution allowed detection of 0.7 to 15 ppm PAH from the gauze saturated with isopropanol. If necessary, a second dilution was made to increase the detectable range to 140 ppm (Kikani et al. 1994). The alkaline phosphatase hapten-enzyme conjugate was added to the diluted sample and to a negative reference solution. The analyte detector used had a discrete sample reaction zone (sample zone) as well as a negative control reference reaction zone (reference zone), each of which contained latex particles that had been coated with affinity-purified antibody. Five drops of prepared test sample were added to the sample zone, and 5 drops of negative reference solution were added to the reference zone of the detector. After 3 minutes, each solution had passed through the immobilized antibody and had been absorbed into the detector by capillary action. Polynuclear aromatic hydrocarbon in the sample competed with the hapten-enzyme conjugate for sites on

the immobilized antibodies. To remove any unbound hapten conjugate, 2 drops of rinse solution were added to each reaction zone. To produce a color endpoint for the immunoassay, 2 drops of alkaline phosphatase color forming substrate were added to each reaction zone which then reacted with the antibody bound hapten conjugate. As the concentration of PAH in the sample increased, the color endpoint decreased in intensity. A hand-held dual-beam reflectometer was then used to compare color intensity of the sample zone to the reference zone. The concentration of PAH in the isopropanol saturated gauze was then calculated from a preprogrammed standard curve and displayed by the reflectometer. The sensitivity, specificity, and predictive values of the test were calculated by standard methods (Martin et al. 1988).

RESULTS AND DISCUSSION

The mean PAH levels detected for each of the exposure groups and sampling times using the adaptation of the Quantix system are presented in Table 1. Since the Quantix system has been calibrated for detection of PAH in 8.4 g of soil, the quantitative data are most useful for comparisons of relative exposures among individuals. To place these relative concentrations in context, 25 µl aliquots (n = 5 of each) of ANS crude oil and Bunker C fuel oil were inoculated onto gauze, and 3.16 ppm (10.4% coefficient of variation) and 3.61 ppm (6.0% coefficient of variation) were detected by the immunoassay, respectively. The detected decrease in PAH concentrations over time is expected to mimic the natural situation due to animal grooming and environmental weathering of the petroleum product in question. In this study, all oil-exposed mink groomed both the Bunker C fuel oil and the ANS crude oil from their coats; the winter weather conditions post-exposure also presumably expedited the decrease in PAH concentrations on the external surfaces of the animals. Obviously, the observed rate of decrease in PAH concentration during a response to an oil spill will vary widely with the composition of the specific petroleum product released into the environment, the behavior of the species affected (e.g. fastidiousness), the environmental factors at the time of the incident (wind speed, water temperature, etc.), and the behavior of the individual animals exposed. Although sea otters may encounter more severe environmental conditions than the mink in this study, we speculate that the experimental exposure dose and duration of exposure is considerably less than would be the expected in an environmental incident. Therefore, we conclude that the simulation of exposure in this model should underestimate or accurately represent the situation in the early stages of an environmental disaster similar to the *Exxon Valdez* incident.

The results of the assay's performance are detailed in Table 2. At 48 hours and 1 week post-exposure the adaptation of the Quantix system was able to quantitatively detect petroleum hydrocarbons on all of the exposed animals and did not falsely detect hydrocarbons on any of the unexposed mink (100% sensitivity and specificity). It is important to note that by 1 week post-exposure, no oil was detectable with the naked eye or to the touch. Like sea otters, the mink were grooming the oil from their coats continuously, and the oil which remained on the fur was undergoing a natural

weathering process. Even 3 weeks after the single 60-second exposure, the system was able to detect 8 of 11 exposed animals in the Bunker C group [73% sensitivity at 3 weeks post-exposure; 95% confidence interval (0.39,0.93)], and 2 of 11 crude oil-exposed mink were correctly classified [18% sensitivity at three weeks post-exposure; 95% confidence interval (0.03, 0.52)]. The greater sensitivity to the Bunker C fue1 oil was not surprising since it contained a higher concentration of PAH than did the crude oil (5098.4 mg/g Bunker C vs. 4370.8 mg/g ANS for a measured suite of 16 PAH). At 3 weeks post-exposure, all 11 control animals were correctly classified as negative as well [100% specificity; 95% confidence interval (0.76, 1.00)].

Because the test was 100% specific at all time points, and thus there were no false positive results, the positive predictive value (the probability of being exposed when testing positive) was 100% at all time points. While the negative predictive value (the probability of being unexposed when testing negative) was also 100% at 48 hours and 1 week post-exposure, it decreased 3 weeks after exposure because of the decrease in sensitivity. The negative predictive value is also influenced by prevalence of exposure (the proportion of oiled animals in the population). For example if 50% of the animals tested at 3 weeks post exposure were actually exposed, only 40% testing negative will not have been exposed. This negative predictive value improves as the prevalence of exposure decreases because there are fewer truly exposed animals to be misclassified.

The Quantix immunoassay has been validated by gas chromatography/mass spectrometry (GC/MS) methods and found to have comparable reproducibility over the linear range of the assay to that obtained using United States Environmental Protection Agency SW-846 methods 8015 and 8270 (Kikani, et al. 1994). One major advantage of the field application of this immunoassay over traditional GC/MS methods is that quantitative results are available in a clinically relevant time period; that is, up to 5 samples may be tested within a 30 minute time frame. Therefore within hours of an oil spill event, animals could be captured, sampled, and released or relocated if determined to be unexposed by the test. Cost efficiency is another advantage; while GC/MS costs are often upwards of $1000 per sample, the immunoassay cost is only about $40 per sample. Finally, GC/MS methods require highly trained personnel operating relatively expensive equipment, whereas the immunoassay application requires only a limited amount of training for field personnel. In training these personnel, it is important to stress that the site of sampling should be selected based upon the individual animal being evaluated. For example, marine birds are more likely to contact oil with their breast plumage while sea otters are more likely to contact oil in multiple locations, and areas chosen for testing should include those which are more difficult to groom. The system's commercially available reagents have a six month shelf-life and are delivered via Federal Express same-day or next-day service, therefore making reagents readily available in case of an oil spill. Because of its increased efficiency over traditional methods and the excellent performance observed in the controlled trial, this immunoassay has promise as a tool for reducing the financial costs of an oil spill.

Perhaps more importantly, the assay may result in a decrease in the morbidities and mortalities associated with the stress of unnecessarily rehabilitating unexposed animals, especially since most sea otters and other wildlife that are candidates for rehabilitation will be captured within a week of contact with surface oil.

Table 1. Mean (Standard Error of the Mean) PAH levels (ppm) detected by the adaptation of the Quantix™ Portable Workstation on swabs of fur from experimentally exposed mink.

Exposure Group	Time from Exposure		
	48 hours	1 week	3 weeks
Control	0 (0)	0 (0)	0 (0)
Bunker C Fuel Oil	119.0 (8.60)	65.33 (16.63)	2.36 (0.69)
Crude Oil	83.40 (20.20)	15.23 (5.45)	0.41 (0.28)

Table 2. Performance of the adaptation of the Quantix™ Portable Workstation for PAH detection as a field assay for petroleum product exposure on fur.

Petroleum Product		Time from Exposure		
		48 hours	1 week	3 weeks
Bunker C Fuel Oil	Sensitivity	100%	100%	73%
	Specificity	100%	100%	100%
Crude Oil	Sensitivity	100%	100%	18%
	Specificity	100%	100%	100%

Acknowledgments. Exposures were part of a large-scale study intended to maximize the benefits to wildlife and to limit the needs for invasive research in the future. This study was supported by a contract from the California Department of Fish and Game Office of Oil Spill Prevention and Response (FG3460 OS), as well as the Department's Petroleum Chemistry Laboratory. The authors wish to thank John Gorham, Bruce Hammock, and Saturnino Yanga for their technical support and Michael Joyce of ARCO Alaska for the donation of ANS crude oil.

REFERENCES

Estes JA (1991) Catastrophes and conservation: lessons from sea otters and the *Exxon Valdez.* Science 254: 1596

Goh KS, Spurlock F, Lucas AD, Kollman W, Schoenig S, Braun AL, Stoddard P, Biggar JW, Karu AE, Hammock BD (1992) Enzyme-linked immunosorbent assay (ELISA) of simazine for Delhi and Yolo soils in California. Bull Environ Contam Toxicol 49: 348-353

Kikani BB, Twamley C, Crawford JO, Hobbib GC, Rittenburg JH (1994) Comparison of immunoassay for polynuclear aromatic hydrocarbon measurement in soil against EPA Method 8270. Amer. Chem. Soc. Meeting Abstracts, San Diego, California, March 13-18, 1994

Lipscomb TP, Harris RK, Moeller RB, Pletcher JM, Haebler RJ, Ballachey BE (1993) Histopathologic lesions in sea otters exposed to crude oil. Vet Pathol 30:1-11

Martin SW, Meek AH, Willeberg P (1988) Veterinary epidemiology: Principles and methods. Iowa State University Press, Ames, Iowa, pp 63-69

Monnett C, Rotterman LM, Stack C, Monson D (1990) Post-release monitoring of radio-instrumented sea otters in Prince William Sound. US Fish and Wldlife Service, Biological Report 90(12):400-420

Rittenburg JH, Stocker DR McCaffrey CR (1993) Application of an immunoassay field test kit for measuring BTEX in gasoline contaminated samples. Fed Environ Restoration Conf Proc, pp 343-348

Stocker DR, Miller SM, Rittenburg JH, Twamley C, Lal BS, Grothaus GD (1993) Analysis of gasoline in soil and water using a rapid on-site immunoassay system. Calabrese EJ and Kostecki PT (eds), In: Hydrocarbon contaminated soils, CRC Press, Boca Raton, Florida, pp 167-179.

Wren, CD (1991) Cause-effect linkages between chemicals and populations of mink (*Mustela vison*) and otter (*Lutra canadensis*) in the Great Lakes Basin. J Toxicol Environ Health 33:549-585