

USE OF CELLULOSE FILTER PAPER TO QUANTIFY WHOLE-BLOOD MERCURY IN TWO MARINE MAMMALS: VALIDATION STUDY

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ABSTRACT: Whole blood (WB) is commonly used to assess mercury (Hg) exposure in mammals, but handling and shipping samples collected in remote areas can be difficult. We describe and validate use of cellulose filter paper (FP) for quantifying WB total Hg concentration. Advantec Nobuto[®] FP was soaked with bottledose dolphin (*Tursiops truncatus*) or harbor seal (*Phoca vitulina*) WB (collected between March and July 2012), then air dried. Untreated blood-soaked FPs were analyzed or were eluted with phosphate-buffered saline (PBS) and the eluate and PBS-treated FP Hg concentrations were determined. Total Hg from dried blood-soaked FPs, postelution FPs, and PBS-based eluate were compared with total Hg concentrations from WB. Recovery (on a concentration basis) for soaked FP relative to WB was 0.89 ± 0.15 , for postelution FP was 0.86 ± 0.13 , and for eluate (with a correction factor applied) was 0.96 ± 0.23 . Least-squares linear regressions were fit for soaked papers ($y = 1.15x$, $R^2 = 0.97$), postelution FPs ($y = 1.22x$, $R^2 = 0.95$), and for eluate with a correction factor applied ($y = 0.91x + 0.03$, $R^2 = 0.97$) as compared with WB. These data show that FP technology can have a valuable role in monitoring blood Hg concentrations in wildlife populations and FPs have the advantage of being easy to use, store, and transport as compared with WB.

Key words: Biomonitoring, marine mammal, Nobuto filter paper, total mercury, wildlife.

INTRODUCTION

Mercury (Hg) is a nonessential element that occurs naturally in the environment. Mercury is released into the atmosphere via events such as volcanic eruptions and forest fires. Since the industrial revolution, anthropogenic releases of Hg into the environment have increased, mostly through the burning of fossil fuels and via the mining industry, and may occur at concentrations of concern to health in some biota (e.g., Dietz et al. 2009, 2013). Following deposition of atmospheric Hg into marine and freshwater systems, microbial activity (largely sulfate-reducing bacteria) can transform Hg to the highly bioavailable and toxic monomethylmercury (MeHg⁺) (Fitzgerald et al. 2007; Parks et al. 2013). Monomethylmercury can bioaccumulate and biomagnify with trophic levels (Coelho et al. 2013), reaching

particularly high levels in numerous fish species and piscivores (Castoldi et al. 2001; Lemes et al. 2011; Castellini et al. 2012).

After ingestion, MeHg⁺ is absorbed via intestinal epithelium passively and via active uptake (Leaner and Mason 2002), and is nearly completely absorbed. Crossing the intestinal epithelium, MeHg⁺ enters the blood where 99% binds to thiol groups; the remaining 1% is transported to organs via binding to diffusible low-molecular-weight thiols (Rooney 2007). Hence blood is the route of exposure (and distribution) for most target organs (i.e., the central nervous system) and is a reliable indicator of recent MeHg⁺ exposure (Risher and Amler 2005).

A key target organ for MeHg⁺ toxicity is the central nervous system as MeHg⁺ crosses the blood-brain barrier via an amino acid transporter and accumulates in nervous tissue (Kerper et al. 1992; Caito

et al. 2013). Clinical signs of acute toxicity include proprioceptive deficits, abnormal postures, blindness, anorexia, coma, and death (Ekino et al. 2007). High levels of MeHg⁺ have been shown to impair components of the nervous system (Basu et al. 2006, 2007b). There is concern that, particularly in fish-eating wildlife, chronic exposure to MeHg⁺ can result in poor reproductive success (Basu et al. 2007a). There is also concern that Hg levels in wildlife and in humans that subsist on wildlife (particularly in higher latitudes) may be reaching concentrations that can have impacts on behavior and health (e.g., Castoldi et al. 2001; Basu et al. 2009), especially for the fetus and neonate (Castellini et al. 2012; Rea et al. 2013).

Whole blood (WB) is commonly used to assess Hg exposure (Brookens et al. 2007; Knott et al. 2011). Blood is relatively easy to access (relative to target tissues such as the kidney and nervous system), is commonly collected by biologists, veterinarians, and others who work with wildlife in the field, and is a good tissue for determining Hg status in wild animal populations. Hair is easily accessible and used for monitoring Hg status in wildlife and is more useful for long-term (chronic) mercury assessment, as hair Hg concentration represents the average concentration of Hg in circulating blood (Budtz-Jorgensen et al. 2004).

There are long-term mercury monitoring programs in place for wildlife, particularly fish (US EPA 2012), and monitoring sometimes follows contamination events (Alvarez et al. 2013). Monitoring programs for humans exist as well (Alaska Epidemiology Bulletin 2013; ANTHC 2013). However, blood is less commonly used for biomonitoring due to relative difficulty (compared with hair) with collection, storage, and transport. Collection in the field can be problematic, especially in remote locations with limited processing and preservation capabilities. The development of a blood-sampling regime that can be easily used in the field by scientists,

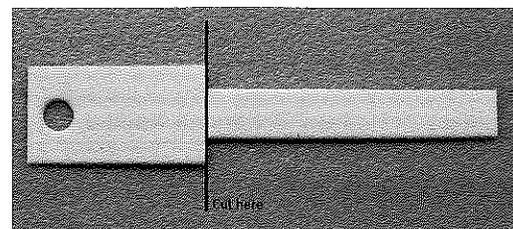


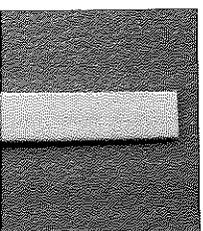
FIGURE 1. Single Nobuto filter paper strip. Black line indicates where strip should be cut after soaking in blood and drying, and before elution (Fig. 2).

hunters, fishermen, or other trained people would facilitate clinical, research, and biomonitoring efforts. We describe the use of cellulose filter paper (FP) for collection of blood in the field and subsequent analysis of total Hg concentration in various FP matrices in comparison with WB collected in standard blood collection tubes.

MATERIALS AND METHODS

Filter paper and samples

Nobuto[®] cellulose FP (Advantec, Dublin, California, USA) was purchased from Cole-Parmer (Vernon Hills, Illinois, USA) and was used for all investigations (Fig. 1). Filter paper was used singly or fashioned into combs of five to six papers for use in the field (Curry et al. 2011). Whole-blood samples were collected between March and July 2012 from wild harbor seals (*Phoca vitulina*) brought to The Marine Mammal Center (Sausalito, California USA, Marine Mammal Protection Act permit 932-1905/MA-009526) for rehabilitation and from long-term resident bottlenose dolphins (*Tursiops truncatus*) live-captured, sampled, and released after health assessments in Sarasota Bay, Florida during May and July 2012 by staff from the Chicago Zoological Society (Wells et al. 2004; National Marine Fisheries Service Scientific Research Permit 15543, Institutional Animal Care and Use Committee 11-09-RW1). Blood samples were collected into BD (Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) Vacutainers[™] containing K₂-ethylenediaminetetra-acetic acid as an anticoagulant. The narrow absorbing ends of 10–12 FP were soaked in WB (approximately 100 μ L/strip) after collection and FP was air-dried overnight. The fluid sample of WB was stored frozen (-20 C). For each animal, dried FP



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METHODS

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samples were shipped overnight at room temper-
ature in a sealed plastic bag with paper towels
layered between each sample and blood samples
were shipped accompanied by freezer packs to
the Wildlife Toxicology Laboratory at the Univer-
sity of Alaska Fairbanks, Fairbanks, Alaska, USA.

Sample preparation

Before chemical analysis, control ($n=10$, no
blood) and blood-soaked FP were freeze-dried
for 48 hr in a Labconco FreeZone 6 Plus
freeze dryer (Kansas City, Missouri, USA).
The narrow absorbing ends of FP_C (control)
and FP_{WB} (soaked, WB) were cut (using a
disposable razor blade) at the junction of the
narrow and wide ends (Fig. 1) and weighed to
determine the dry mass of blood on each
paper (mass WB = mass FP_{WB} - average
mass FP_C). All 60 FP_C and three FP_{WB} from
each individual animal sample set were
analyzed directly for total mercury concentra-
tion ([THg]). The [THg] was calculated on the
basis of the mass of mercury (nanograms) and
mass of blood (~100 mg) on each strip. Three
more FP_{WB} from each individual animal
sample set were separately eluted according to
the protocol developed by Curry et al.
(2011). Each FP_{WB} was cut into five to seven
pieces into a 2-mL preweighed cryogenic tube
(Thermo Scientific, Waltham, Massachusetts,
USA) using stainless steel iris scissors. Each
strip was then covered with 400 µL of
phosphate-buffered saline (PBS; Gibco, Carls-
bad, California, USA) with 1% penicillin-
streptomycin (Gibco). Each cryogenic tube
was agitated to ensure that FPs were soaked,
and were eluted overnight (16 hr) at 4 C.

After 16 hr, approximately 200 µL of eluate
(E) were removed from each cryovial using a
micropipettor. Eluate was transferred to a 1.5-
mL microcentrifuge tube (Fisher Scientific,
Waltham, Massachusetts, USA) and held at
-50 C until analysis. Postelution FPs (includ-
ing ~200 µL of remaining eluting buffer) were
again freeze dried for 48 hr. After drying, each
cryovial (containing postelution FP pieces) was
weighed to determine the final weight of the
postelution paper (FP_E).

Mercury analysis

All samples (WB, FP_C, FP_{WB}, FP_E, and E)
were analyzed for [THg] on a Milestone
DMA-80 direct mercury analyzer (Milestone
Inc., Shelton, Connecticut, USA; US EPA
method 7473) using a 16-point calibration
curve from 0.25 ng to 400 ng, similar to Knott
et al. (2011). Samples were analyzed in
triplicate when possible (i.e., when there were
enough FPs for each sample). Single FPs (for

FP_E a single FP included five to seven cut
pieces) were analyzed in nickel sample boats
and WB (~100 µL) and eluates (100 µL) were
analyzed in quartz sample boats. The detection
limit using this method was 5 ng/g for 100 µL
of blood or eluate and 2.5 ng/g for 200 µL of
eluate.

Quality control included a 10-ng (1 µg/g)
liquid calibration standard (Perkin Elmer, Wal-
tham, Massachusetts, USA, item N9300133),
and DORM-3 (National Research Council
Canada, Ottawa, Ontario, Canada) and DOLT-
4 (National Research Council Canada) certified
standards analyzed in triplicate in each DMA80
run. Recoveries were 94.6±5.2% (10 ng),
102.2±4.4% (DORM-3, reference range 0.382±
0.060 mg/kg), and 100.1±6.8% (DOLT-4, refer-
ence range 2.58±0.22 mg/kg).

Calculations and statistics

Data were managed in Microsoft Excel, and
statistics were performed using program R (R
Development Core Team 2013). Least-
squares linear regressions were fit to FP_{WB},
FP_E, and E compared with WB. Confidence
intervals (95%) for slopes were constructed,
and slopes were compared with a test value of
1 using R. Student's paired *t*-tests were used to
compare [THg] means of FP_{WB}, FP_E, and E
with WB.

Whole-blood data were converted to a dry-
weight basis using the proportion of dry matter
in WB. For some calculations and statistics wet
weight concentrations are reported; for others,
dry weight concentrations. To determine the
dry weight of blood from each species, 100 µL
of WB were weighed, freeze-dried for 48 hr,
and reweighed. The dry blood weights were
24.9±1.8% for harbor seals and 20.5±0.7%
for bottlenose dolphins.

A correction factor was applied to eluate
samples to estimate the original WB (wet)
[THg] (Fig. 2). The elution process involves
adding 400 µL of PBS (~0.400 g) to strips
(FP_{WB}) containing dried components (0.2–
0.25 g) from approximately 100 µL (~0.100 g)
of blood. Therefore a correction factor (CF)
was estimated for each sample as follows:

$$CF = \text{mass of E} / \text{mass of WB}_{\text{wet}} = (0.400 \text{ g} + \text{weight [in g] of dry blood on FP}_{\text{WB}}) / 0.100 \text{ g}$$

This correction factor was then applied to
eluate [THg]:

$$E_{CF} = E \times CF \approx \text{WB (wet)}$$

This correction factor result was compared
with the original WB (wet) [THg].

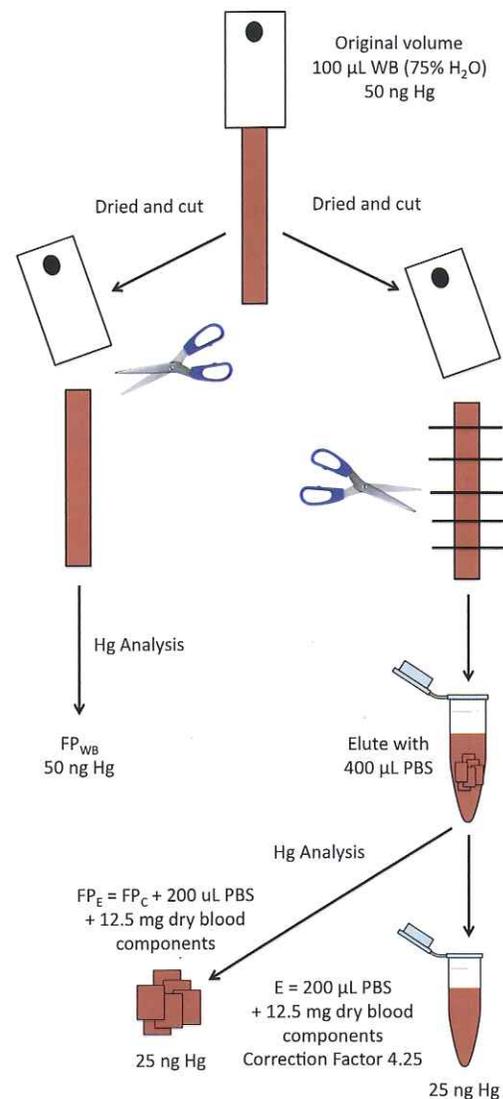


FIGURE 2. A conceptual model of the elution process. Each filter paper is soaked in approximately 100 μ L of whole blood (WB) (original volume). After drying, approximately 20–25 mg of dry blood products remain on filter paper (FP_{WB}). These FP_{WB} can be analyzed for total mercury concentration [THg] directly, or eluted as follows. The dry blood products on FP_{WB} are eluted in 400 μ L of phosphate-buffered saline (PBS), 200 μ L are collected as eluate (the remaining 200 μ L remain soaked into the filter paper [FP_E]). FP_E or the eluate (E) can then be analyzed for [THg].

RESULTS

The average weight of the narrow part (Fig. 1) of FP_C is 0.0466 ± 0.002 g ($n=10$).

Single control FPs not soaked with blood were below the detection limit of the DMA-80 (0.5 ng/FP, $n=10$). Mean [THg] values (on a concentration basis) for WB, FP_{WB} , FP_E , and E are summarized in Table 1. FP_{WB} , FP_E , and E [THg] relative to [THg] in WB in matched samples are summarized in Figure 3. For dolphins, the relative proportion of [THg] in FP_{WB} and FP_E compared with WB is 0.87 ± 0.08 and 0.82 ± 0.13 , respectively. For harbor seals, the relative proportion of [THg] in FP_{WB} and FP_E compared with WB was more variable at 0.95 ± 0.42 and 0.92 ± 0.32 , respectively. The mean difference between the proportion of [THg] FP_{WB} compared with WB is 0.04 ($P < 0.001$), between FP_E and WB is 0.05 ($P < 0.001$), and there is no mean significant difference between E_D WB ($P=0.4$; paired t -tests).

Figure 4 shows [THg] WB regressed on FP_{WB} , FP_E , and E values. Data for WB, FP_{WB} , FP_E , and E are presented on a wet-weight basis. The R^2 for blood-soaked FP is 0.97, for postelution FP is 0.95, and for eluate (with correction factor applied) is 0.97. A 95% confidence interval for the slope is 1.12–1.19 for WB regressed on FP_{WB} , 1.18–1.32 for WB regressed on FP_E , and 0.89–0.97 for WB regressed on E. Tests for each slope (H_0 : slope=1 or $y=x$) indicates $P < 0.01$ for each regression (Fig. 4).

DISCUSSION

We used blood-soaked FP samples to assess mercury concentrations in the blood of bottlenose dolphins and harbor seals. The values for WB total mercury for bottlenose dolphins and harbor seals from our study populations (Table 1) are within the ranges previously reported (Brookens et al. 2007; Woshner et al. 2008).

Advantec Nobuto FP strips are uniform in size and weight (0.0446 ± 0.002 g), and their [THg] is below the detection limit of a DMA80 (< 0.5 ng). Our data support that cellulose FP soaked in WB and air-dried is an accurate and reproducible way

TABLE 1. Mean, range, SD, and sample number (*n*) for total mercury concentration [THg] in bottlenose dolphin (*Tursiops truncatus*, *n*=25) and harbor seal (*Phoca vitulina*, *n*=34) whole blood (WB), filter paper soaked in whole blood (FP_{WB}), postelution filter paper (FP_E), and eluate (E) samples.

Species	WB (μg/g)		FP _{WB} (μg/g)		FP _E (μg/g)		E (μg/g)	
	Wet ^a	Dry ^b	Wet ^b	Dry ^a	Wet ^b	Dry ^a	Wet ^a	CF ^c Applied ^b
Bottlenose dolphin								
Mean	0.48	2.39	0.41	2.06	0.39	1.97	0.12	0.50
SD	0.33	1.66	0.28	1.42	0.27	1.42	0.08	0.35
Range	0.12–1.34	0.61–6.71	0.09–1.15	0.47–5.75	0.09–0.97	0.45–5.67	0.03–0.35	0.13–1.51
Harbor seal								
Mean	0.16	0.64	0.14	0.56	0.13	0.55	0.03	0.13
SD	0.11	0.42	0.10	0.38	0.09	0.36	0.02	0.09
Range	0.03–0.45	0.12–1.78	0.03–0.42	0.12–1.67	0.04–0.41	0.14–1.66	0.01–0.11	0.03–0.46

^a Measured.
^b Calculated.
^c CF=correction factor.

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to quantify WB [THg] for some mammals. Overall recoveries on a concentration basis are very high, ranging from 82 to 95%, when compared with WB concentration for FP_{WB}, FP_E, and E_{CF} (Fig. 3). Additionally, with *R*² values of 0.97, 0.95, and 0.97 respectively for FP_{WB}, FP_E, and E_{CF} (Fig. 4), WB mercury concentration can be easily estimated from dried or

eluted samples, provided [THg] is high enough to be detected.

This technique promises to be valuable to scientists, wildlife managers, veterinarians, and others needing a simple, inexpensive, and highly effective method for collecting blood samples for mercury analysis in combination with other assays. Perhaps even more important, these FPs could be distributed to hunters and used in the field to increase the scope of wildlife-monitoring programs. Programs aimed at developing community-based wildlife health-monitoring programs exist (Brook et al. 2009; ANHSC 2013), and distribution of FP sample kits (including instructions and prepaid shipping labels) through outlets like these would benefit mercury and other disease/health-monitoring efforts around the globe (Curry et al. 2011).

Our findings demonstrate that mercury in blood elutes readily, and our methods allow half of the eluate and roughly half of the mercury to remain with the postelution filter paper (Fig. 4). We also show that Hg-associated dry components of blood likely distribute in a similar way by using a correction factor that demonstrated results with a strong correlation to WB [THg]. Because mercury is bound to sulfhydryl groups on hemoglobin molecules

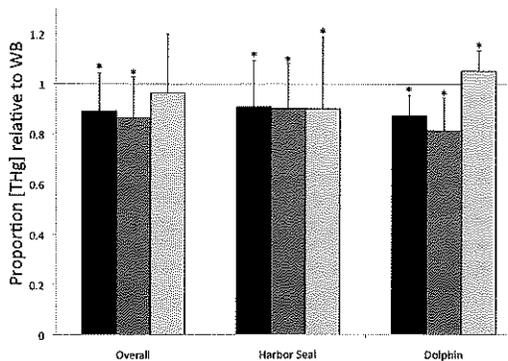


FIGURE 3. Proportion of total mercury concentration [THg] (μg/g) in filter paper with whole blood (black bars), eluted filter paper (medium gray bars), and eluate (light gray) relative to whole blood (WB=1) for bottlenose dolphin (*Tursiops truncatus*, *n*=25) and harbor seal (*Phoca vitulina*, *n*=34) samples. Error bars indicate 1 SD from the mean. * Indicates significant difference in means of paired samples when compared with WB as gold standard (*P*<0.05).

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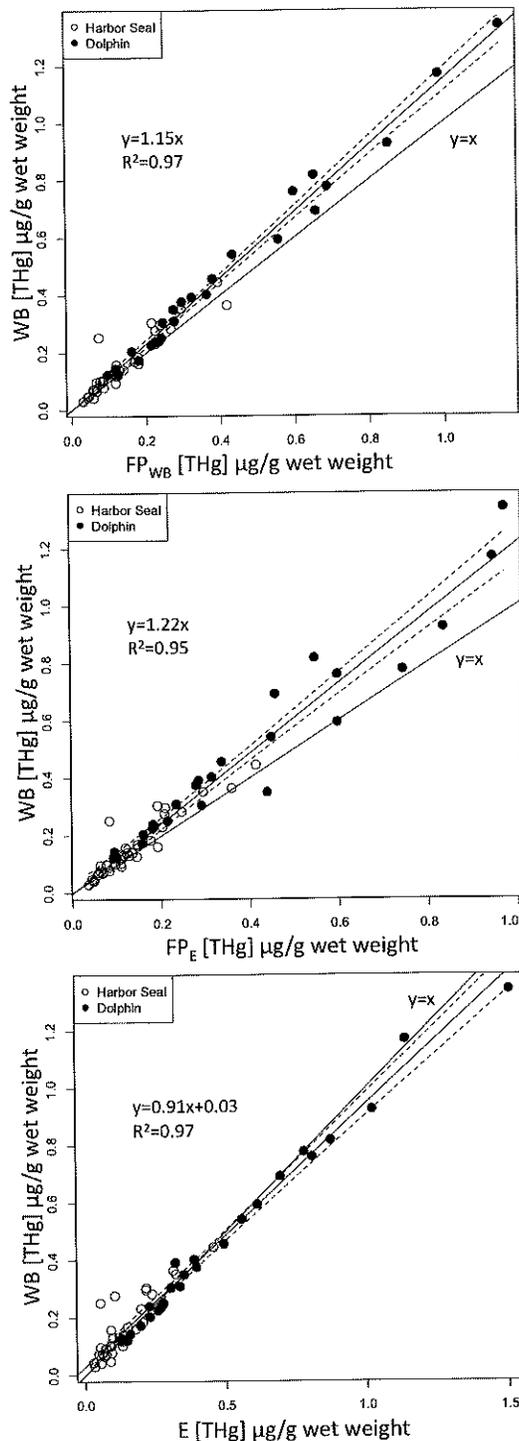


FIGURE 4. Linear regressions of whole blood (WB) on filter paper (FP) with WB (FP_{WB}), FP with eluate (FP_{E}), and eluate only (E) (with dilution factor applied). All slopes are not equal to 1. Dashed lines are a 95% confidence band for the slope. A line of unity ($y=x$) is shown in each panel.

(Weed et al. 1962), we hypothesize that the hemoglobin is following this same pattern and is moving into the eluate, and half of that remains on the FP_{E} with the residual 200 μL of buffer. On the basis of this we have developed a conceptual model of the elution process describing the utility of predicting WB [THg] directly using blood-soaked FP_{WB} and indirect methods that use certain postelution products (FP_{E} , E; Fig. 2).

Although blood is not as easy to collect as hair, FP technology facilitates blood collection and makes it easier to store and ship air-dried blood. Hair provides a long-term picture of mercury status (Budtz-Jorgensen et al. 2004), whereas blood represents short-term exposure, and is the route of exposure for target organs (the central nervous system and kidneys). The combination of dried FP and hair samples, both of which can be stored at room temperature and shipped under ambient conditions, will allow wildlife scientists to obtain a more complete picture of the mercury status in populations of interest.

The designed use of these FPs is for protein (antibody) preservation for antibody detection (serology). We have shown the added advantage of being able to use FP_{WB} , FP_{E} , or E for quantifying mercury in WB. Previous studies have used FP eluate to validate serologic use in wildlife populations (Curry et al. 2011). We emphasize the excellent correlations between [THg] in WB and both FP_{E} and E (Fig. 4). Thus, one can utilize the FP eluate as intended for serology, and use any remaining FP_{WB} or FP_{E} to quantify mercury. This type of use could be a significant advantage if the available blood volume is limited, either in small species or in situations where hunters or wildlife professionals are unwilling or unable to obtain large quantities of blood.

One unknown factor pertains to the shelf life of these samples. All of our analyses were conducted within 8 mo of collecting samples on FP. It would be

hypothesize that the same pattern of mercury in whole blood, and half of the mercury in the residual blood. On the basis of this we developed a model of the utility of using blood-mercury methods that use filter paper (FP_E, E₁) as easy to collect and store, facilitates blood sampling, is easier to store and transport, provides a long-term stability status (Budtz-Jørgensen, 2004), whereas blood mercury exposure, and is a good target for target organs (liver and kidneys). The use of dried FP and hair samples can be stored at room temperature and shipped under ambient conditions will allow wildlife sampling to be more complete and representative of status in popula-

tion. These FPs is for preservation for anti-mercury (anti-Hg). We have shown that being able to use filter paper for quantifying mercury in whole blood has been used for FP use in wildlife (Hansen et al. 2011). We found that correlations between both FP_E and E₁ can utilize the FP for serology, and use of FP_E to quantify mercury use could be a good alternative to the available blood mercury in small species hunters or wildlife sampling or unable to collect blood.

It pertains to the samples. All of our samples were collected within 8 mo of capture. It would be

important to see if similar results would be obtained with long-term storage. However, we do not anticipate volatilization or degradation to be significant for [THg] measures as compared with more vulnerable components such as antibodies.

This FP technique promises to be broadly applicable wherever field sampling of WB for [THg] is needed. The strips can be air dried, do not need to be refrigerated, and theoretically have a long, stable shelf life once samples are collected. This method will be particularly useful in monitoring [THg] in subsistence foods in remote Alaskan communities, where Alaska native peoples often subsist on fish-eating marine mammals. Application of this technology to human fish-consumer blood sampling, in conjunction with hair-monitoring programs, should also be considered.

ACKNOWLEDGMENTS

We thank The Marine Mammal Center and the Chicago Zoological Society's Sarasota Dolphin Research Program staff and volunteers for collecting WB and FP samples. Dolphin blood samples were collected during health assessments funded by Dolphin Quest and the Office of Naval Research. We thank Jennifer Yordy and Kristina Cammen for blood-processing assistance during the dolphin health assessments, and John Harley, Megan Templeton, and Gary Lose for assistance in the laboratory. Analytical work was funded by the Rural Alaska Monitoring Program funded via the Alaska Native Tribal Health Consortium from a grant from the US Fish and Wildlife Service, Arctic Landscape Conservation Consortium.

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Submitted for publication 12 August 2013.

Accepted 26 November 2013.