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Persistent Organic Pollutant Determination in Killer Whale Scat Samples: Optimization of a Gas Chromatography/Mass Spectrometry Method and Application to Field Samples

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Abstract Biologic sample collection in wild cetacean populations is challenging. Most information on toxicant levels is obtained from blubber biopsy samples; however, sample collection is invasive and strictly regulated under permit, thus limiting sample numbers. Methods are needed to monitor toxicant levels that increase temporal and repeat sampling of individuals for population health and recovery models. The objective of this study was to optimize measuring trace levels (parts per billion) of persistent organic pollutants (POPs), namely polychlorinated-biphenyls (PCBs), polybrominated-diphenyl-ethers (PBDEs), dichlorodiphenyltrichloroethanes (DDTs), and hexachlorocyclobenzene, in killer whale scat (fecal) samples. Archival scat samples, initially collected, lyophilized, and extracted with 70 % ethanol for hormone analyses, were used to analyze POP concentrations. The residual pellet was extracted and analyzed using gas chromatography coupled with mass spectrometry. Method detection limits ranged from 11 to 125 ng/g dry weight. The described method is suitable for *p*,*p*′-DDE, PCBs-138, 153, 180, and 187, and PBDEs-47 and 100; other POPs were below the limit of detection. We applied this method to 126 scat samples collected from Southern Resident killer whales. Scat samples from 22 adult whales also had known POP concentrations in blubber and demonstrated significant correlations ($p < 0.01$) between matrices across target analytes. Overall, the scat toxicant measures matched previously reported patterns from blubber samples of decreased levels in reproductive-age females and a decreased *p*,*p*′-DDE/$\sum$PCB ratio in J-pod. Measuring toxicants in scat samples provides an unprecedented opportunity to noninvasively evaluate contaminant levels in wild cetacean populations; these data have the prospect to provide meaningful information for vital management decisions.

The Salish Sea is an area of Pacific inland waterways that includes Puget Sound and the San Juan Islands of Washington state and southern British Columbia. This diverse and productive ecosystem is riddled with human impacts. The Southern Resident killer whales (SRKW; *Orcinus orca*), which consist of three family groups, or pods, J, K, and L, subsist in these waters during the summer months (May–October) (Hanson et al. 2010). This killer whale ecotype experienced an unexplained 20 % decline in their population census in the late 1990s, which alarmed the public and scientists alike to the possibility that this ecosystem may no longer be able to sustain these carnivores at the top of the food web. The SRKWs were listed as endangered in 2001 and 2005 by Canada and the United States, respectively. Excessive exposure to environmental contaminants was identified as one of the primary risk factors that may be contributing to the population decline

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by the National Marine Fisheries Service in the Southern Resident Killer Whale Recovery Plan (National Marine Fisheries Service 2008); other risk factors included decreased prey availability, vessel effects and sound, and oils spills.

Persistent organic pollutants (POPs) are a group of toxic chemicals that are ubiquitous in the environment and have been associated with adverse health effects (e.g., endocrine disruption) in wildlife populations (Tyler et al. 1998), including studies specific to marine mammals (Jepson et al. 2005; Lahvis et al. 1995; Reijnders 1986; Schwacke et al. 2002). Organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclobenzene (HCB), were banned or their use was restricted in the United States during the 1970s and 1980s. Polychlorinated biphenyls (PCBs), used in capacitors, transformers, and consumer products, such as house paint, were banned from open source use in the United States in the 1970s. Polybrominated diphenyl ethers (PBDEs) have been widely used as a flame retardant in consumer products. The industrial production and importation of two of the three technical mixtures of PBDEs (penta- and octaBDEs) was stopped in the United States in 2004, and United States manufacturers and importers of the remaining form (decaBDE) committed to terminate all uses by the end of 2013 (Environmental Protection Agency 2014). Substantial human and ecological exposures to these POPs continue to occur due to the large quantities that were used historically and continue to be used in certain global regions, as well as their persistence in the environment, resistance to biodegradation, and bioaccumulation in the food chain (Letcher et al. 2010; Li et al. 2006).

Pod, age, and sex-class differences in POP levels have been previously reported in SRKWs based on measures from blubber biopsy samples (Krahn et al. 2007, 2009; Ross et al. 2000). Specifically, the ratio of DDTs to PCBs in blubber samples, used to provide insight into whales' foraging locations, was demonstrated to be higher in K- and L-pods compared to J-pod. This POP ratio pattern in K- and L-pods is often referred to as the "California signature" due to alleged seasonal foraging off of the central California coast where high levels of DDT are still found in the marine environment (Sericano et al. 2014). PCB concentrations are higher in J-pod (male, 1 sample, 41,000 ng/g lipid) compared with the other two pods of SRKWs (K- and L-pod males, four samples, range 24,000–39,000 ng/g lipid) (Krahn et al. 2009). This is considered part of an "urban signature" and is believed to be associated with the more frequent foraging of J-pod in Puget Sound. The concentration of these contaminants generally increase with age, particularly the POPs more resistant to biotransformation (Boon et al. 1994; Wolkers et al. 2007). Decreased toxicant levels in reproductive-age females (Krahn et al. 2009) have been attributed to mobilization of these compounds from adipose tissue in response to the high-energy needs of lactation (Debier et al. 2003; Krahn et al. 2009; Pomeroy et al. 1996; Ross et al. 2000). Krahn et al. demonstrated maternal transfer of a large portion of the mother's POPs burden during gestation and through breast milk in two mother-offspring pairs of SRKWs; the resulting POP concentrations in the nursing calves were higher than their mother's (Krahn et al. 2009). This same study also demonstrated that female whales reaccumulate POPs between pregnancies once lactation ended and transfer of POPs to a calf ceased. Males have been shown to accumulate toxicants throughout their lifetime (Ross et al. 2000).

Blubber biopsy samples previously collected on the SRKWs demonstrated that levels of POPs in this population exceed a health-effects threshold developed by Kannan et al. (2000) through a risk characterization extrapolated from studies of immunologic and reproductive effects in seals, otters, and mink (Kannan et al. 2000; Krahn et al. 2009; Ross et al. 2000). Numerous modeling efforts have been made to evaluate population consequences in cetacean populations associated with exposures to pollutants, or other factors such as prey abundance (Hall et al. 2006; Hickie et al. 2007; Ward et al. 2009). Ward et al. (2009) quantified the effect of prey abundance on killer whale reproduction and reported SRKW fecundity was highly correlated with Chinook salmon abundance. The authors stated, "It remains unclear what effects contaminants have on killer whale fecundity because appropriate data do not exist." Risk assessments from other cetaceans suggest reproductive impairment is apparent at the current levels of contaminants (Hall et al. 2006; Schwacke et al. 2002). Hall et al. (2006) estimated the population consequence of PCB exposure in bottlenose dolphins (*Tursiops truncates*) using PCB levels reported in bottlenose dolphins from Sarasota Bay, Florida (Wells et al. 2005), coupled with dose–response data (Reddy et al. 2001) and age-specific fecundity and survival data from a mark recapture study (Wells and Scott 1990), and reported decreased calf survivorship associated with maternal PCB accumulation. The authors acknowledged the predictions were limited by model naivety and parameter uncertainty and emphasized the need for more data collection.

To date, collection of biological samples for toxicant analyses in wild killer whale populations has been limited to analysis of blubber biopsy samples (Krahn et al. 2007, 2009; Ross et al. 2000; Ylitalo et al. 2001) and tissues (e.g., blubber, liver) collected during necropsies of whales that have washed ashore (Kajiwara et al. 2006; Kannan et al. 1993). While the toxicant information obtained from biopsy samples has been invaluable (Alava et al. 2012; Hickie et al. 2007), collection is invasive and strictly regulated under permit. As such, this sampling method restricts sample size, and the cross-sectional study design generally only includes one sample per whale over all

sampling periods. Current methods to monitor temporal and annual trends of toxicant levels in the SRKW population include stable tissue archives (Becker et al. 1997), but these reserves are limited and new methods would help to ensure comprehensive contaminant data are available for population monitoring and health assessment models. Noninvasive measurement of POPs in killer whale feces provides one solution, due to its availability in the ecosystem and relative ease of collection (Ayres et al. 2012; Hanson et al. 2010). A total of 266 scat samples were collected between 2010 and 2013; thus, the limiting factor in this study was not sample availability but funds to evaluate the samples. The similar congener profiles of PCBs demonstrated in the blood, internal fat, and feces of bottlenose dolphins (captive) and otters (Marsili et al. 1995; Van den Brink and Jansman 2006), and the detection of specific POP congeners and metabolites in the feces of river otters, rats, mice, and sheep (Elliott et al. 2008; Hakk et al. 2009; Vrecl et al. 2005), indicate that circulating POP concentrations can be reliably measured in feces. However, to our knowledge, no study has evaluated toxicant levels in scat from killer whales or any other wild cetacean population.

The primary objective of the current study was to optimize a gas chromatography/mass spectrometry method to measure trace levels of POPs (PCBs, PBDEs, DDTs, and HCB) in killer whale scat samples. The second objective was to apply these methods to samples collected from SRKWs in the Salish Sea to evaluate whether scat POP levels are reflective of measures from blubber samples.

Materials and Methods

Sample Collection

SRKWs appear with regularity in the areas around the San Juan Islands and Puget Sound between spring (mid-May) and fall (mid-October). Sample collection occurred in these inland waters of Washington State and British Columbia, collectively referred to as the Salish Sea, between May and October from 2010 to 2013 (Supplemental Fig. 1).

SRKWs were located and identified based on dorsal fin shape and saddle patch pattern (CWR 2015). Detection dogs were trained to locate SRKW scat floating on the water's surface (Ayres et al. 2012; Hunt et al. 2004; Rolland et al. 2006), taking advantage of the dog's remarkable ability to locate SRKW scat samples at distances compatible with the "Be Whale Wise" guidelines (>200 yards away). Samples were scooped off of the surface of the water using a 1-L polypropylene beaker, collected in a 50-mL polypropylene tube, immediately centrifuged using a small field centrifuge, and all seawater was decanted. The remaining pellet was frozen on dry ice and remained frozen until processed in the lab (within 6 months of collection). In the lab, samples were thawed, homogenized (by combining multiple collection tubes from the same sample in a glass beaker and stirring thoroughly with a stainless steel spatula), centrifuged at 1092 relative centrifugal force (RCF), decanted, and swabbed for genetic analysis to identify species, sex, pod, and individual identification by National Oceanic and Atmospheric Administration's Northwest Fisheries Science Center (NOAA NWFSC) (Ford et al. 2011). Genetic material was not compromised by undergoing a freeze/thaw cycle. As such, samples were swabbed in the lab, instead of the field, to ensure adequate material was collected and sterile technique was employed. One genetic match per sample assured sample collection was from one animal.

All samples analyzed were originally collected to evaluate a combination of fecal hormone measures (thyroid and glucocorticoid) to assess inadequate prey and increased vessel traffic on this endangered population (Ayres et al. 2012). As such, following homogenization samples were freeze-dried, weighed to 80 mg dry weight, and hormones were extracted using 70 % ethanol (15 mL). The ethanol extract was decanted and stored at −20 °C for hormone analyses (Ayres et al. 2012; Wasser et al. 2010, 2000). The remaining fecal pellet was stored at −20 °C for toxicant analyses. A total of 266 viable scat samples were collected May through October from 2010 to 2013. Analysis of all samples was cost prohibitive; as such, 126 samples were selected for analysis of toxicant concentrations. Selection of samples was restricted to those with a mass ≥0.07 g that also were successfully genotyped to confirm individual identity ($n = 165$) (two additional samples were selected from suspected pregnant females, based on progesterone profiles). The final sample selection was based on a priori hypotheses to evaluate seasonal and annual changes in toxicant concentration, and variation based on reproductive status, with no consideration of whether data were available on POP levels in blubber.

The Southern Resident killer whale population, including births and deaths, has been well documented since 1976 by the Center for Whale Research (CWR 2013). These data were used to evaluate age and sex class characteristics of individual whales.

Analysis for Persistent Organic Pollutants

Chemicals and Reagents

All chemicals, solvents, and reagents used were pesticide-grade. All glassware, aluminum foil, stainless steel spatulas, and other materials used were triple rinsed in acetone (Fisher Scientific, Pittsburgh, PA) or methylene chloride

(Fisher Scientific) prior to use to remove trace organics. Florisil and sodium sulfate were triple rinsed in methylene chloride, then baked at 130 °C for 24 h. Florisil was deactivated by adding 1.2 % of conditioned deionized water based on dry weight. Silica (Mallinckrodt, 100 mesh powder) was rinsed three times with methylene chloride followed by drying in an oven at 160 °C for 3 h. Acidic (concentrated sulfuric acid, 44 % w/w) and basic (1 N sodium hydroxide, 23 % w/w) silica were prepared from the cleaned silica (Environmental Protection Agency 2007).

The individual PBDE and PCB congeners reported in this study follow the International Union of Pure and Applied Chemistry (IUPAC) numbering system for PCBs. A stock solution of native compounds was prepared by dissolving the individual congeners PBDEs-28, 47, 99, 100, 153, 154 (Wellington Laboratories, Ontario, Canada), PCBs-95, 99, 149, 187 (Accustandard, New Haven, CT), *p*,*p*′-dichlorodiphenyldichloroethylene (*p*,*p*′-DDE; the predominant metabolite of DDT) (Supelco, St. Louis, MO), and HCB (Accustandard), and a PCB mixture, including PCBs-101, 118, 138, 153, 180 (PCB Congener Standard #2, Restek, Bellefonte, PA), in toluene (Omnisolv®). Calibration curve solutions were made by dilution in toluene, with concentrations ranging from 0.5 to 100 ppb. Target analyte selection was based on pilot sample analyses performed by NOAA NWFSC using a gas chromatography coupled with mass spectrometry (GC/MS) method described in Sloan et al. (2014).

A surrogate standard mixture (250 ng/mL of each compound) was prepared in toluene from individual isotope-labelled standards PCB-118 (Wellington Laboratories), *p*,*p*′-DDE (Cambridge Isotope Laboratories, Tewksbury, MA), and HCB (Cambridge Isotope Laboratories), a PCB mixture including isotope-labelled PCBs-101, 138, 153, 180 (Cambridge Isotope Laboratories), and individual native congeners PBDEs-30, 77, 105, 140 (Wellington Laboratories). This standard was used to monitor analyte recoveries and for internal standard quantification using isotope dilution methods. Native standards with the same number of bromines but different substitutions were selected as surrogates for PBDE internal standards rather than labelled compounds, because negative chemical ionization (NCI) fragments both to ^{79}Br and ^{81}Br (Eljarrat et al. 2002). The PBDE native surrogates were selected to have different retention times on the GC system, allowing them to be distinguished from the target analytes. A similar fragmentation to halogen occurred with PCBs-52 and 95. As such, PCB-52 was dropped from the analysis because an appropriate internal standard surrogate was not available, and PCB101-$^{13}C_{12}$, an isotope-labelled homologue for PCB-95, was selected as the new surrogate for PCB-95.

Extraction and Purification

Scat sample extraction, purification, and quantification methods were developed using procedures modified from EPA methods 3630C and 1614 (Environmental Protection Agency 1996, 2007). Frozen fecal pellets from the hormone extraction were thawed and spiked with 20 µL surrogate standard (250 ng/mL) before extraction. Sodium sulfate (500 mg) and florisil (500 mg) were added to the samples to remove residual water and polar compounds, respectively, along with 20 mL of methylene chloride. The sample extracts were sonicated in a water bath at room temperature for 30 min, centrifuged for 10 min at 2910 RCF, and decanted into a glass collection tube. This extraction step was repeated with an additional 20 mL of methylene chloride, and the two extracts were combined. The extract volume was reduced to approximately 1 mL at 45 °C under a gentle stream of nitrogen, and solvent-exchanged to hexane (Fisher Scientific).

The extract was loaded onto a 5 mL disposable polypropylene column (Thermo Scientific, Rockford, IL) packed from bottom to top with 200 mg basic silica (23 % w/w), 50 mg silica, and 300 mg acidic silica (44 % w/w). The target compounds were eluted using 11 mL of hexane:methylene chloride (50:50, v/v). The final sample was solvent exchanged to 100 µL toluene under a gentle stream of nitrogen and stored in the dark at −20 °C until analysis.

Analysis

All sample extracts and standards were analyzed using GC/MS (Agilent 7890A) operating in the NCI mode, using methane as the reagent gas, with selected ion monitoring (SIM) to enhance sensitivity prior to instrumental analysis. The instrument was fitted with a DB-XLB column (30 m length, 0.25 mm film thickness, 0.25 µm i.d., with Duraguard, 10 m) (Agilent, Santa Clara, CA). The port was operated in splitless mode, fitted with a glass liner, and delivered 1 µL by auto-injection. The carrier gas was helium. Chromatographic separation was achieved using an initial temperature of 60 °C with a 2 min hold, ramping to 240 °C at a rate of 30 °C per minute with a 2 min hold, then ramping to 340 °C at a rate of 10 °C per minute with a 5 min hold. The total run time per sample was 25 min.

Each analytical batch (two total) contained: a ten-point standard calibration curve at the beginning and end of each run; a repeat run of one point from the calibration curve every 15 samples to check instrument maintenance (calibration check vial); and a laboratory procedure blank for each extraction group (every 11 samples) to monitor contamination (i.e., the presence of artifact POPs arising from laboratory associated procedures). One sample was repeated in each analytical batch to test the instrument for

accuracy and precision, and a toluene blank was run to detect solvent contamination. Sample concentrations were calculated for the individual POPs using the standard calibration curve. The reproducibility of the response factors in the calibration check vials were checked for each series of samples analyzed and were typically ±10 % the expected values. Any measured responses in the procedure lab blanks were subtracted from the responses measured in samples from the same extraction group. All final values are reported as ng/g dry fecal weight.

Ethanol Extracts

Eight archived 70 % ethanol extracts were analyzed for POPs to ensure that previous extraction of the fecal pellet with 70 % ethanol for hormone analyses (Ayres et al. 2012) would not affect the POP concentrations obtained from the subsequent methylene chloride extraction of the fecal pellet (i.e., extraction method optimized in this study). Four archived ethanol extracts from methylene chloride extracted fecal pellets with low POP concentration levels, and four ethanol extracts from samples with high POP concentrations, were selected to test that POPs extracted by the ethanol were relative across a range of sample toxicant concentrations. A total of 5 mL of ethanol extract per sample was spiked with 20 µL of 250 ng/mL surrogate standard to monitor recovery and for quantification using isotope dilution methods. The volume of each ethanol extract was reduced to approximately 1 mL at 45 °C under a gentle stream of nitrogen. Sodium sulfate was added to thoroughly cover and saturate the remaining liquid, which was assumed to be mostly water, and 5 mL of hexane was added. The resulting hexane extract was run through a 5 mL Pasteur pipet column packed with sodium sulfate to ensure complete removal of water. The sample extract volume was reduced to 1 mL under a gentle stream of nitrogen. Final extracts were loaded onto solid-phase extraction columns and analyzed by NCI-GC/MS using the protocol outlined above for the fecal pellets. Analyte concentration measures in the procedure blank were subtracted from the sample measures for all compounds except for PCBs, which were not representative of the scat samples. PCB levels were negligible in all procedure blanks for the extraction and purification of the sample pellets, therefore laboratory contamination was not a concern. All final values are reported as ng/mL.

Blubber Biopsy Samples

Toxicant levels previously measured in 22 blubber biopsy samples from adult (≥10 years old) SRKWs, and associated whale identification data, were provided by NOAA NWFSC. Blubber biopsy samples were collected in the United States and Canada between May 2006 and January 2013. All samples were analyzed using techniques described elsewhere (Sloan et al. 2014). All blubber biopsy samples were analyzed for the same target analytes as measured in the scat samples. The average period of time between collection of blubber and scat samples was 3 years, 2 months (range 0 year, 1 month to 7 years, 2 months).

Method Detection Limit and Limit of Quantification

The method detection limit and percent recovery experiment included three standards covering the range of expected values in the samples based on the pilot sample analyses performed by NOAA NWFSC (described above): 0.5, 5, and 50 ppb. In total, 100 µL of each standard was added (i.e., spiked) to an 80 mg fecal pellet made of pooled fecal samples representing all age-sex classes and pods in the SRKW population, previously extracted in 70 % ethanol, and stored at −20 °C. Each standard across the expected range was measured with six replicates; six matrix blanks were also evaluated. Coefficient of variation (%CV) was calculated as the percent of dividing the standard deviation by the mean. Method detection limit (MDL) and limit of quantification (LOQ) were calculated by multiplying the standard deviation by 3.365 and 10, respectively. MDL, LOQ, and mean recovery (with matrix subtracted out) were quantified using an external standard calibration method because the internal standard method was not available at the time of this experiment.

Statistical Methods

Toxicant concentrations measured in the eight ethanol extracts (ng/mL) were compared to the toxicant concentrations measured in the methylene chloride extracted fecal pellets (ng/g dry scat) from the same samples using linear regression analysis. All values were log-transformed to achieve normality. This was the only analysis using the toxicant measures in the ethanol extracts. All other analyses only use the toxicant measures in the methylene chloride extracted fecal pellets.

Toxicant concentrations were measured in 126 scat samples, 124 of these samples had a genotype confirmed individual identity representing 52 unique whales. Twenty-two unique adult whales had both blubber and scat toxicant measures available. Blubber and scat toxicant measures of individual congeners and compounds were log-transformed to achieve normality and compared using mixed effects linear regression models adjusted for repeat sampling on individual whales by modeling individual whale as a random effect. This comparison was restricted to adult whales to minimize variation due to the growth dilution effect of

toxicants in juvenile whales (Hickie et al. 2007), which would be exacerbated by the variable period of time between collection of blubber and scat samples. Toxicants in scat were evaluated by individual congener and class of compound ($\sum$PCBs; $\sum$PBDEs; and *p*,*p*′-DDE), as well as by ratio of *p*,*p*′-DDE/$\sum$PCBs. The adjusted mean and standard error of toxicant measures were compared by year of sample collection, pod, and age–sex class. Age–sex class was defined as: juveniles (<10 years old; either sex), adult males (≥10 years), reproductive females (≥10 to <40 years), and post-reproductive age female whales (≥40 years). Effects were estimated using a mixed effects model, to account for repeat sampling on individual whales. Potential covariates included sex, age, pod, and Julian date of sample collection. Final model selection was based on AICc (Akaike Informational Criterion) score. All statistical analyses were performed using SAS v9.3 (SAS Institute Inc, Cary, NC).

Results and Discussion

The target analytes, retention times, and quantification and confirmation ions are listed in Table 1. PCB95 and PCB99, each using PCB101-$^{13}C_{12}$ as a surrogate standard, were dropped as target analytes because the %CV for recovery of the 5 ppb spike was 22.7 and 21.4 %, respectively, indicating this was not an appropriate surrogate for either compound. An external standard method was also tested, but the %CV for recovery of the 5 ppb spike was still high, 23.0 and 21.8 %, respectively, indicating there was too much matrix interference for these compounds to be quantified at 5 ppb, the concentration level in the middle of the range of expected values.

The mean recoveries for the remaining target analytes (7 PCBs, 6 PBDEs, *p*,*p*′-DDE, and HCB) ranged from 61 to 101 % with a 50 ppb spike, and 84–135 % with a 5 ppb spike for all compounds except for HCB, which had a 224 % recovery (Table 2). The recovery for the 0.5-ppb spike was inflated (15–835 %) likely due to high variability in the recovered compounds due to matrix interference on the GC system. The MDL ranged from 11 to 20 ng/g for individual PCBs, 16–37 ng/g for individual PBDEs, and was 124 ng/g for *p*,*p*′-DDE and 125 ng/g for HCB. Low toxicant concentrations in these mass limited samples as well as matrix interference may limit the usefulness of this method for whales with a low toxicant burden. Future studies would benefit from a larger sample mass.

Significant correlations were demonstrated when comparing the POP concentrations in the eight archived hormone ethanol extracts with the associated fecal pellet extracted with methylene chloride for *p*,*p*′-DDE, PCBs-118, 149, 138, 153, 180, and 187, and PBDEs-47 and 100 ($R^2 > 0.80$; $p < 0.05$; Table 3). This finding indicates that extraction of the fecal pellet with 70 % ethanol, prior to the subsequent methylene chloride extraction of the fecal pellet for toxicant analysis, is proportional to the total contaminant level thus the effect of contaminant removal from the ethanol extraction step is consistent between samples. Therefore, no additional ethanol extracts were analyzed for toxicant concentrations. HCB, PCB101, PBDE28, PBDE99, PBDE153, and PBDE154 did not demonstrate a significant linear association and were excluded from the final analyses out of concern of nonuniform extraction by the ethanol extraction step across different toxicant concentration levels. PCB149 was retained, although the

Table 1 List of persistent organic pollutant target analytes quantified by NCI-GC/MS-SIM with surrogate standard, retention time (RT), quantification ion (Quant Ion), primary confirmation ion (Primary conf ion), and secondary confirmation ion (Secondary conf ion)

Target analyte	Surrogate standard	RT	Quant ion	Primary conf ion	Secondary conf ion
HCB	HCB-$^{13}C_6$	9.13	284	286	282
PCB101	PCB101-$^{13}C_{12}$	11.84	326	328	324
p,*p*′-DDE	*p*,*p*′-DDE-d8	12.32	318	316	–
PCB118	PCB118-$^{12}C_{12}$	13.08	326	328	324
PBDE28	PBDE30	13.15	79	81	–
PCB153	PCB153-$^{13}C_{12}$	13.33	360	362	358
PCB149	PCB153-$^{13}C_{12}$	12.82	360	362	358
PCB138	PCB138-$^{13}C_{12}$	13.86	360	362	–
PCB180	PCB180-$^{13}C_{12}$	15.08	394	396	398
PCB187	PCB180-$^{13}C_{12}$	14.04	394	396	392
PBDE47	PBDE77	15.31	79	81	–
PBDE100	PBDE105	16.87	79	81	–
PBDE99	PBDE105	17.34	79	81	–
PBDE154	PBDE140	18.52	79	81	–
PBDE153	PBDE140	19.30	79	81	–

–, not applicable

Table 2 Method detection limit and percent recovery of persistent organic pollutants measured in killer whale scat samples

Target analyte	PCB 101	PCB 118	PCB 138	PCB 149	PCB 153	PCB 180	PCB 187	PBDE 28	PBDE 47	PBDE 99	PBDE 100	PBDE 153	PBDE 154	*p,p′*-DDE	HCB
Spike (ng/mL)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	5	5	5	5	0.5	0.5
Mean[a]	0.0959	0.0562	0.0908	0.0080	0.1109	0.0354	0.0284	0.0239	0.0750	0.2841	0.3356	0.1771	0.2742	0.6498	0.8387
Standard deviation[a]	0.0072	0.0033	0.0057	0.0008	0.0073	0.0020	0.0012	0.0030	0.0070	0.0226	0.0373	0.0231	0.0343	0.0900	0.0739
Coefficient of variation (%)	7.5	6.0	6.2	9.9	6.6	5.6	4.3	12.5	9.3	8.0	11.1	13.0	12.5	13.8	8.8
MDL (ng/g)	19.3	15.8	19.0	15.8	20.3	13.4	11.4	16.1	22.5	26.5	37.1	33.5	34.1	124.4	125.0
LOQ (ng/g)	37.9	25.1	35.8	41.0	39.9	18.9	15.5	26.4	45.3	68.1	102.5	86.5	79.1	361.3	356.3
Mean, 0.5 ppb spike (ng/mL)[b]	0.49	0.73	0.67	0.07	0.94	1.16	1.15	1.48	1.48	1.01	0.83	2.08	0.54	1.06	4.17
Mean, 5 ppb spike (ng/mL)[b]	4.52	4.19	4.26	5.38	4.51	4.58	5.56	5.16	5.60	5.98	6.76	5.01	5.64	5.72	11.19
Mean, 50 ppb spike (ng/mL)[b]	32.64	32.50	32.91	46.50	32.82	33.13	46.25	35.25	41.64	50.49	61.01	33.23	38.71	30.66	31.74
% Recovery, 0.5 ppb spike	99	147	133	15	188	232	230	296	295	203	166	416	107	212	835
% Recovery, 5 ppb spike	90	84	85	108	90	92	111	103	112	120	135	100	113	114	224
% Recovery, 50 ppb spike	65	65	66	93	66	66	93	70	83	101	122	66	77	61	63

[a] Response values, not quantified using calibration curve

[b] Concentration levels calculated using an external standard curve

Table 3 Comparison of target analytes measured in eight fecal samples extracted first with 70 % ethanol (extract for hormone level analyses), followed by methylene chloride extraction of the fecal pellet (method optimized in this paper to measure POP levels)

Target analyte	R^2	Coefficient of variation (%)	*p* value
HCB	0.002	10.1	0.911
p,p′-DDE	0.98	5.6	<0.001
∑6PCBs	0.95	7.7	<0.001
∑4PCBs	0.95	7.6	<0.001
PCB101	0.02	50.9	0.734
PCB118[a]	0.89	9.9	0.002
PCB149[a]	0.86	28.1	0.003
PCB138	0.98	5.5	<0.001
PCB153	0.98	6.3	<0.001
PCB180	0.97	8.1	<0.001
PCB187	0.96	8.7	<0.001
∑2PBDEs	0.82	8.6	0.002
PBDE28	0.24	157.3	0.216
PBDE47	0.81	8.9	0.002
PBDE99	0.21	50.1	0.260
PBDE100[b]	0.95	13.7	<0.001
PBDE153	0.20	122.5	0.264
PBDE154	0.06	56.0	0.570

∑6PCBs = PCB118, PCB149, PCB138, PCB153, PCB180, PCB187

∑4PCBs = PCB138, PCB153, PCB180, PCB187

∑2PBDEs = PBDE47, PBDE100

[a] One outlier excluded from statistical model

[b] Two outliers excluded from statistical model

28.1 % CV indicates a possible non-linear association across varying levels of toxicant concentrations.

Killer whale fecal toxicant concentrations (ng/g dry weight) measured in the methylene chloride extracted fecal pellets were compared to concentrations of cumulative and individual congeners of PCBs (Σ4PCBs, ∑6PCBs: PCBs-118, 149, 138, 153, 180, and 187), PBDEs (∑2PBDEs: PBDEs-47 and 100) and *p,p′*-DDE in blubber biopsy samples (ng/g lipid adjusted) from the same whale ($n = 22$ unique pairs; Fig. 1). The linear associations, adjusted for multiple scat samples collected per whale, were significant ($p < 0.01$) for *p,p′*-DDE [slope estimate, 1.018; standard error (SE), 0.190], both PBDE congeners (∑2PBDE slope estimate, 0.477; SE, 0.160), and all PCB congeners measured except for PCB118 ($p = 0.067$) (data not shown). The finding for PCB118 may be explained by this congener being more readily metabolized than the other PCBs measured; therefore, samples may not reflect bioaccumulation of this compound (Boon et al. 1994). A new metric, ∑4PCBs (cumulative measure of PCBs - 138, 153, 180, 187) was created excluding PCB118 based on the non-significant correlation with the blubber biopsy samples

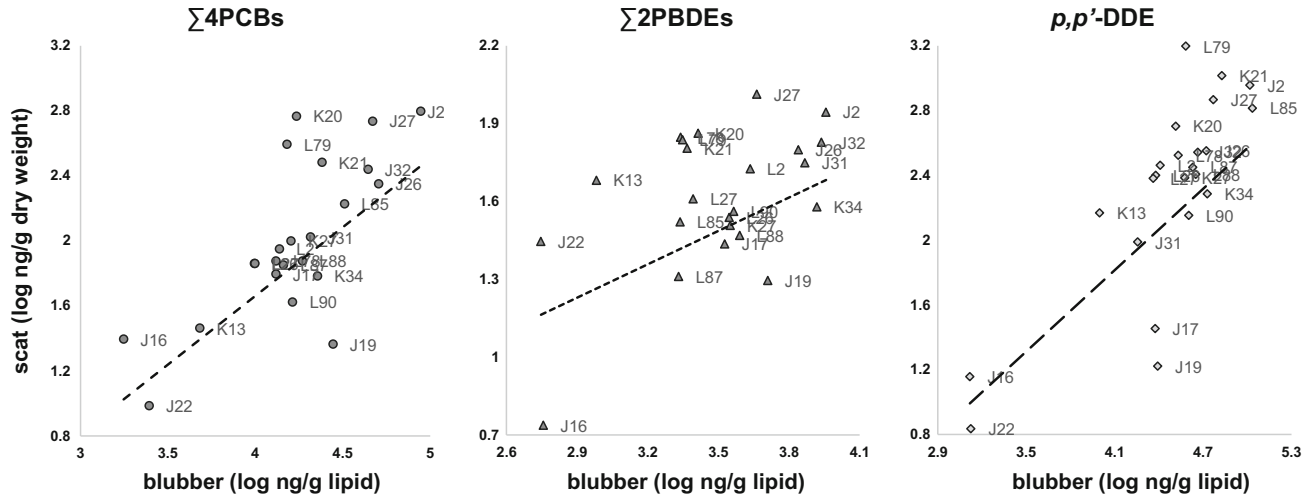

Fig. 1 Matched comparison of ∑4PCB (slope estimate, 0.896; $p < 0.001$), ∑2PBDE (slope estimate, 0.477; $p < 0.01$), and p,p'-DDE (slope estimate, 1.018; $p < 0.001$) levels in blubber (ng/g lipid) and scat (ng/g dry weight) samples from 22 unique adult Southern Resident killer whales

analysis and PCB149 based on the possible nonlinear association across toxicant concentration levels in the ethanol extract analysis. A comparison of the resulting ∑4PCBs metric in the blubber and scat samples from the same whale was statistically significant ($p < 0.001$) (slope estimate, 0.896; SE, 0.181) (data not shown; Fig. 1). Inclusion of neither years between blubber and scat sample collection from the same whale nor age-sex class improved the fit of the data to the model.

Sample analyses performed by NOAA NWFSC were on "unprocessed" samples that had not undergone lyophilization or ethanol treatment. Linear associations of 39 samples that were analyzed using both the protocol at NOAA NWFSC (Sloan et al. 2014) and the protocol outlined in this paper were significant for ∑4PCB, ∑2PBDEs, and p,p'-DDE (all $p < 0.001$; data not shown). This demonstrates any loss to volatilization during lyophilization or through the ethanol treatment does not affect the relative amount of toxicants in the samples. Nonetheless, unprocessed (fresh) samples should be used when possible to minimize concern of altered measurement of toxicant levels; in the least, an internal standard should be added to the sample prior to processing to correct for potential loss of target analytes.

Application of the optimized methods to measure POPs in methylene chloride extracted fecal pellets in 124 SRKW scat samples from 52 unique whales collected between May and October, 2010–2013, showed a statistically higher ratio of p,p'-DDE/∑4PCBs in K pod (geometric mean (GM), 2.7; 95 % confidence interval (CI), 2.2–3.4) and L pod (3.3; 2.8–3.9), compared to J pod (1.2; 1.0–1.4) (Fig. 2). This ratio in scat reflects findings from the same ratio in blubber previously used to demonstrate pod differences in toxicant levels to provide insight into whales'

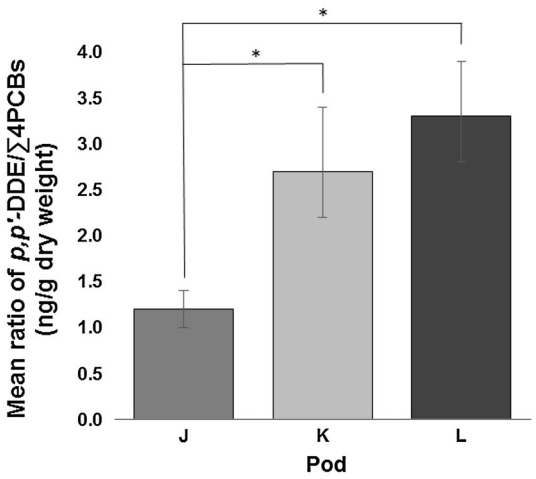

Fig. 2 Ratio of p,p'-DDE/∑4PCBs in 124 killer whale scat samples collected 2010–2013, adjusted for Julian day, age-sex class, and repeat samples collected from the same whale *$p < 0.001$

foraging locations (Krahn et al. 2007). There was no statistical difference in ∑4PCBs, ∑2PBDEs, or p,p'-DDE concentrations between years or pods, adjusted for Julian day, age-sex class, and repeat sampling on individual whales (Table 4). However, there was a suggested increase in PCBs in J pod and p,p'-DDE in K- and L-pod similar to previous reports on blubber biopsy samples (Krahn et al. 2007). Due to the persistent nature of these compounds a difference in toxicant level by year was not expected during a 4-year study. Statistically lower levels of POPs in reproductive females (age ≥10 to <40) compared with juveniles, adult males, and post-reproductive age females was apparent when evaluating the concentration by age-sex class. The GM for ∑4PCBs in females age ≥10 to <40

Table 4 Descriptive data of 124 killer whale scat samples from 52 unique whales collected between May and October, 2010-2013, analyzed for persistent organic pollutant concentrations

	Number of samples	Number of unique whales	∑4PCBs (ng/g dry wt)			∑2PBDEs (ng/g dry wt)			*p,p′*-DDE (ng/g dry wt)		
			Geometric mean[b]	95 % CI		Geometric mean[b]	95 % CI		Geometric mean[b]	95 % CI	
Year											
2010	18	16	130.0	77.0	219.5	26.7	20.1	35.5	243.7	140.5	422.7
2011	47	38	129.5	90.2	185.9	64.0	52.8	77.5	321.1	220.2	468.4
2012	33	23	115.9	76.1	176.7	43.9	35.0	55.0	218.8	140.7	340.1
2013	26	22	75.0	47.5	118.4	34.9	27.3	44.6	156.0	96.6	251.8
Pod											
J	58	19	146.0	93.6	227.6	56.2	44.5	71.0	174.3	109.9	276.3
K	27	13	120.4	68.5	211.5	43.2	31.8	58.8	320.6	178.2	577.0
L	39	20	86.9	55.7	135.4	37.6	29.5	48.0	288.9	181.8	459.3
Age–sex class[a]											
Juvenile	22	12	123.4	68.8	221.3	55.3	39.7	77.1	291.9	158.5	537.8
Reproductive age female	46	20	41.7	27.0	64.5	29.0	22.8	37.0	55.4	35.2	87.3
Post-reproductive female	22	7	237.2	117.4	479.4	60.8	41.4	89.2	596.2	287.3	1237.7
Adult male	34	15	136.5	82.0	227.2	43.3	32.6	57.6	363.8	213.7	619.3

∑4PCBs = PCB138, PCB153, PCB180, PCB187

∑2PBDEs = PBDE47, PBDE100

[a] Two whales switched from Juvenile to Adult male category during the 4-year study

[b] All geometric means adjusted for Julian day and age-sex class when not as a main effect, and repeat samples from individual whale

was 41.7 ng/g dry wt (95 % CI 27.0–64.5), whereas the GM for juveniles was 123.4 ng/g dry wt (95 % CI 68.8–221.3). This pattern is similar to the previously reported finding from blubber biopsy samples and is likely related to the maternal transfer of these toxicants during gestation and lactation (Debier et al. 2003; Krahn et al. 2009; Pomeroy et al. 1996; Ross et al. 2000). Likewise, the elevated levels in post-reproductive females (≥40 years) (GM, 237.2; 95 % CI 117.4–479.4) and adult males (≥10 years) (136.5; 82.0–227.2) also fit the expected pattern based on the bioaccumulation of these pollutants. These reported values exhibit measures in scat samples that have undergone lyophilization and ethanol extraction treatments. Any loss from these treatments has been demonstrated to be proportional to the total contaminant levels in the samples; therefore, between-sample evaluations are considered acceptable comparisons of these relative values. However, any application of these measures to metrics outside of this study should be made with caution.

POPs are lipophilic compounds that are resistant to biological degradation. As top-level predators, cetaceans are highly susceptible to POPs due to biomagnification from contamination in the food chain (Hickie et al. 2007; USEPA 2002). Accumulated POPs are stored predominantly in the adipose tissue, as measured by blubber biopsy samples. The significant linear association ($p < 0.01$) of toxicant measures in the blubber and measures in the scat for all classes of compounds indicate measures in the scat reflect accumulated levels of these contaminants. The SRKW population feed almost exclusively on salmonids, particularly Fraser River Chinook salmon (*Oncorhynchus tshawytscha*), between spring and fall (Ford and Ellis 2006; Ford et al. 1998; Hanson et al. 2010). Chemical analyses have revealed the current tissue levels of POPs in Fraser River Chinook are at concerning levels (Kelly et al. 2011; O'Neill et al. 2006). The extent toxicant measures in the scat also reflect elimination of toxicants in the prey is not known. Further study evaluating the toxicant profiles of Fraser River Chinook salmon and other potential prey sources would allow for the calculation of a Metabolic Index that would quantify relative bioaccumulation in the food chain to indicate whether the toxicants measured in the scat are from a prey or blubber source (Wolkers et al. 2004).

Conclusions

Measuring trace levels of toxicants in scat samples provides an unprecedented opportunity to noninvasively evaluate contaminant levels in wild cetacean populations, as well as monitor temporal and annual trends in toxicant levels across broad geographic landscapes. Using our current sample collection method (a single detection dog team), up to 80 viable scat samples were successfully collected each year. The collection of multiple scat samples per whale across seasons and years allow for the evaluation of important questions, such as how prey availability affects the concentration and profile of circulating toxicants in the endangered SRKW population. Additionally, the concurrent measurement with other physiologic indices obtained from these scat samples, such as nutritional, stress, and reproductive hormones (Ayres et al. 2012), may enable the assessment of associated physiologic impacts of toxicant exposures on population health. The incorporation of these data into population recovery and risk assessment models has the prospect to provide meaningful information for vital management decisions to improve and maintain the health of our shared marine environment.

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