Good Practices When Handling Fish Tissue to Avoid Data Reliability Issues: Results from Sample Wrapping and Holding Time Studies

Harry McCarty[1], John Healey[2], Blaine Snyder[3], and Tara Cohen[3]

[1] General Dynamics Information Technology

[2] USEPA, Office of Water, Office of Science and Technology, Standards and Health Protection Division

[3] Tetra Tech Center for Ecological Sciences

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The presenter is *not* an EPA employee, but a contractor.

Fish Tissue Contaminant Study Background

- Fish tissue contaminant studies require careful sample handling and storage practices to reduce the chance of cross-contamination and maintain sample integrity and quality
- Beginning with the 2000-2004 National Lake Fish Tissue Study, EPA provided sampling kits that included large sheets of solvent-rinsed aluminum foil to be used to wrap individual fish specimens
- The concern was that some of the 268 analytes being measured in that first study might have been used in the aluminum foil manufacturing process
- Solvent rinsing small pieces of foil in the lab was a common practice that was carried over to the foil used in the fish sampling protocols

Background (continued)

- Holding times for fish study samples were based on a consensus of the study planners, and set at one year from the time the whole fish was prepared for analysis (e.g., scaled, filleted, homogenized, and aliquoted for analyses).
- This facilitated the centralized preparation of whole fish, as well as allowing better “batching” of sample aliquots sent to multiple analytical laboratories
- EPA also archived extra containers of homogenized tissue from each sample in the event of breakage or lab accidents, or in the longer-term, if there was later interest in other contaminants.

The Concerns with Foil

Solvent rinsing of the foil has proven costly and challenging

- The established cleaning protocol is labor- and space-intensive and many laboratories have no interest in the effort
- Prices in the last 25 years have varied widely and unpredictably, from $4 to over $16 per 18"x48" sheet
- Those prices do not include the cost of the materials (bulk rolls of heavy-duty foil and the sterile bags) that were supplied to the labs.
- National-scale surveys with 200-500 sites and 5+ fish per site require lots of foil, at a cost of up to $100 per site!
- Other fish monitoring programs (e.g., GLNPO) have stopped using foil altogether

The Concerns with Archiving Samples

- Long-term frozen storage of archived samples requires significant investment in suitable freezer space and is energy intensive
- Labor associated with managing and inventorying an archive is not trivial
- While the archive process is “forward thinking,” long-term planning is needed.
  - How many jars are really needed? (At one point, OST was storing over 10,000 jars from just one study!)
  - Are there any data to demonstrate that the quality and integrity of samples can be maintained for years?

♫ *10,151 Jars of Fish in the Freezer, 10,151 Jars of Fish …* ♫

←This, times 2,
became this →

Change is Hard

“But we’ve always done it that way!”

But Change Is Easier with Data …

- Therefore, in 2023, OST began two related studies:
- The first study investigated the presence of mercury, PCBs, and various PFAS on three types of aluminum foil:
  - Previously prepared name-brand heavy-duty foil that had been rinsed with methylene chloride, dried in a muffle furnace, folded, and stored in a plastic bag for shipment to the field
  - Unrinsed name-brand heavy-duty foil purchased locally for the study
  - Unrinsed generic heavy-duty foil purchased locally for the study
- Three separate rinsates were prepared in triplicate from 6"x6" sections of foil by immersing both sides of the foil in:
  - Reagent water, for mercury
  - Methanol, for PFAS
  - Methylene chloride, for PCBs

Study Design (continued)

- The second study was a retrospective evaluation of holding times that involved new analyses of archived homogenized tissue samples that were previously analyzed for mercury, PCB congeners, and PFAS
- The samples were drawn from 6 previous OST studies spanning 3 to 22 years of frozen storage, with 12 samples/study that covered a wide range of reported concentrations and the most common fish species. The sample jars were either 250-mL or 500-mL in size.
- To minimize potential effects of freezer burn, each partially thawed archive jar was subsampled using a stainless steel conical sampling tool, and any discolored tissue near the top or the bottom of the core was removed
- Single results from the original analyses and the new analyses were compared to assess effects of long-term storage

Analytical Methods

- The analytical methods used for both the foil rinsate samples and the holding time samples were consistent with the analysis techniques used in the original studies:
  - Method 1631E was used for mercury, with sample preparation by Method 1631B, Appendix A
  - Method 1668C was used for PCB congeners
  - Method 1633 was used for PFAS
    - The tissue portion of Method 1633 was based on the sample preparation and LC/MS/MS procedures developed by the laboratory that first analyzed OST samples in 2008
    - The list of PFAS studied by OST evolved over time, from 13 strictly perfluorinated compounds to the 40 PFAS in Method 1633, so the results represent different time spans for different analytes
- All of the samples were analyzed with the method-specified QC samples and the results thoroughly reviewed

Foil Study Results for Mercury

Foil Type	Replicate #	Result (ng/L)	Mean	SD
Name Brand – Rinsed	1	0.37	0.42	0.0866
Name Brand - Rinsed	2	0.52		
Name Brand - Rinsed	3	0.37		
Name Brand - Unrinsed	1	0.99	0.70	0.3119
Name Brand - Unrinsed	2	0.74		
Name Brand - Unrinsed	3	0.56		
Generic – Unrinsed	1	0.38	0.46	0.1471
Generic - Unrinsed	2	0.29		
Generic - Unrinsed	3	0.60		
Solvent Blank (Reagent water)	--	ND (<0.2)	NA	NA

ANOVA Results for Mercury

Source of Variation	SS	df	MS	F	P-value	F_{crit}
Between Groups	0.23349	2	0.116744	4.40176	0.06658	5.14325
Within Groups	0.15913	6	0.026522			
Total	0.39262	8				

The null hypothesis is that the rinsates for all three types of foil have the same mercury concentrations.

The calculated value for F=4.40176, which is less than the F_{crit} of 5.14324, so the null hypothesis cannot be rejected and the conclusion is that **there is no statistically significant difference** between the mercury concentrations in the rinsates from the three types of foil that might be used to wrap fish samples.

Foil Study Results for PCB Congeners

- All of the rinsate samples and the solvent blank were analyzed for all 209 PCB congeners (as 162 results for individual congeners or coeluting groups of congeners)
- No congeners were reported in the solvent blank
- Despite detection limits at single-digit picogram levels, only 1 congener was found in any of the foil samples
- PCB-178 was reported at 2.97 pg/sample in just one rinsate from the generic unrinsed foil
- Therefore, **no statistical analyses of the PCB results were required** relative to the use of foil to wrap fish samples

Foil Study Results for PFAS

- All of the rinsate samples and the solvent blank were analyzed for the 40 target analytes in Method 1633
- Of those 40 target analytes, only 6 were detected, at 0.1 to 7.7 ng/sample in any of the rinsates or the solvent blank:
  - PFBA (in all 9 rinsates and the solvent blank)
  - PFPeA (in all 9 rinsates and the solvent blank)
  - PFHxA (in all 9 rinsates and the solvent blank)
  - PFHpA (in all 9 rinsates and the solvent blank)
  - PFOA (in 8 rinsates and the solvent blank)
  - PFNA (in 5 rinsates)
- The remaining 34 analytes were never detected, including PFOS!

Blank Subtraction for PFAS

- The PFAS solvent blank results were similar to, and sometimes higher than, the rinsate results, suggesting that the methanol may have been the source of 5 of the 6 PFAS detected in the rinsates. Therefore, we performed a blank subtraction.
- We performed the statistical analyses on *both* the original results and the blank-subtracted results. But when the blank correction resulted in a negative value, we set that result to a non-detect, using a zero.
- ANOVAs for all 6 reported PFAS showed similar results to those for mercury **– there were no statistically significant differences between in the rinsates from the three types of foil that might be used to wrap fish samples**, using either the original results and the blank-subtracted rinsate results.

Worst-Case Scenario Assessments

- The mean Mercury and PFAS results from the rinsates from the 6"x6" foil squares of each foil type were scaled up to the 18"x48" sheets of foil that are sent to the field
- Our very conservative “worst-case scenario assessment” assumes that:
  - All of the contaminant on a full sheet of foil is only on the side of the foil in contact with the fish (versus immersing the test pieces completely)
  - All of the contaminant is somehow transferred to the fillet portion of the fish, even though most of the foil never touches the fish directly
  - Rinsing and scaling the fish at the start of preparation does not remove any of the transferred contaminant from the surface of the fish
  - All of the transferred contaminant might end up in a 50-g bulk quantity of homogenized fillet tissue used to create aliquots for the analysis labs (50 g approximates the smallest mass of tissue in any recent OST studies, while most homogenates were *much* larger)

Mercury Foil Assessment

Foil Type	Mean Mass on Full Sheet (ng)	Worst-case Transfer to 50-g Sample (ng/g)	Lowest MDL (ng/g)	Detectable?
Name Brand - Rinsed	2.016	0.04032	0.09	**No**
Name Brand - Unrinsed	3.664	0.07328	0.09	**No**
Generic - Unrinsed	2.032	0.04064	0.09	**No**

The lowest fish tissue mercury result from 868 samples in OST's three most recent studies was 4.5 ng/g, 2 orders of magnitude greater than the worst-case estimate.

Therefore, mercury is not a concern, regardless of the type of foil used to wrap fish samples.

PFAS Foil Assessment

- Similar worst-case assessments were made using the blank-corrected results for the 6 PFAS detected in the rinsate samples.
- For 5 of the 6 PFAS, the worst-case estimates were 2 to 13 times *lower* than the lowest MDLs from recent OST studies, and thus those PFAS would not be detectable.
- **The exception was for PFPeA**:

Foil Type	Worst-Case (ng/g)	Lowest MDL (ng/g)	Detectable?
Name Brand - Rinsed	0.1244	0.077	Yes
Name Brand - Unrinsed	0.0969		Yes
Generic – Unrinsed	0.0801		Yes, barely

The lowest fish tissue PFPeA result from 868 samples in OST's three most recent studies was 0.115 ng/g, and it was only one of three PFPeA hits in those 868 samples.

Implications for Future OST Studies

- The results of this small study indicate that the aluminum foil used to wrap fish samples is not a likely source of the mercury, PCB, or PFAS found in OST's samples, whether it is solvent-rinsed or not.
- The substantial costs of providing solvent-rinsed foil for wrapping samples might be avoided with no loss of data quality.
- If the use of foil to wrap fish samples is still important, the logistics of providing samplers with a small roll of off-the-shelf foil are much simpler and could save up to $85 per site (e.g., over $42,000 for a study with 500 sites).

Holding Time Study Considerations

- Historically, EPA studied holding times in aqueous samples because analytes might be lost from the sample over time due to volatilization, reactions with other sample components, microbial degradation, and other processes.
- Waiting too long to analyze a sample could make it appear to meet a compliance limit when it really does not.
- In contrast, in OST's fish studies, the analytes of interest are PBTs - persistent, bioaccumulative, and toxic pollutants. As such, they are not likely to be "going away" in tissue samples with time, especially in a freezer.
- Even for mercury, which *might* volatilize from a water sample in 28 days, in fish, it is incorporated into the tissue, likely as methyl mercury, and bound to proteins

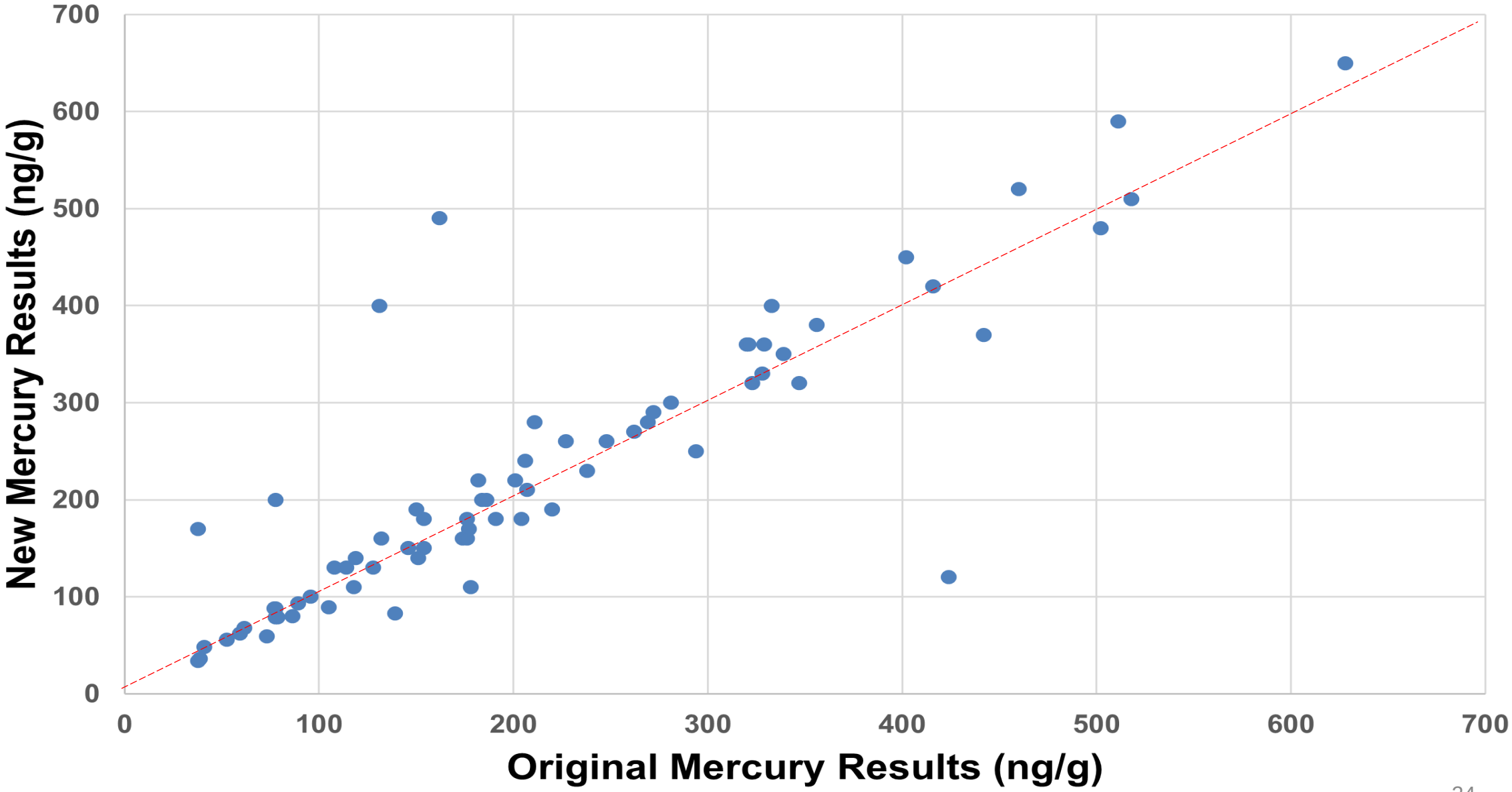
Considerations (continued)

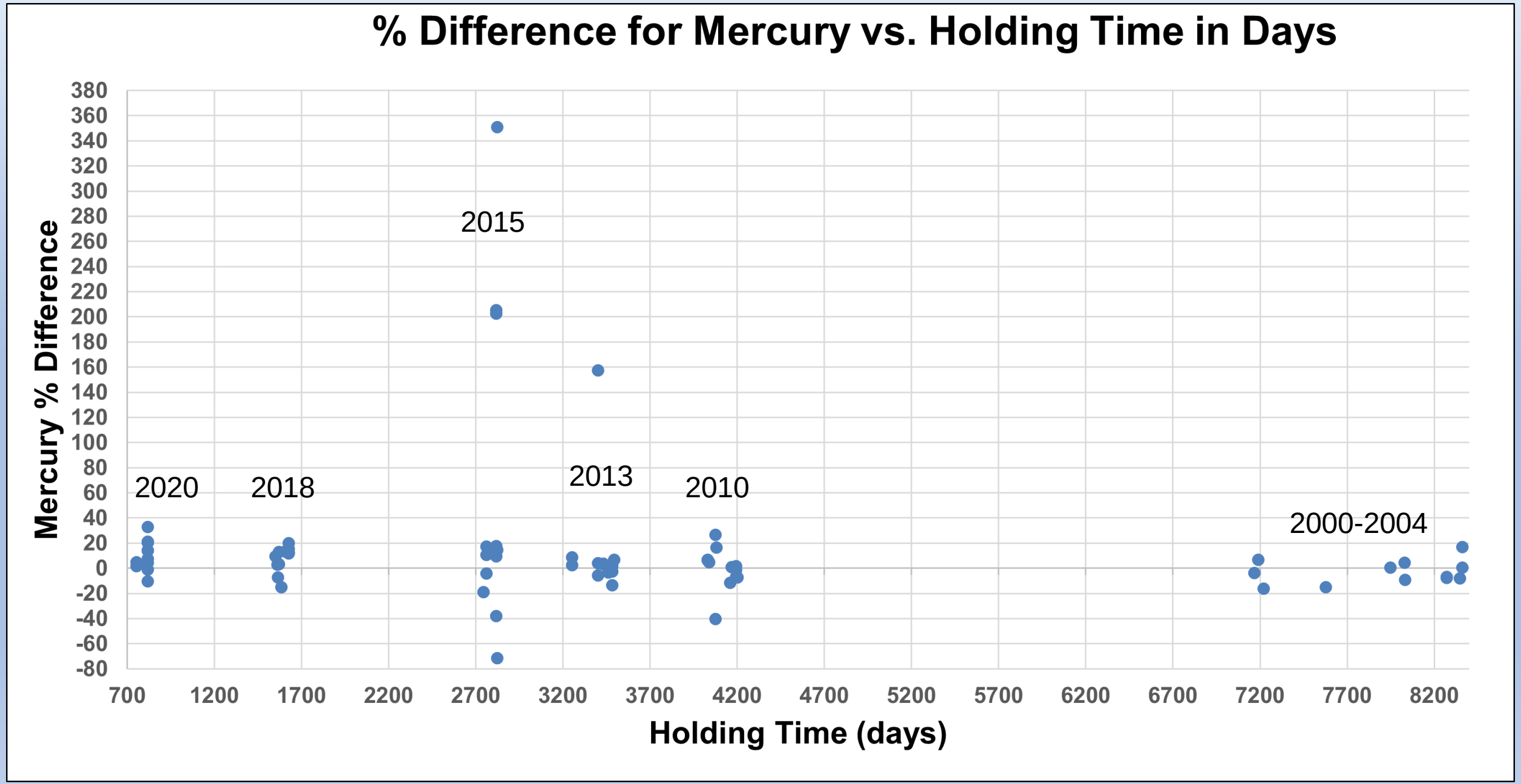
- The most likely effect of long-term storage of frozen tissue samples is an *increase* in reported sample concentrations due to the loss of moisture that dries out the tissue and reduces its “wet” weight.
- We’ve all seen ice crystals inside a freezer bag full of meat that was stored for too long. That ice is not likely to contain any contaminants, but the frozen meat is drying out over time and the weight on the label is no longer accurate
- But how much of a change will there be over time?

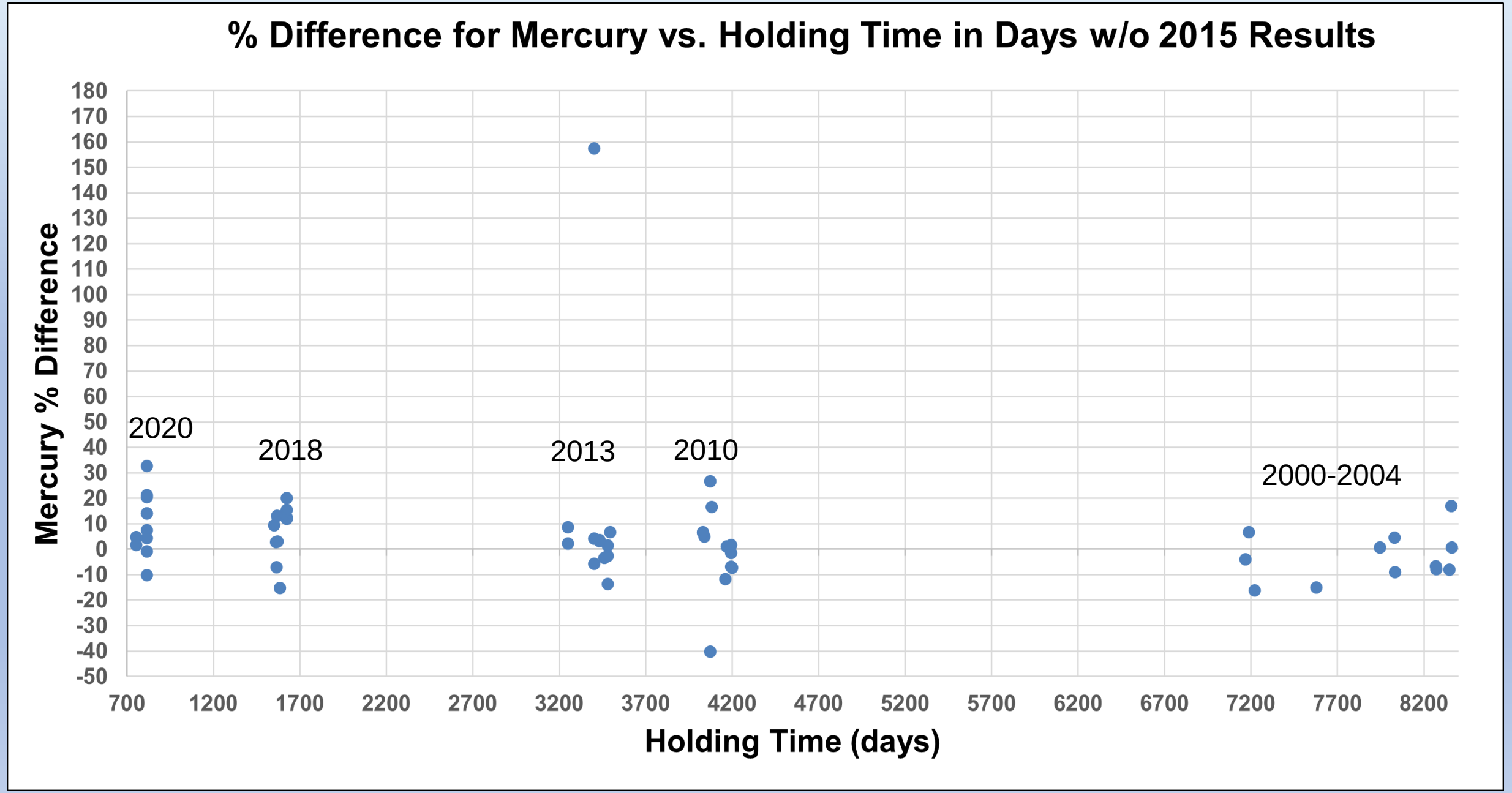
Retrospective Study

- This is a “retrospective” study because back in 2000, no one envisioned the need to assess holding times over such a long term, so replicate samples were *not* set aside initially and *not* analyzed at prescribed internals over time.
- Instead, we identified suitable frozen archived samples from 6 OST studies that were collected between 2000 and 2020.
- We have two sets of mercury and PCB results for 72 samples that were held about 700 to 8,400 days between analyses
- OST started PFAS analyses later, so 60 of those same samples have two sets of PFAS results for 13 to 40 target PFAS that were held about 700 to 4,500 days between analyses
- The easiest way to examine the results is to plot the new and original results against one another, as shown for mercury

Plot of Original and New Mercury Results

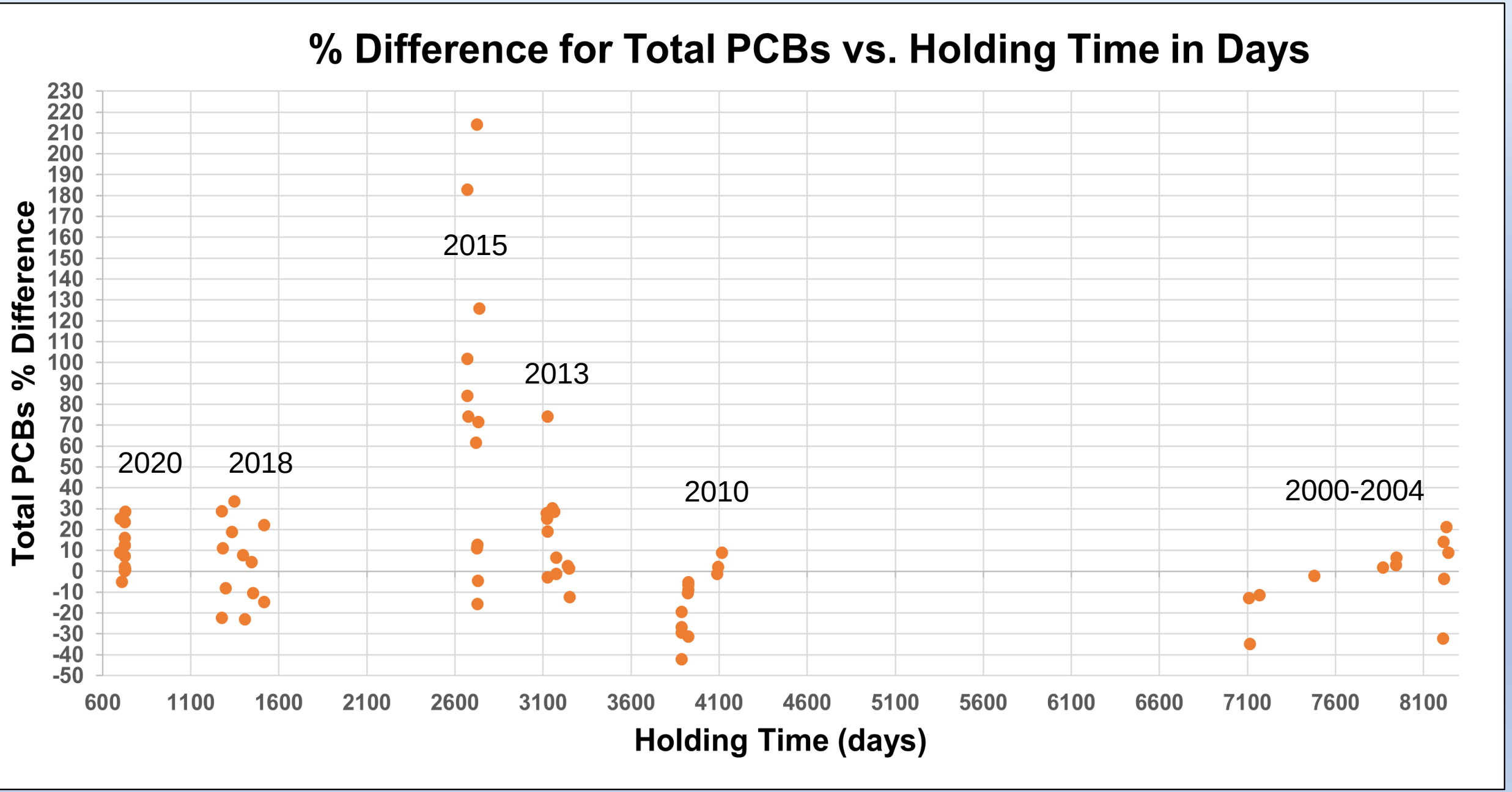



% Difference for Mercury vs. Holding Time in Days
Mercury % Difference
380
360
340
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
0
-20
-40
-60
-80
2015
2020
2018
2013
2010
2000-2004
700
1200
1700
2200
2700
3200
3700
4200
4700
5200
5700
6200
6700
7200
7700
8200
Holding Time (days)


% Difference for Mercury vs. Holding Time in Days w/o 2015 Results
Mercury % Difference
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
2020
2018
2013
2010
2000-2004
700
1200
1700
2200
2700
3200
3700
4200
4700
5200
5700
6200
6700
7200
7700
8200
Holding Time (days)

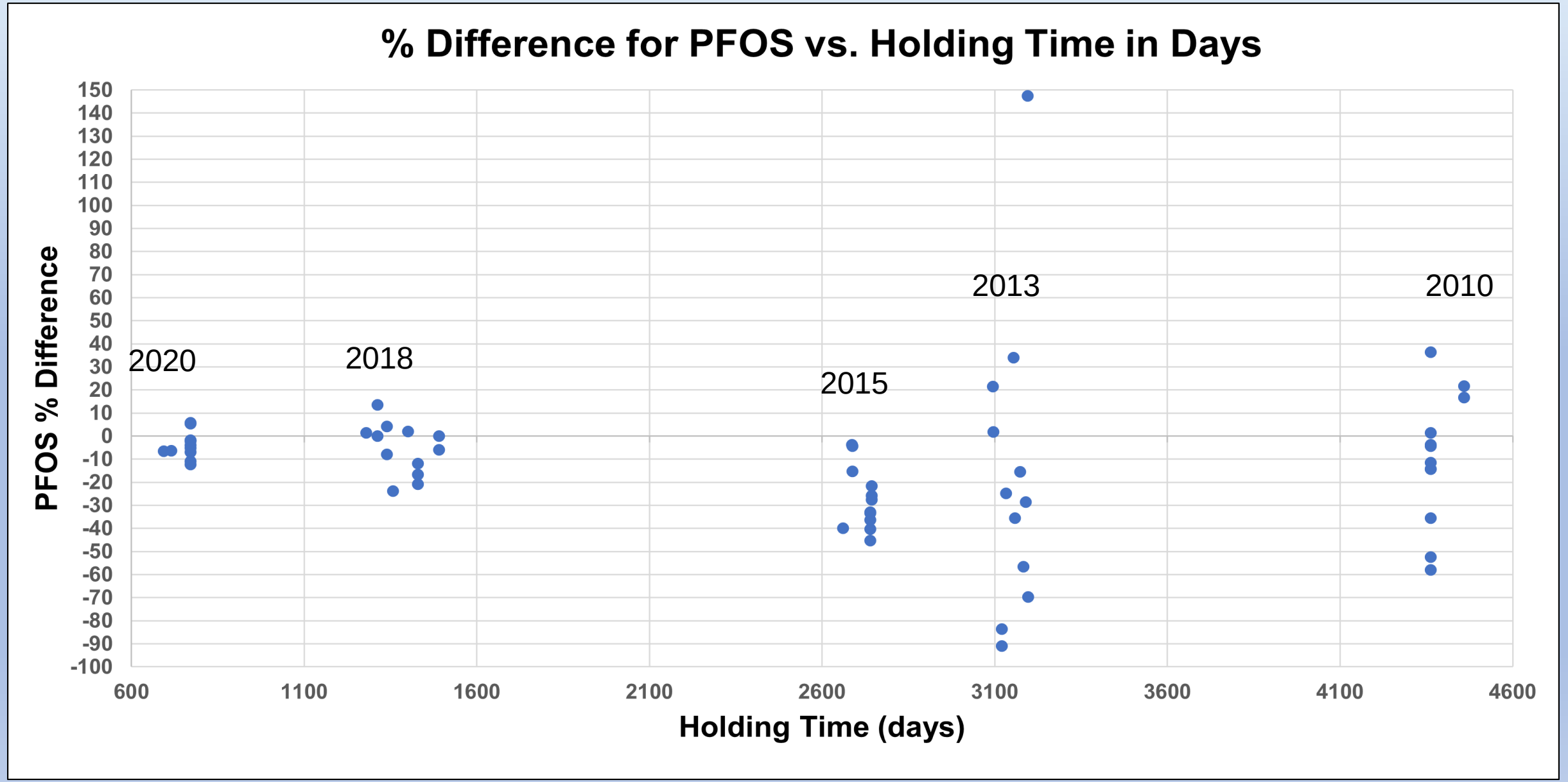
PCB Congeners

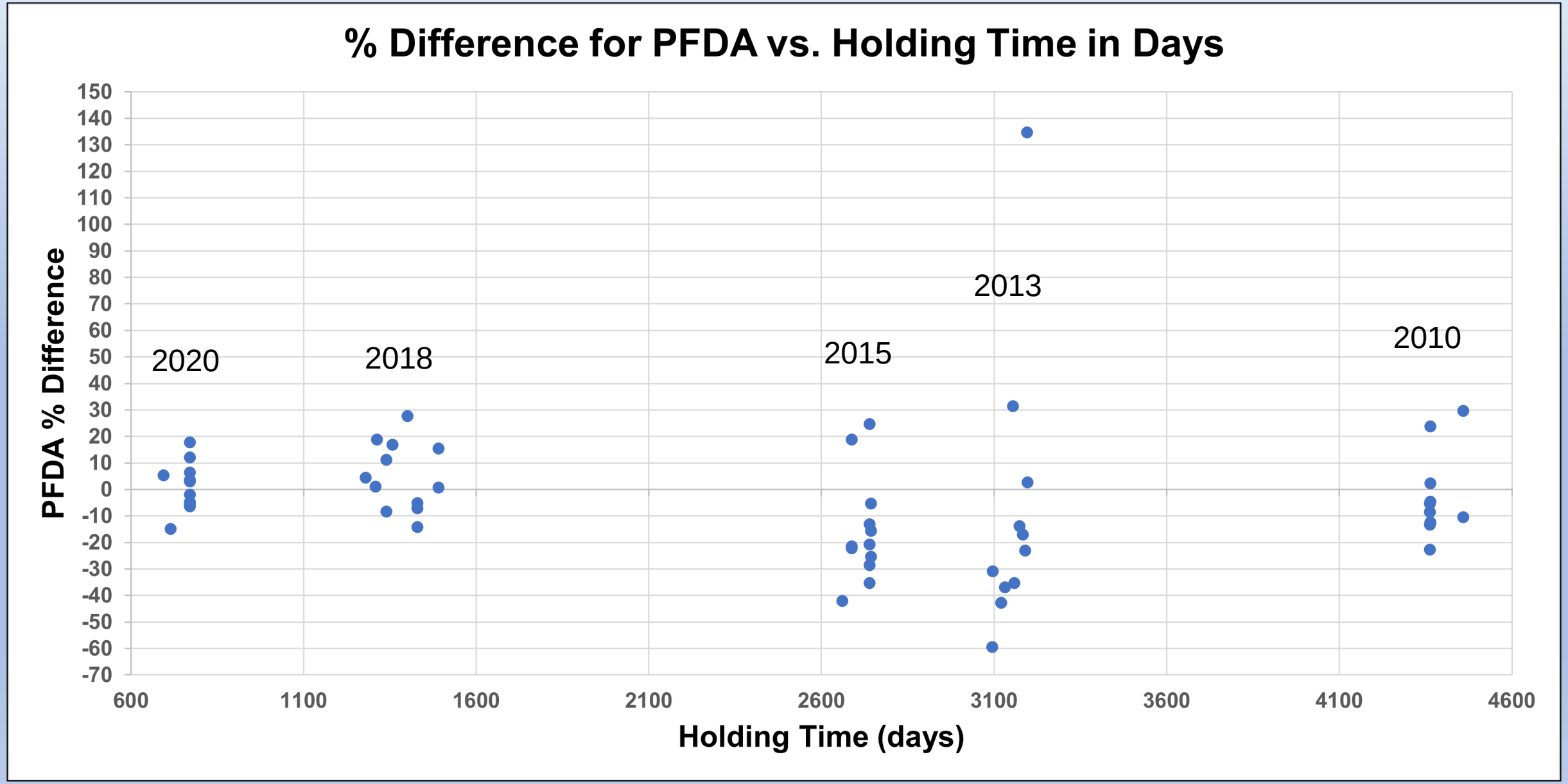
- We evaluated a large number of congeners that were found in the majority of the study samples
- It is not practical (nor polite) to try to display the plots for all 209 congeners
- For the purpose of this presentation, we have focused on the “Total PCBs” results, in part because those totals are the basis for OST’s assessments of the risks associated with eating fish containing PCBs
  - OST calculates the “Total” by summing the concentrations of all detected congeners or coeluting groups of congeners, and using 0 for any non-detects
  - The original Total PCB results for the subset of samples in this study ranged from 1 to 1,265 n/g


% Difference for Total PCBs vs. Holding Time in Days
Total PCBs % Difference
Holding Time (days)
2020
2018
2015
2013
2010
2000-2004
230
220
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
600
1100
1600
2100
2600
3100
3600
4100
4600
5100
5600
6100
6600
7100
7600
8100

PFAS

- As with the PCBs, we will not present data for all of the analytes
- Because the analyte lists for the successive studies increased as more authentic analytical PFAS standards and labeled analogs became available, we were able to examine the results for different analytes across different timeframes
- Although they used the same basic techniques, the PFAS methods in the earliest studies were far less standardized than Method 1633, which may have introduced more variability
- For the purpose of this presentation, we are focusing on PFOS, which has been found in nearly all the fish samples we have had analyzed since 2010, and was present in all of this study's samples, so the effects of holding time on PFOS can be assessed over the longest timeframe in this study.


% Difference for PFOS vs. Holding Time in Days
PFOS % Difference
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
-80
-90
-100
600
1100
1600
2100
2600
3100
3600
4100
4600
Holding Time (days)
2020
2018
2015
2013
2010


% Difference for PFDA vs. Holding Time in Days
PFDA % Difference
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
-20
-30
-40
-50
-60
-70
2020
2018
2015
2013
2010
600
1100
1600
2100
2600
3100
3600
4100
4600
Holding Time (days)

Holding Time Study Conclusions

- For mercury, samples might be held for over 20 years and still produce mercury results within ± 20% of the presumed true value, and with a 14% positive bias, on average. That positive bias drops to 2.4% without the 5 outlier values from the 2015 study.
- For PCBs, without those 2015 samples, samples might be held for over 20 years and still produce Total PCB results within ± 30% of the presumed true value, with a mean positive bias of only 2.8%.
- For PFAS, the samples covered a shorter timeframe, but samples may be held for up to 4 years and the PFOS results will still fall within ± 20%. The other commonly detected PFAS (largely the PFCAs) show similar patterns.
- Therefore, the current 1-year holding times are more than reasonable and samples archived for much longer could be useful to illustrate trends.

For Further Information, Contact:

John Healey
USEPA Office of Water
Office of Science and Technology
Healey.john@epa.gov

or go to:

https://www.epa.gov/choose-fish-and-shellfish-wisely/studies-fish-tissue-contamination

Questions?