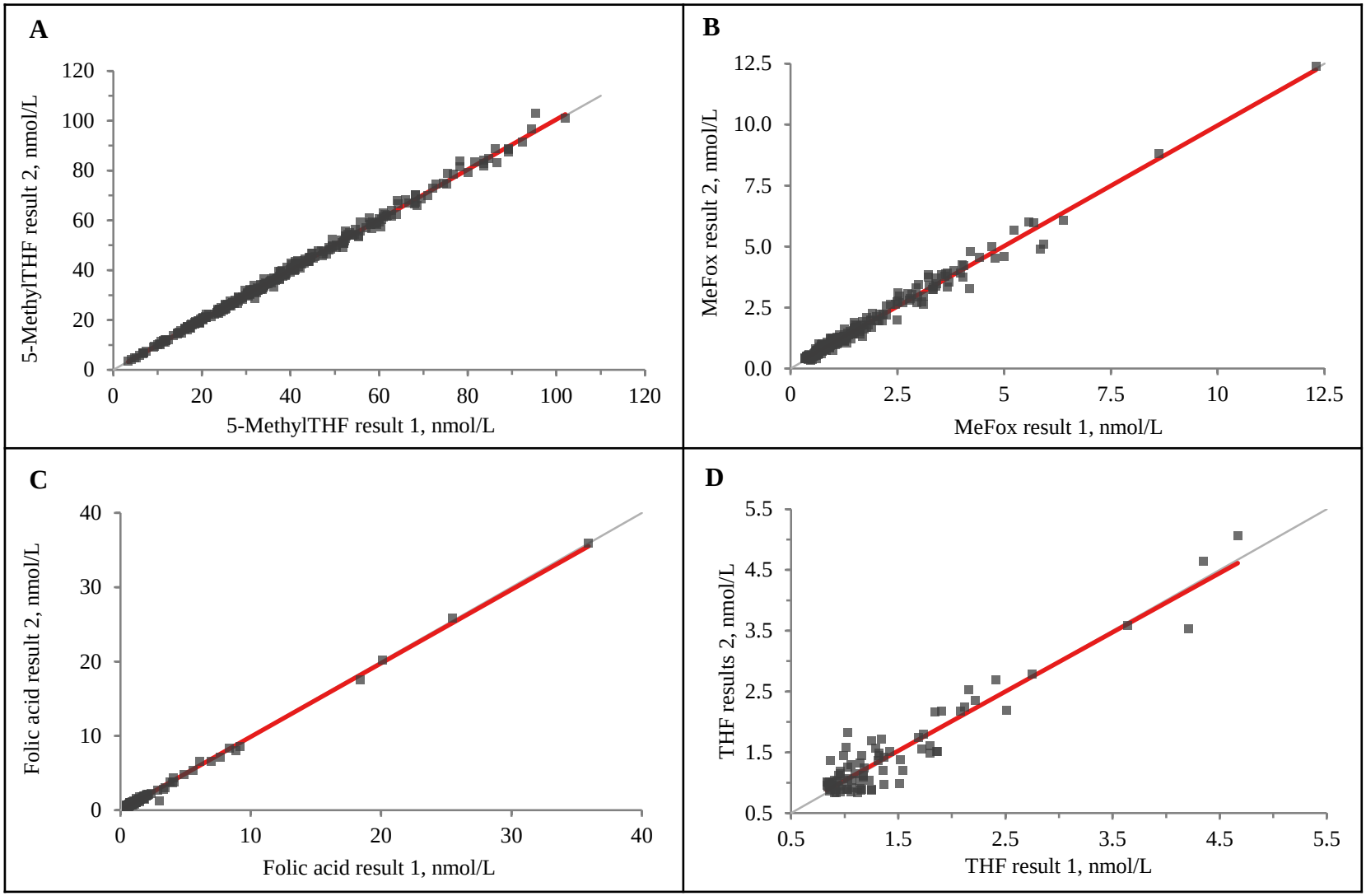
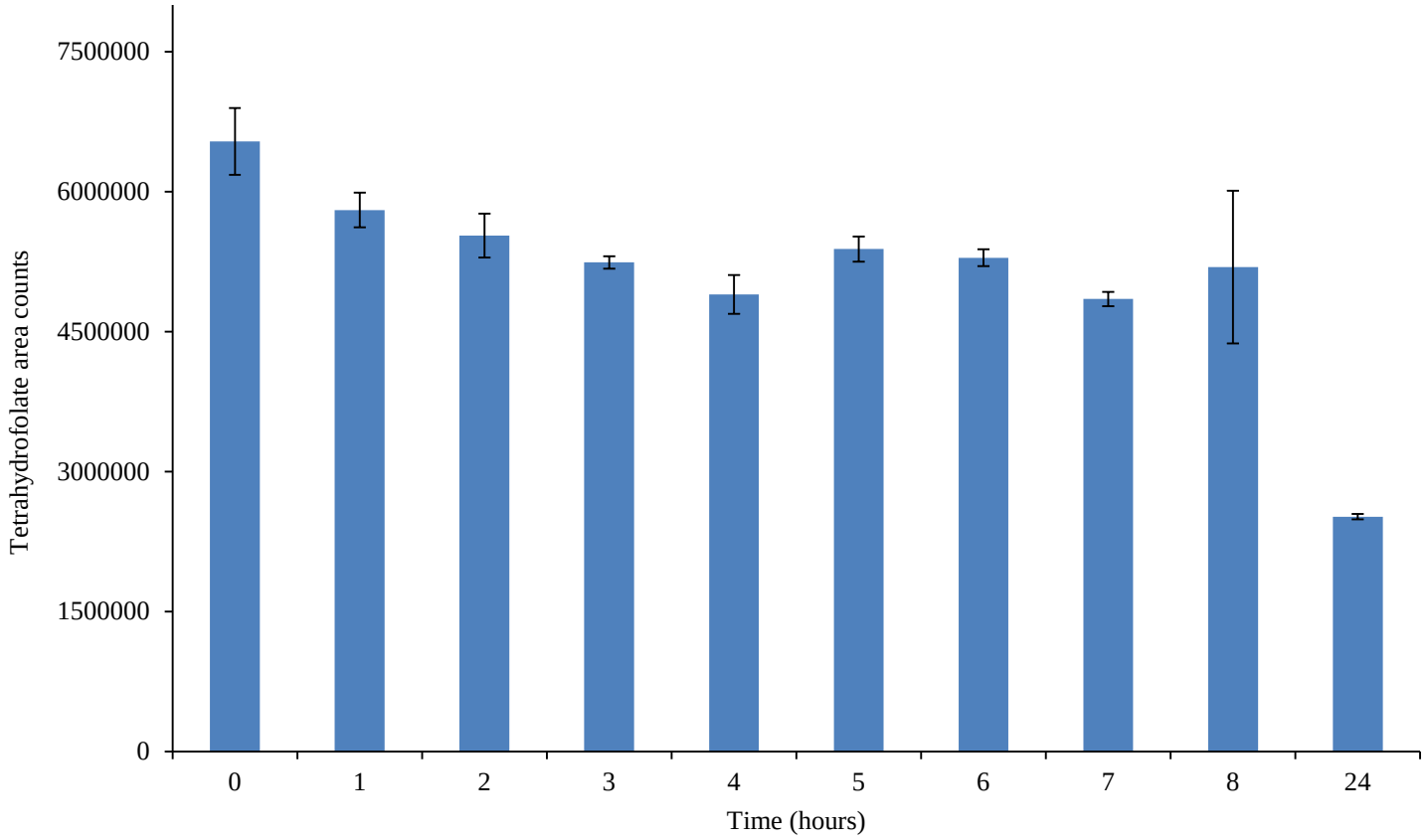
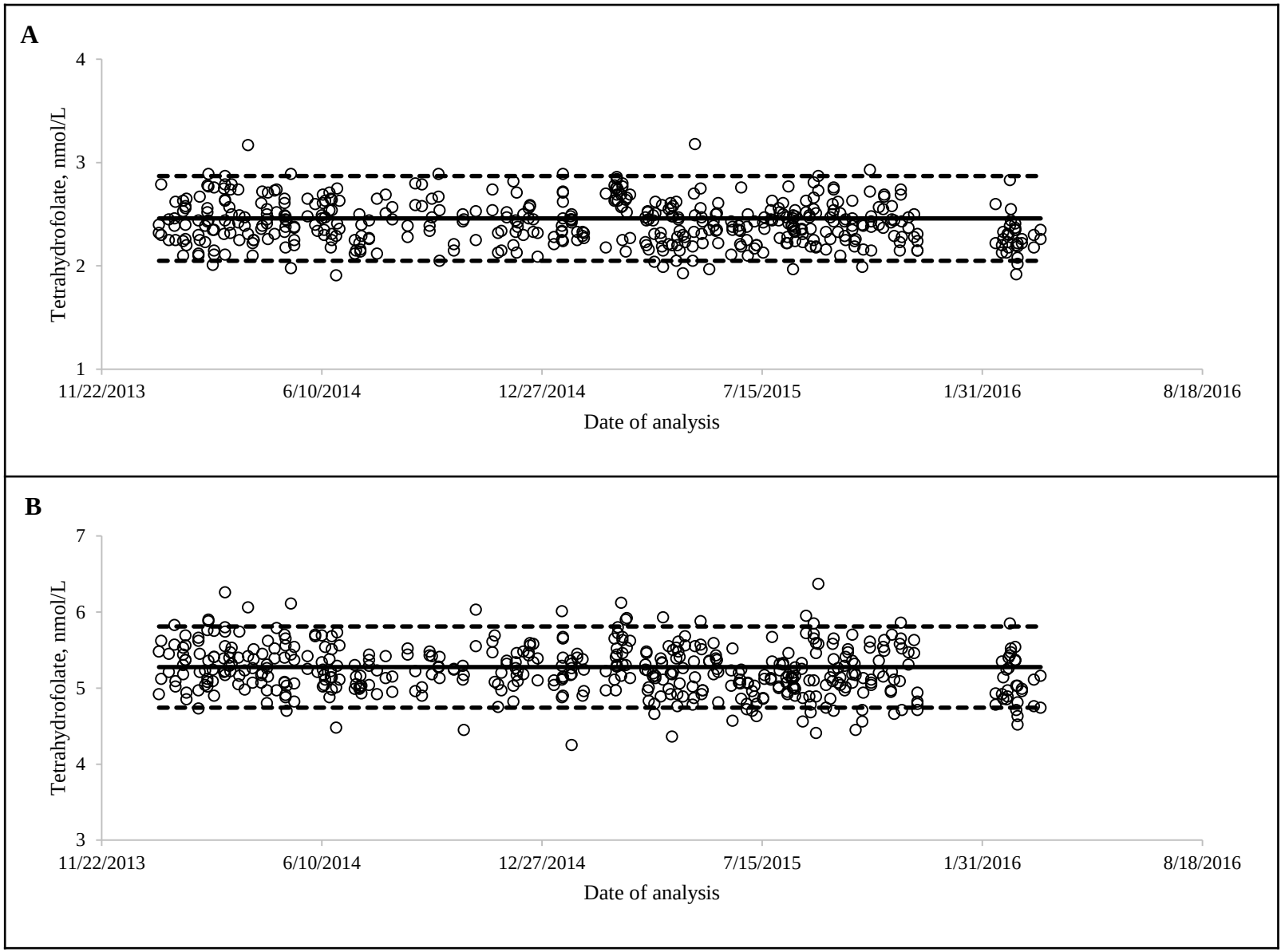
Supplemental Figure 1. Scatter plot of the relative change in concentrations between the initial and repeat analysis for each serum folate form as a function of the time between the 2 measurements in convenience samples from a large CDC study. Samples contained 5 g/L L-ascorbic acid added at the time the serum was prepared. Sample size was 301 for 5-methylTHF (panel A), 211 for MeFox (panel B), 214 for folic acid (panel C), and 81 for THF (panel D). 5-MethylTHF, 5-methyl-tetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α- hydroxy-5-methylTHF; THF, tetrahydrofolate.



Supplemental Figure 2. Change in tetrahydrofolate area counts over time. A neat solution (2 µmol/L THF in 0.5% ascorbic acid) was kept in a closed vial at room temperature for up to 24 hours. From that vial, a 100 nmol/L calibrator was prepared in sample solvent hourly for direct HPLC-MS/MS analysis (0-8 hours) and after 24 hours. Folic acid in these samples corresponded to ~0.5% of the THF amount up to 7 h, and increased slightly to ~0.8% of the THF amount at 24 h, potentially indicating some oxidation of THF to folic acid.



Supplemental Figure 3. Serum tetrahydrofolate concentrations in 2 quality control pools (medium level, panel A; high level, panel B) containing 5 g/L ascorbic acid and measured over the course of 2 years, from January 2014 to March 2016. Solid line represents the pool mean concentration and dashed lines represent the ± 2 SD acceptability limits.



Supplemental Table 1. Recovery of tetrahydrofolate added to serum QC pools prepared with and without ascorbic acid

Sample	Spike (nmol/L)	No additional AA added[a] Measured[c] (nmol/L)	No additional AA added[a] Recovery[d] (%)	Additional AA added[b] Measured[c] (nmol/L)	Additional AA added[b] Recovery[d] (%)
Pools prepared with ascorbic acid[e]					
Low QC pool A	0	0.18[f] ± 0.00	-	0.18[f] ± 0.00	-
Low QC pool A + spike	20	18.1 ± 0.50	89.6 ± 2.50	17.8 ± 0.40	88.3 ± 2.02
Medium QC pool A	0	2.26 ± 0.05	-	1.95 ± 0.08	-
Medium QC pool A + spike	20	20.2 ± 0.38	89.9 ± 1.67	19.8 ± 0.32	89.7 ± 1.07
High QC pool A	0	4.79 ± 0.32	-	5.14 ± 0.33	-
High QC pool A + spike	20	22.5 ± 0.75	88.4 ± 5.35	22.8 ± 0.15	84.4 ± 1.06
Pools prepared without ascorbic acid[g]					
Low QC pool B	0	0.18[f] ± 0.00	-	0.18[f] ± 0.00	-
Low QC pool B + spike	20	2.86 ± 0.19	13.4 ± 0.94	17.1 ± 0.06	84.8 ± 0.29
Medium QC pool B	0	0.20[f] ± 0.04	-	0.22[f] ± 0.06	-
Medium QC pool B + spike	20	9.40 ± 0.48	46.0 ± 2.54	18.2 ± 0.15	89.9 ± 1.67
High QC pool B	0	0.49 ± 0.07	-	0.79 ± 0.03	-
High QC pool B + spike	20	8.33 ± 0.16	39.2 ± 1.12	17.7 ± 0.21	88.4 ± 5.35

[a] No additional ascorbic acid added to the sample at the time of sample preparation

[b] Additional ascorbic acid (5 g/L) added to the sample at the time of sample preparation

[c] Values represent mean ± SD of 3 replicates

[d] Recovery represents mean ± SD and was calculated as the measured concentration difference between the spiked and unspiked sample divided by the nominal concentration of the spike

[e] 5 g/L ascorbic acid was added to each serum QC pool at the time of pool preparation in 2013 and the pools were stored at -70°C; in 2017, an aliquot of each pool was thawed and spiked with 1 level of tetrahydrofolate

[f] LOD for THF: 0.25 nmol/L; measured concentrations <LOD replaced with an imputed value of LOD divided by square root of 2

[g] No ascorbic acid was added to either serum QC pool at the time of pool preparation in 2006 and the pools were stored at -70°C; in 2017, an aliquot of each pool was thawed and spiked with 1 level of tetrahydrofolate

Supplemental Table 2. Long-term folate stability in serum pools stored at -70°C for ≥4 years

Analyte[a]	Pool material[b]	Ascorbic acid added[c]	Folate spiked[d] (nmol/L)	Mean ± SD [*n*] concentration Initial[e] (nmol/L)	Current[f] (nmol/L)	Relative change[g] (%)
5-MethylTHF	2013 Low	Yes	No	14.1 ± 0.21 [54]	13.9 ± 0.56 [6]	-1.3
	2013 Medium	Yes	No	25.9 ± 0.68 [54]	26.5 ± 0.31 [3]	2.3
	2013 High	Yes	No	49.9 ± 0.94 [54]	49.8 ± 0.80 [3]	-0.2
	2011 Low	Yes	No	17.9 ± 0.45 [20]	18.3 ± 0.44 [5]	2.3
	2011 Medium	Yes	No	32.3 ± 1.02 [20]	33.8 ± 0.32 [3]	4.6
	2011 High	Yes	No	47.6 ± 1.23 [20]	49.0 ± 0.40 [3]	3.0
	2009 Low	No	No	14.5 ± 0.37 [54]	14.6 ± 0.07 [2]	0.7
	2009 Medium	No	No	31.8 ± 0.92 [54]	32.2 ± 0.28 [2]	1.1
	2009 High	No	No	46.6 ± 1.61 [54]	45.5 ± 0.14 [2]	-2.3
	2006 Low	No	No	6.28 ± 0.36 [62]	6.47 ± 0.23 [5]	3.0
	2006 Medium	No	No	22.1 ± 1.47 [62]	23.6 ± 0.95 [5]	6.8
	2006 High	No	No	35.7 ± 2.62 [62]	38.7 ± 1.26 [5]	8.3
MeFox	2013 Medium	Yes	No	2.40 ± 0.07 [54]	2.58 ± 0.13 [3]	7.5
	2013 High	Yes	No	2.86 ± 0.06 [54]	3.11 ± 0.13 [3]	8.7
	2011 Medium	Yes	No	1.51 ± 0.13 [20]	1.46 ± 0.04 [3]	-3.3
	2011 High	Yes	No	2.96 ± 0.11 [20]	2.86 ± 0.09 [3]	-3.4
Folic acid	2013 Medium	Yes	4.5	4.03 ± 0.30 [54]	3.96 ± 0.07 [3]	-1.7
	2013 High	Yes	8.0	9.04 ± 0.71 [54]	8.75 ± 0.23 [3]	-3.2
	2011 Medium	Yes	No	3.88 ± 0.35 [20]	3.86 ± 0.12 [3]	-0.5
	2011 High	Yes	No	7.96 ± 0.53 [20]	7.50 ± 0.21 [3]	-5.8
	2009 Medium	No	No	3.38 ± 0.31 [54]	3.01 ± 0.01 [2]	-10.9
	2009 High	No	No	8.04 ± 0.74 [54]	7.43 ± 0.01 [2]	-7.6
	2006 Medium	No	1.0	1.45 ± 0.18 [62]	1.52 ± 0.08 [5]	4.5
	2006 High	No	5.0	5.33 ± 0.51 [62]	5.35 ± 0.10 [5]	0.3
THF	2013 Medium	Yes	2.0	2.44 ± 0.18 [54]	2.26 ± 0.05 [3]	-7.4
	2013 High	Yes	5.0	5.39 ± 0.22 [54]	4.79 ± 0.32 [3]	-11.1
	2011 Medium	Yes	2.0	1.37 ± 0.20 [20]	1.08 ± 0.12 [3]	-21.2
	2011 High	Yes	4.0	4.58 ± 0.48 [20]	3.63 ± 0.08 [3]	-20.8
5-FormylTHF	2013 Medium	Yes	1.0	1.22 ± 0.06 [54]	1.19 ± 0.01 [3]	-2.5
	2013 High	Yes	2.0	2.32 ± 0.08 [54]	2.28 ± 0.01 [3]	-1.7
	2011 Medium	Yes	1.0	0.58 ± 0.08 [20]	0.54 ± 0.03 [3]	-6.9
	2011 High	Yes	2.0	2.32 ± 0.15 [20]	2.15 ± 0.09 [3]	-7.3
5,10-MethenylTHF	2013 Medium	Yes	2.0	1.96 ± 0.11 [54]	2.19 ± 0.16 [3]	11.7
	2013 High	Yes	5.0	4.74 ± 0.24 [54]	4.99 ± 0.04 [3]	5.3
	2011 Medium	Yes	2.0	1.41 ± 0.11 [20]	1.54 ± 0.05 [3]	9.2
	2011 High	Yes	5.0	4.32 ± 0.19 [20]	4.35 ± 0.33 [3]	0.7

[a] 5-FormylTHF, 5-formyltetrahydrofolate; 5,10-methenylTHF, 5,10-methenyltetrahydrofolate; 5-methylTHF, 5-methyltetrahydrofolate; MeFox, pyrazino-s-triazine derivative of 4α-hydroxy-5-methylTHF; THF, tetrahydrofolate

[b] Year indicates when the material was prepared; low, medium, or high indicates the level of the pool

[c] Yes indicates that ascorbic acid (5 g/L) was added to the pool at the time of preparation

[d] Concentration of minor folate forms added to the pool at time of preparation to obtain moderate concentrations

[e] Measurement of the pool material over ≥10 days during initial characterization after pool preparation

[f] Measurement of the pool material over 1 or 2 days in late 2017

[g] Calculated as relative difference between current and initial measurement