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Proficiency Test Final Report
AQA 21-05
Chlorophyll a in Water

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Accredited for compliance with ISO/IEC 17043

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SUMMARY

This report presents the results of the proficiency testing study AQA 21-05 – Chlorophyll a in Water. The study covered the measurement of chlorophyll a and pheophytin a in water. Pheophytin a was included in this study as a measure of chlorophyll a degradation.

Two samples were prepared: Samples S1 and S2 - each consisted of one filter.

Thirty two laboratories registered to participate and all submitted results.

The assigned value was the robust average of participants' results. The associated uncertainty was estimated from the robust standard deviation of the participants' results.

The outcomes of the study were assessed against the aims as follows:

i. compare the performance of participant laboratories and assess their accuracy;

Laboratory performance was assessed using both z-scores and E_n-scores.

Of 55 z-scores, 49 (89%) were satisfactory with $|z| \leq 2.0$.

Of 55 E_n-scores, 34 (62%) were satisfactory with $|En| \leq .01$.

ii. evaluate the laboratories' methods used in determination of chlorophyll a in water;

There was no significant difference between chlorophyll a results from acetone extraction and chlorophyll a results from ethanol and methanol extraction.

iii. compare the performance of participant laboratories with their past performance;

Despite lower concentrations in this study, participants performed better over time.

iv. develop the practical application of traceability and measurement uncertainty and provide participants with information that will be useful in assessing their uncertainty estimates.

Of 76 numerical results, 66 were reported with an expanded measurement uncertainty.

A large number of participants were wrongly reporting an estimate of uncertainty expressed as a *value* to a result expressed as a *range* (e.g. less than the level of reporting).

v. produce materials that can be used in method validation and as control samples.

The chlorophyll a PT samples are homogeneous and well characterised, both by in-house testing and from the results of the proficiency round. A long term stability study conducted over two years found no significant changes in the level of chlorophyll a overtime if stored frozen. These samples can be used for quality control, method development and method validation. Surplus test samples from this study are available for sale.

1 INTRODUCTION

1.1 NMI Proficiency Testing Program

The National Measurement Institute (NMI) is responsible for Australia's national measurement infrastructure, providing a range of services including a chemical proficiency testing program.

Proficiency testing (PT) is: 'evaluation of participant performance against pre-established criteria by means of interlaboratory comparison.'[1] NMI PT studies target chemical testing in areas of high public significance such as trade, environment and food safety. NMI offers studies in:

- inorganic analytes in soil, water, food and pharmaceuticals;
- pesticide residues in fruit and vegetables, soil and water;
- petroleum hydrocarbons in soil and water;
- PFAS in soil, water, biota and food;
- allergens in food;
- controlled drug assay; and
- folic acid in flour.

2 STUDY AIMS

The aims of the study were to:

- compare the performance of participant laboratories and assess their accuracy;
- evaluate the laboratories' methods used in the determination of chlorophyll a in water;
- compare the performance of participant laboratories with their past performance;
- develop the practical application of traceability and measurement uncertainty;
- provide participants with information that will be useful in assessing their uncertainty estimates; and
- produce materials that can be used in method validation and as control samples.

2.1 Study Conduct

The conduct of NMI proficiency tests is described in the NMI Chemical Proficiency Testing Study Protocol.[2] The statistical methods used are described in the NMI Chemical Proficiency Statistical Manual.[3] These documents have been prepared with reference to ISO Standard 17043[1] and The International Harmonized Protocol for Proficiency Testing of (Chemical) Analytical Laboratories.[4]

NMI is accredited by the National Association of Testing Authorities, Australia (NATA) to ISO 17043 as a provider of proficiency testing schemes. This scheme is within the scope of NMI's accreditation.

The choice of the test method was left to the participating laboratories with the following stipulations: (1) all procedures were to be carried out under subdued light to prevent photo-decomposition, and (2) use 90% (v/v) acetone as the extraction solution.

3 STUDY INFORMATION

3.1 Selection of Matrices and Analytes

The study was based on participants' expressions of interest and was intended to help laboratories to assess their methods for chlorophyll a measurements in water.

3.2 Participation

Thirty two laboratories registered to participate and all submitted results.

The timetable of the study was:

Invitation issued:	30 March 2021
Samples dispatched:	27 April 2021
Results due:	14 May 2021
Interim report issued:	17 May 2021

3.3 Test Material Specification

Two samples were provided for analysis.

Samples S1 and S2 consisted of one glass fibre filter each.

Participants were asked to report results as they would normally report them to a client in units of µg/L. The sample description in the instruction letter was " 1L of water was filtered through 0.45 µm glass fibre filter. The glass fibre filter was placed in an airtight brown container, wrapped in aluminium foil and stored frozen in the dark." The full sample preparation procedure is presented in Appendix 1.

3.4 Laboratory Code

All laboratories that agreed to participate were assigned a confidential code number.

3.5 Sample Preparation, Analysis and Homogeneity Testing

Homogeneity testing was subcontracted to ChemCentre and was conducted for chlorophyll a in Samples S1 and S2. The preparation and analysis are described in Appendix 1. The sample was found to be sufficiently homogeneous for the assessment of participants' results.

3.6 Stability of Analytes

Stability testing was subcontracted to ChemCentre and was conducted for chlorophyll a in the study samples. This is described in Appendix 3. The samples were found to be sufficiently stable for the assessment of participants' results.

The outcomes from a long term stability study for chlorophyll a conducted using the PT samples from a previous study over two years are also presented in Appendix 4.

3.7 Sample Storage, Dispatch and Receipt

Samples S1 and S2 were stored at -20°C and dispatched by courier on 27 April 2021.

A description of the test samples, instructions to participants, and a form for participants to confirm the receipt of the test sample were sent with the sample.

An Excel spreadsheet for the electronic reporting of results was emailed to participants.

3.8 Instructions to Participants

Participants were instructed as follows:

- Participants are advised to start analyses as soon as they receive the samples; if this is not possible then the samples should be stored in a freezer.
- Participants are asked to record the date when the analyses were conducted.
- All procedures should be carried out under subdued light to prevent photo-decomposition.
- Quantitatively analyse the samples using your normal test method but use 90% (v/v) acetone as extraction solution.

- Report results as you would report to a client. This is the figure that will be used in all statistical analysis in the study report.
- For each analyte in each sample, report the expanded measurement uncertainty associated with your analytical result (e.g. 5.02 ± 0.51 µg/L).
- Participants are asked to analyse and report results in units of µg/L.

SAMPLE S1		SAMPLE S2	
Test	Approximate Conc. Range µg/L	Test	Approximate Conc. Range µg/L
chlorophyll a	<10	chlorophyll a	10-50
pheophytin a	NA	pheophytin a	NA

NA-not available

- Please send us the requested details regarding the test method and the basis of your uncertainty estimate.
- Return the completed results sheet by email (proficiency@measurement.gov.au).

3.9 Interim Report

An interim report was emailed to participants on 17 May 2021.

4 PARTICIPANT LABORATORY INFORMATION

4.1 Test Method Summaries

Summaries of test methods are transcribed in Table 1 and Table 2.

Table 1 Methodology

Lab. Code	Method Reference	Disruption Method	Extraction Time	Extraction Agent	Vol (mL)
1	Inhouse-based on APHA 10200H	grinding	24 hours	90% acetone	10
2	APHA10200-H	grinding		90% acetone	10
3	In-house (derived from APHA 10200 H)	Agitation at 75°C	5 min	90% Ethanol	10
4	ISO 1991	heating the sample at 75 degrees	5 minutes	ethanol	20
5	APHA (online edition) 10200 H	sonication	Overnight	90% acetone	
6	APHA 10200 H 23rd Ed.	grinding	1 min	90% acetone	10
7	APHA 10200H	sonication	10 minutes	90% acetone	10
8*	ISO 10260 (1992) for chlorophyll a and phaeophytin	Vortex at 1800rpm	60 seconds	96% ethanol	10
9	APHA 10200-H	grinding	no set time, just until ground up very well	90% acetone	10
10*	APHA 10200H	Shaking	3 minutes in 86 C- 88 C water bath	90% methanol	15
11	APHA 10200H Chlorophyll	tissue homogeniser	until filter was homogenised	90% acetone	extracts made up to 20
12	APHA Method 10200H			90% acetone	10

Lab. Code	Method Reference	Disruption Method	Extraction Time	Extraction Agent	Vol (mL)
13*	ISO 10260:1992 Rev 2017	Nil	24hr extraction in dark, in fridge @ 4°C	90% acetone	15
14	APHA 21st Edition, 2005, 10200H	grinding	2 Hours	Acetone	10
15	APHA 10200H	sonication	minimum 2 hours	90% acetone	5
16*	ISO 10260 (1992) for chlorophyll a and phaeophytin	Vortex @ 1800 rpm	60 seconds	96% Ethanol	10
17	APHA 10200 H 23rd Ed.	grinding	1 min	90% acetone	10
18*	APHA 10200-H	grinding	24 hours	90% acetone	10
19	APHA 10200H	sonication	1 minute	90% acetone	8
20	APHA 10200H	Sonicator	30 min	Acetone	20
21*	SCOR-UNESCO	sonication	25min	90% acetone	10
22	Standard Methods for the Examination of Water and Wastewater, APHA. Method 10200 H.	Shaking	1 min	90% acetone	20
23*	APHA 10200 H Chlorophyll (trichromatic method for result calculation)	sonication	2 x 10mins	90% acetone	10
24	APHA 10200 H	grinding	2 minutes	90% acetone	10
25*	APHA 10200H (Modified) 23rd ed.2017	sonication	20 Hours	90% acetone	10
26*	Standard Methods for the Examination of Water and Wastewater. APHA. 10200 H Chlorophyll	sonication	20 minutes	90% acetone	10
27	APHA Method 10200 H Chlorophyll	grinding	2 minutes grinding, steep 2 hours	90% acetone	8
28*	APHA 23rd Edition 10200H	grinding	approximately 1 minute	Acetone solution (mix of 90% acetone and saturated magnesium carbonate)	9
29	APHA 10200 H.2-b (Chlorophyll a)	grinding	12 hours	90% acetone	10
30	10200 H, APHA AWWA (2012)	grinding	20 secs in 5 sec pulses	90% acetone	20
31	APHA 10200H	grinding	1 minute	90% acetone	10
32	American Public Health Association (APHA) - Standard Methods for the Examination of Water and Wastewater, Method 10200 H.	grinding	2 hours	90% acetone	10

*Additional information in Table 2

4.2 Additional Method Information

Participants had the option to report additional information for each sample analysed. These are transcribed in Table 2.

Table 2 Additional Method information

Lab Code	Additional Information
8	This laboratory used 96% ethanol as the extraction solvent as it the solvent used for the routine method.
10	Samples were intact at the time of receipt. S1 filter had more loosen cellulose fibres than S2 filter, that could contribute to S1 background to be higher for spectrometry analysis, S1 was centrifuged little longer to avoid this interference. Extraction of analytes was trouble free, hence longer sonication or grinding was not required. Shaking was sufficient to recover pigments. Measurement technique for Pheophytin a: Spectrophotometer.
13	Samples S1 and S2: Please note our usual extraction method is to use 90% ethanol, cold extracted not 90% acetone as recommended. We used 90 % acetone just for this trial. Methodology: Magnesium carbonate was not used.
16	The laboratory used 96% ethanol as the solvent for this proficiency round (it is the solvent used for the routine method).
18	Methodology: Subtract the 750nm OD reading from the reading before (OD 664nm) and after acidification (OD 665nm). Using the corrected values calculate chlorophyll 'a' and pheophyton 'a' as follows: Chlorophyll 'a' ug/L = 26.7 (664b - 665a) x V1/ V2 x L Pheophyton 'a' = 26.7 [1.7 (665a) - 664b] x V1 / V2 x L Where V1 = Volume of extract (millilitres) V2 = Volume of sample (litres) L = Light path length 664b anf 665a = optical density of 90% acetone extract before and after acidification respectively.
21	Methodology: Based on trichromatic equations.
23	Methodology: Samples were sonicated for 10 mins at room temperature, frozen for 2 hrs, sonicated for another 10 mins at room temperature, centrifuged for 2 mins to clarify.
25	Sample S1: Chlorophyll a uncensored results of 0.6 ug/L; Pheophytin a uncensored result of 0.8 ug/L. Methodology: Chlorophyll a (g/m3) = (V_e/V_{sample} * 26.7) * ($A664_b$ - $A665_a$); Pheophytin a (g/m3) = (V_e/V_{sample} * 26.7) * (1.7 * $A665_a$ - $A664_b$); Where; V_e is the volume of extractant (10mL), V_{sample} is the volume filtered (1000mL), $A664_b$ is the absorbance at 664 before acidification, $A665_a$ is the absorbance at 665 after acidification.
26	Methodology: Chlorophyll a (mg/L) = [11.85(OD664nm-OD750nm) – 1.54(OD647nm-OD750nm) – 0.08(OD630nm-OD750nm)]*10mL/1L.
28	Methodology: Pheophytin calculated from chlorophyll a.

4.3 Instruments Used for Measurements

The instruments measurement methods reported by participants are presented in Appendix 6.

4.4 Basis of Participants' Measurement Uncertainty Estimates

Participants were requested to provide information about the basis of their uncertainty estimates. Those returned are transcribed in Table 3.

Table 3 Basis of Uncertainty Estimate

Lab. Code	Approach to Estimating MU	Information Sources for MU Estimation[a]		Guide Document for Estimating MU
		Precision	Method Bias	
1	Top Down - precision and estimates of the method and laboratory bias	Control Samples - RM		Armishaw 2002-3
2	Top Down - precision and estimates of the method and laboratory bias	Control Samples Duplicate Analysis Instrument Calibration	CRM Instrument Calibration Laboratory Bias from PT Studies	ISO/GUM

Lab. Code	Approach to Estimating MU	Information Sources for MU Estimation[a]		Guide Document for Estimating MU
		Precision	Method Bias	
3	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis Instrument Calibration	Instrument Calibration Matrix Effects Laboratory Bias from PT Studies	Eurachem/CITAC Guide
4	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis Instrument Calibration	Laboratory Bias from PT Studies	Eurachem/CITAC Guide
5	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis		**technical guide
6	Standard deviation of replicate analyses multiplied by 2 or 3	Control samples - RM Duplicate Analysis	Matrix Effects	Nata Technical Note 33
7	Bottom Up (ISO/GUM, fish bone/ cause and effect diagram)	Control Samples - SS Duplicate Analysis Instrument Calibration	CRM Instrument Calibration Matrix Effects Laboratory Bias from PT Studies Recoveries of SS Standard Purity	NMI Uncertainty Course
8	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis Instrument Calibration	Instrument Calibration Laboratory Bias from PT Studies	Eurachem/CITAC Guide
9	Top Down - precision and estimates of the method and laboratory bias	Control Samples - RM Duplicate Analysis Instrument Calibration	Instrument Calibration Matrix Effects	Eurachem/CITAC Guide
10*	Standard deviation of replicate analyses multiplied by 2 or 3	Control Samples Duplicate Analysis Instrument Calibration	Instrument Calibration	Inhouse
11	Bottom Up (ISO/GUM, fish bone/ cause and effect diagram)	Control Samples - CRM Duplicate Analysis	CRM Instrument Calibration	ISO/GUM
12	Top Down - precision and estimates of the method and laboratory bias	Control Samples - RM		NATA Tech Note
13	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis Instrument Calibration	Instrument Calibration Laboratory Bias from PT Studies	NATA Techical Note 33
14	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis Instrument Calibration	Instrument Calibration	Eurachem/CITAC Guide
15	Standard deviation of replicate analyses multiplied by 2 or 3	Control Samples - RM Duplicate Analysis		Nordtest Report TR537
16	Top Down - reproducibility (standard deviation) from PT studies used directly	Duplicate Analysis	Instrument Calibration Laboratory Bias from PT Studies	Eurachem/CITAC Guide
17	Standard deviation of replicate analyses multiplied by 2 or 3	Control samples - RM Duplicate Analysis	Matrix Effects	Nata Technical Note 33
18	Bottom Up (ISO/GUM, fish bone/ cause and effect diagram)	Control samples - RM Duplicate Analysis Instrument Calibration	Instrument Calibration Laboratory Bias from PT Studies	Eurachem/CITAC Guide
19	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis	CRM	old NATA tech note

Lab. Code	Approach to Estimating MU	Information Sources for MU Estimation[a]		Guide Document for Estimating MU
		Precision	Method Bias	
20	Top Down - precision and estimates of the method and laboratory bias	Control samples - RM Instrument Calibration	Instrument Calibration	Eurolab Technical Report No1/2007
21	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis	Instrument Calibration	NMI Uncertainty Course
22	Standard deviation of replicate analyses multiplied by 2 or 3	Duplicate Analysis	Laboratory Bias from PT Studies	ISO/GUM
23	Standard deviation of replicate analyses multiplied by 2 or 3	Duplicate Analysis	Instrument Calibration	Paul Armishaw, Australian Government Analytical Laboratories, Western Australia, AGAL Public Interest Program, public interest report series number 2002-3 (June 2002). Estimating measurement uncertainty in an afternoon: a case study.
24	Bottom Up (ISO/GUM, fish bone/ cause and effect diagram)			Eurachem/CITAC Guide
25*	Standard deviation of replicate analyses multiplied by 2 or 3	Duplicate Analysis	Instrument Calibration	**technical guide
26	Standard deviation of replicate analyses multiplied by 2 or 3	Control Samples - RM Duplicate Analysis	Laboratory Bias from PT Studies	ISO/GUM
27	Bottom Up (ISO/GUM, fish bone/ cause and effect diagram)	Control Samples - SS Duplicate Analysis		NMI Uncertainty Course
28	Top Down - precision and estimates of the method and laboratory bias	Duplicate Analysis		**technical guide
29*		Instrument Calibration	Instrument Calibration	
30	Top Down - precision and estimates of the method and laboratory bias	Control samples - CRM	CRM	ISO/GUM
31*	Professional judgment	Instrument Calibration	Instrument Calibration	In-house method
32	Top Down - precision and estimates of the method and laboratory bias	Control Samples - CRM Duplicate Analysis	CRM	

[a] RM = Reference Material, CRM = Certified Reference Material, SS =Spiked Samples. *Additional information in Table 4. **redacted to preserve confidentiality.

4.5 Additional Uncertainty Information

Participants had the option to report additional information for each sample analysed. These are transcribed in Table 4.

Table 4 Additional Uncertainty Information

Lab Code	Additional Information
10	Uncertainty calculated from proven routine chlorophyll method using duplicate analysis among trained analysts.
25	UoM is based on ISO 17025, Standard Specific Criteria and EURACHEM/CITAC Guide.
29	Measurement Uncertainty not calculated.
31	MU calculations were based on EURACHEM/CITAC Guide, ISO/GUM and NATA Technical Note 33.

4.6 Participant Comments on this PT Study or Suggestions for Future Studies

The study co-ordinator welcomes comments or suggestions from participants about this study or possible future studies. Such feedback may be useful in improving future studies. Participants' comments are reproduced in Table 5.

Table 5 Participants' Comments

Participants' Comments	Study Co-ordinator's Response
Difficult to open sample. Ice had partially melted during transit around the outside of the sample, then when refrozen prior to testing the next day the water had created a solid block of ice around the sample.	Freezing is a preservation procedure used for many tests. The procedure used by most laboratories is to defrost the sample overnight at 4ºC.
Why do the instruction specify "Quantitatively analyse the samples using your normal test method but use 90% (v/v) acetone as extraction solution" when we do not use 90% acetone as extraction solution. This is a deviation from our method.	Measurement of chlorophyll a in water is an empirical measurement – where the method of extraction defines the measurand. With testing laboratories each using different extraction reagents at different concentrations and in different combinations, each could be considered to be measuring a different measurand that is their version of chlorophyll a in water. This lack of uniformity in the procedures can make it difficult to compare participants' results. The participating laboratories were asked to analyse the sample using their normal measurement technique but with 90% acetone as the extraction solution (the most popular method used for this test).

5 PRESENTATION OF RESULTS AND STATISTICAL ANALYSIS

5.1 Results Summary

Participant results are listed in Tables 6 to 9 with resultant summary statistics: robust average, median, maximum, minimum, robust standard deviation (SD_{rob}) and robust coefficient of variation (CV_{rob}). Bar charts of results and performance scores are presented in Figures 2 to 5.

An example chart with interpretation guide is shown in Figure 1.

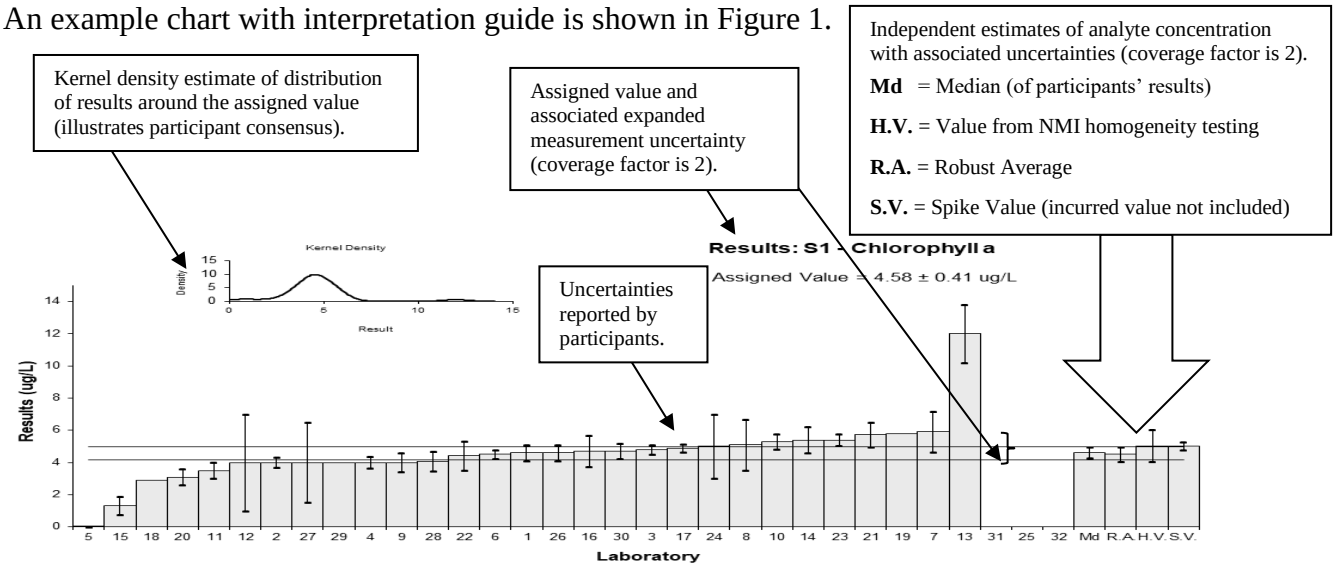


Figure 1 Guide to Presentation of Results

5.2 Outliers and Extreme Outliers

Outliers were results less than 50% and greater than 150% of the robust average and were removed before assigned value calculation. Extreme outliers were obvious blunders, such as those with incorrect units, decimal errors, or results from a different proficiency test item (gross errors) and were removed for calculation of summary statistics.[3, 4]

5.3 Assigned Value

An example of an assigned value calculation using data from the present study is given in Appendix 2. The assigned value is defined as: 'the value attributed to a particular property of a proficiency test item.'[1] In this study, the property is the mass concentration of analyte. Assigned values were the robust average of participants' results; the expanded uncertainties were estimated from the associated robust standard deviations. [4, 5]

5.4 Robust Average

The robust averages and associated expanded measurement uncertainties were calculated using the procedure described in 'Statistical methods for use in proficiency testing by interlaboratory comparisons, ISO13528:2015(E)'.[5]

5.5 Robust Between-Laboratory Coefficient of Variation

The robust between-laboratory coefficient of variation (robust CV) is a measure of the variability of participants' results and was calculated using the procedure described in ISO 13528:2015(E).[5]

5.6 Target Standard Deviation

The target standard deviation (σ) is the product of the assigned value *(X)* and the performance coefficient of variation (PCV). This value is used for calculation of participant z-score and provides scaling for laboratory deviation from the assigned value.

$\sigma = (X) * PCV$ Equation 1

It is important to note that the PCV is a fixed value and is not the standard deviation of participants' results. The fixed value set for PCV is based on the existing regulation, the acceptance criteria indicated by the methods, the matrix, the concentration level of analyte and/or on experience from previous studies. It is backed up by mathematical models such as Thompson Horwitz equation.[6]

5.7 z-Score

An example of z-score calculation using data from the present study is given in Appendix 2.

For each participant's result a z-score is calculated according to Equation 2 below:

$$z = \frac{(\chi - X)}{\sigma}$$ Equation 2

where:

z is z-score
χ is participant's result;
X is the assigned value
σ is the target standard deviation

A z-score with absolute value (|z|):

- $|z| \leq 2.0$ is satisfactory;
- $2.0 < |z| < 3.0$ is questionable;
- $|z| \geq 3.0$ is unsatisfactory.

5.8 E_n-Score

An example of E_n-score calculation using data from the present study is given in Appendix 2.

The E_n-score is complementary to the z-score in assessment of laboratory performance. E_n-score includes measurement uncertainty and is calculated according to Equation 3 below:

$$E_n = \frac{(\chi - X)}{\sqrt{U_\chi^2 + U_X^2}}$$ Equation 3

where:

E_n is E_n-score
χ is a participant's result;
X is the assigned value
U_χ is the expanded uncertainty of the participant's result
U_X is the expanded uncertainty of the assigned value

An E_n-score with absolute value ($|E_n|$):

- $|E_n| \leq 1.0$ is satisfactory;
- $|E_n| > 1.0$ is unsatisfactory.

5.9 Traceability and Measurement Uncertainty

Laboratories accredited to ISO/IEC Standard 17025:2018[7] must establish and demonstrate the traceability and measurement uncertainty associated with their test results. Guidelines for quantifying uncertainty in analytical measurement are described in the Eurachem/CITAC Guide.[8]

6 TABLES AND FIGURES

Table 6

Sample Details

Sample No.	S1
Matrix.	Water
Analyte.	Chlorophyll a
Units	ug/L

Participant Results

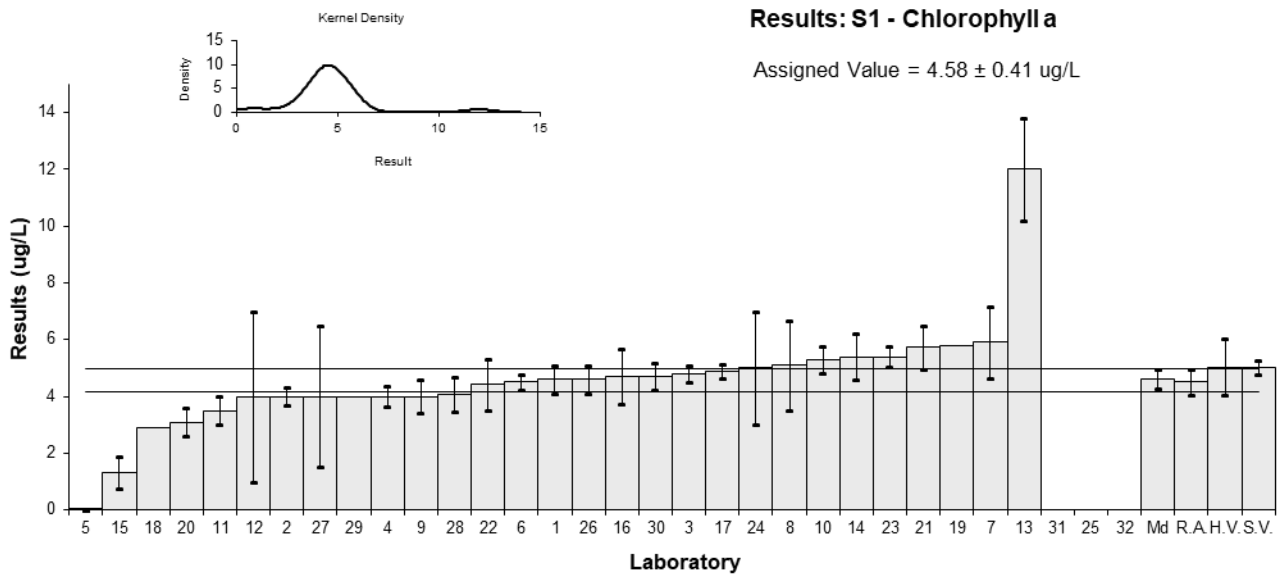
Lab Code	Result	Uncertainty	z-Score	E_n-Score
1	4.6	0.5	0.02	0.03
2	4	0.33	-0.63	-1.10
3	4.8	0.3	0.24	0.43
4	4.0	0.36	-0.63	-1.06
5	0.00396	0.0006	-5.00	-11.16
6	4.5	0.25	-0.09	-0.17
7	5.9	1.25	1.44	1.00
8	5.09	1.57	0.56	0.31
9	4	0.6	-0.63	-0.80
10	5.3	0.48	0.79	1.14
11	3.5	0.5	-1.18	-1.67
12	4	3	-0.63	-0.19
13	12	1.8	8.10	4.02
14	5.4	0.8	0.90	0.91
15	1.3	0.559	-3.58	-4.73
16	4.69	0.98	0.12	0.10
17	4.9	0.25	0.35	0.67
18	2.9	NR	-1.83	-4.10
19	5.8	NR	1.33	2.98
20	3.085	0.5	-1.63	-2.31
21	5.72	0.77	1.24	1.31
22	4.41	0.9	-0.19	-0.17
23	5.4	0.35	0.90	1.52
24	5	2	0.46	0.21
25	<3.0	2.1		
26	4.6	0.5	0.02	0.03
27	4	2.5	-0.63	-0.23
28	4.09	0.61	-0.53	-0.67
29	4	NR	-0.63	-1.41
30	4.7	0.47	0.13	0.19
31	<2	2		
32	<5	1		

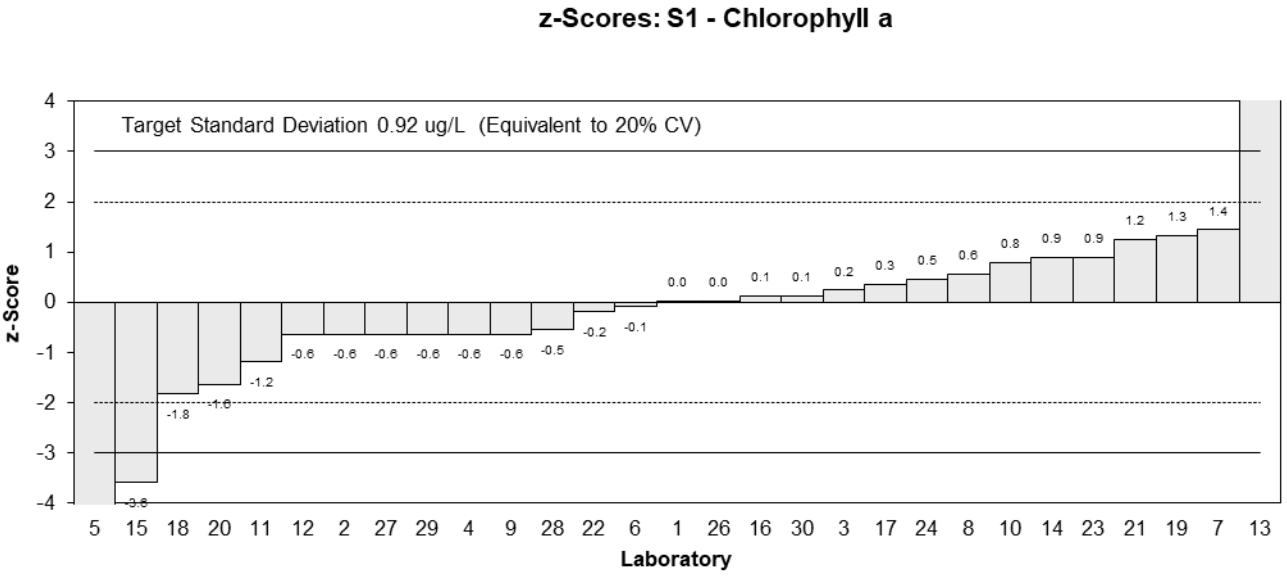
Statistics*

Assigned Value**	4.58	0.41
Spike	5.00	0.25
Homogeneity Value	5.0	1.0
Robust Average	4.57	0.44
Median	4.60	0.34
Mean	4.70	
N	28	
Max.	12	
Min.	1.3	

Robust SD	0.94
Robust CV	21%

*Laboratory 5 removed from statistical calculation (gross error).
**Robust Average excluding laboratories 13 and 15.


Kernel Density
Density
Result
Results: S1 - Chlorophyll a
Assigned Value = 4.58 ± 0.41 ug/L
Results (ug/L)
Laboratory
5 15 18 20 11 12 2 27 29 4 9 28 22 6 1 26 16 30 3 17 24 8 10 14 23 21 19 7 13 31 25 32 Md R.A. H.V. S.V.


z-Scores: S1 - Chlorophyll a
Target Standard Deviation 0.92 ug/L (Equivalent to 20% CV)
z-Score
Laboratory
5 15 18 20 11 12 2 27 29 4 9 28 22 6 1 26 16 30 3 17 24 8 10 14 23 21 19 7 13

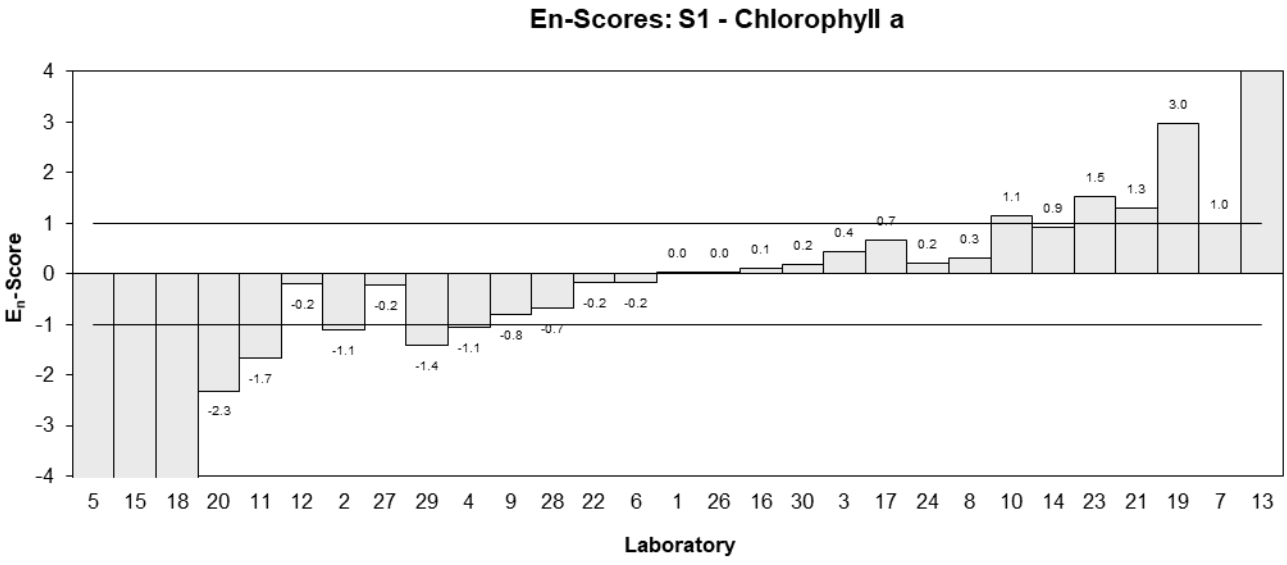

En-Scores: S1 - Chlorophyll a
En-Score
Laboratory
5 15 18 20 11 12 2 27 29 4 9 28 22 6 1 26 16 30 3 17 24 8 10 14 23 21 19 7 13

Figure 2

Table 7

Sample Details

Sample No.	S1
Matrix.	Water
Analyte.	Pheophytin a
Units	ug/L

Participant Results

Lab Code	Result	Uncertainty
1	1.0	0.5
2	<1	0.2
3	NR	NR
4	1.2	0.19
5	<0.004	NR
6	<2.0	0.26
7	NT	NT
8	0.57	0.26
9	1	0.1
10	7.94	NR
11	NT	NT
12	NR	NR
13	<2	NR
14	<0.5	NR
15	NR	0.559
16	0.74	0.24
17	<2.0	0.26
18	2.8	NR
19	NR	NR
20	NR	NR
21	0.64	0.27
22	1.31	0.3
23	NT	NT
24	NT	NT
25	<3.0	2.1
26	NR	NR
27	1	1
28	1.13	0.17
29	3.9	NR
30	NR	NR
31	9.28	1.2
32	<5	1

Statistics

Assigned Value	Not Set	
Spike	Not Spiked	
Robust Average	1.51	0.98
Median	1.00	0.30
Mean	2.50	
N	13	
Max.	9.28	
Min.	0.57	
Robust SD	1.6	
Robust CV	88%	

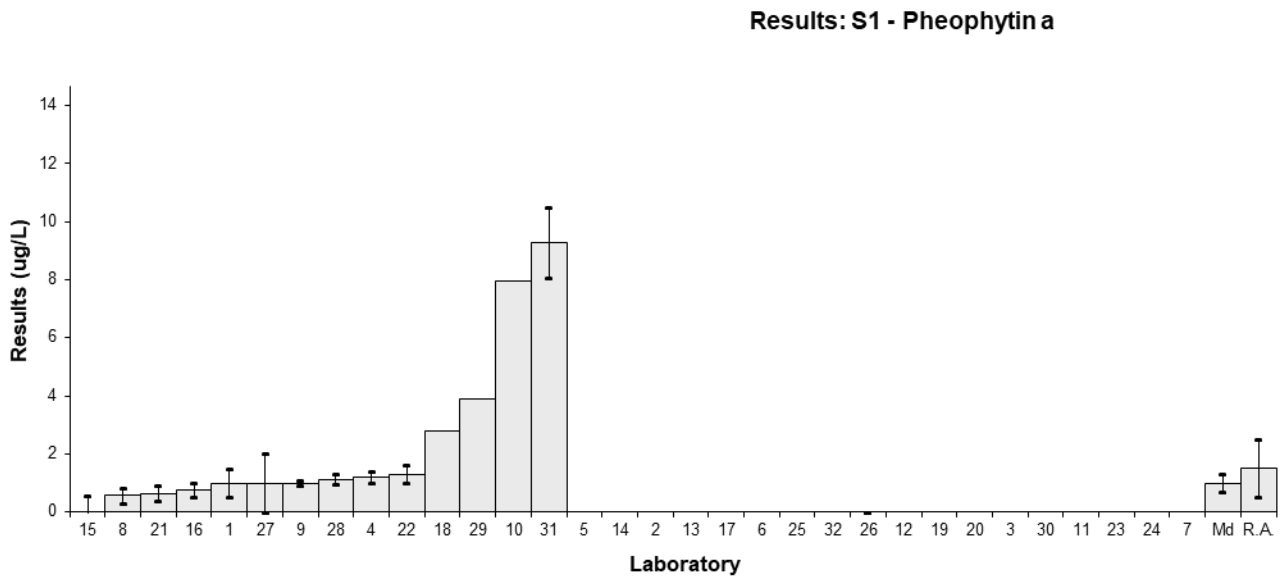

Results: S1 - Pheophytin a
Results (ug/L)
0
2
4
6
8
10
12
14
15 8 21 16 1 27 9 28 4 22 18 29 10 31 5 14 2 13 17 6 25 32 26 12 19 20 3 30 11 23 24 7 Md R.A.
Laboratory

Figure 3

Table 8

Sample Details

Sample No.	S2
Matrix.	Water
Analyte.	Chlorophyll a
Units	ug/L

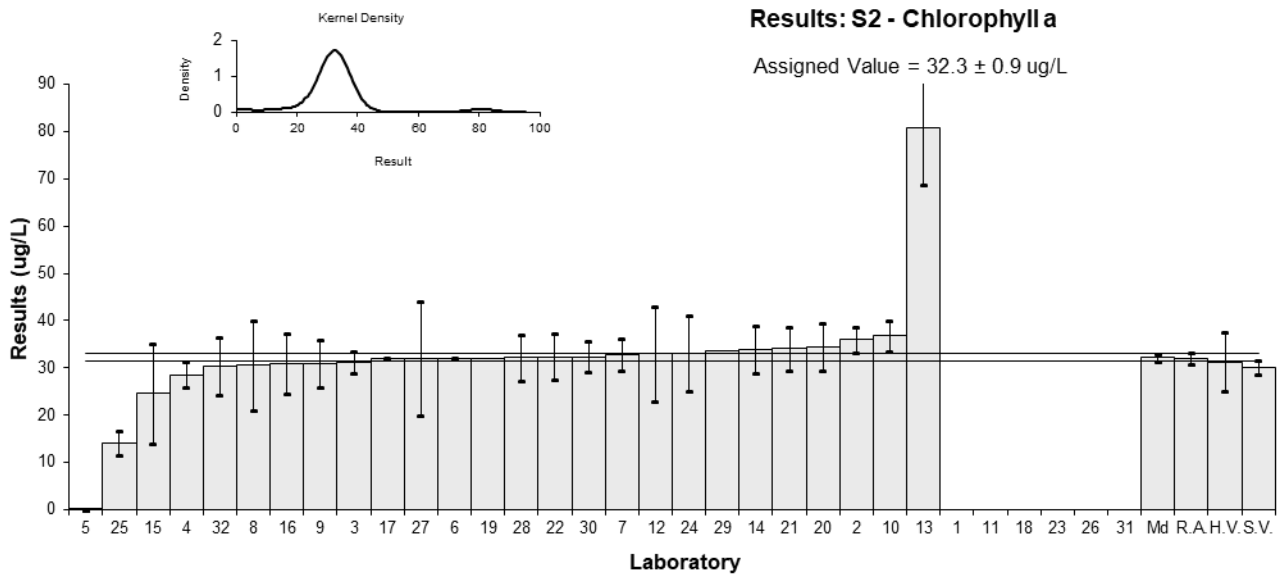
Participant Results

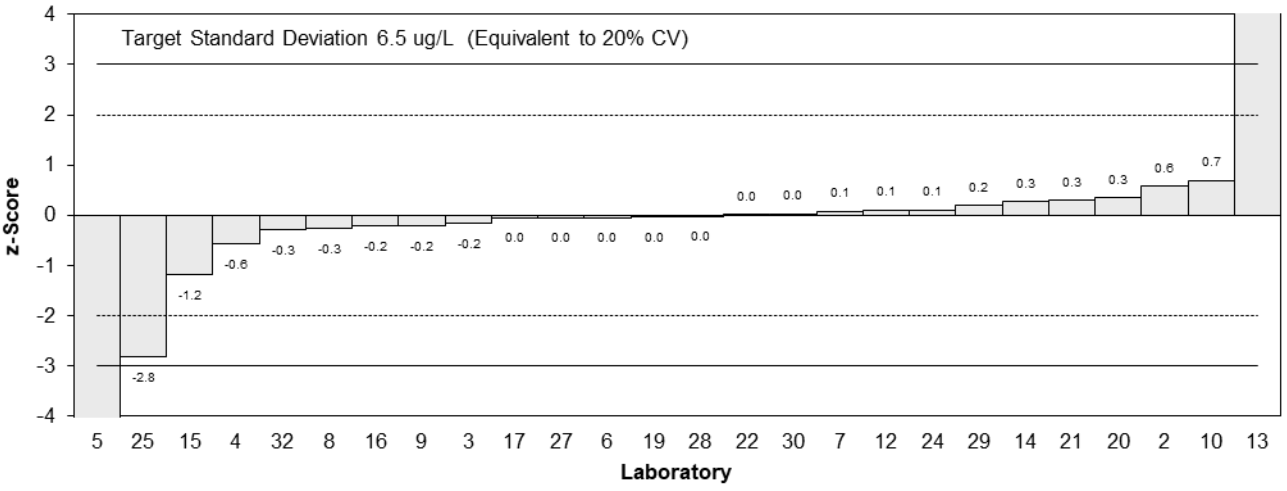
Lab Code	Result	Uncertainty	z-Score	E_n-Score
1	NT	NT		
2	36	2.66	0.57	1.32
3	31.2	2.4	-0.17	-0.43
4	28.6	2.6	-0.57	-1.34
5	0.027	0.004	-5.00	-35.86
6	32	0.25	-0.05	-0.32
7	32.8	3.3	0.08	0.15
8	30.56	9.44	-0.27	-0.18
9	31	5	-0.20	-0.26
10	36.8	3.31	0.70	1.31
11	NT	NT		
12	33	10	0.11	0.07
13	80.7	12.1	7.49	3.99
14	34	5	0.26	0.33
15	24.6	10.58	-1.19	-0.73
16	30.93	6.43	-0.21	-0.21
17	32	0.25	-0.05	-0.32
18	NT	NT		
19	32.1	NR	-0.03	-0.22
20	34.5	5	0.34	0.43
21	34.16	4.6	0.29	0.40
22	32.31	4.9	0.00	0.00
23	NT	NT		
24	33	8	0.11	0.09
25	14.1	2.6	-2.82	-6.61
26	NT	NT		
27	32	12	-0.05	-0.02
28	32.2	4.83	-0.02	-0.02
29	33.5	NR	0.19	1.33
30	32.4	3.24	0.02	0.03
31	NT	NT		
32	30.4	6.09	-0.29	-0.31

Statistics*

Assigned Value**	32.3	0.9
Spike	30.0	1.5
Homogeneity Value	31.3	6.3
Robust Average	32.3	1.1
Median	32.2	0.8
Mean	33.4	
N	25	
Max.	80.7	
Min.	14.1	
Robust SD	2.2	
Robust CV	6.9%	

*Laboratory 5 removed from statistical calculation.
**Robust Average excluding laboratories 13 and 25.


Kernel Density
Density
Result
Results: S2 - Chlorophyll a
Assigned Value = 32.3 ± 0.9 ug/L
Results (ug/L)
Laboratory
5 25 15 4 32 8 16 9 3 17 27 6 19 28 22 30 7 12 24 29 14 21 20 2 10 13 1 11 18 23 26 31 Md R.A. H.V. S.V.


z-Scores: S2 - Chlorophyll a
Target Standard Deviation 6.5 ug/L (Equivalent to 20% CV)
z-Score
Laboratory
5 25 15 4 32 8 16 9 3 17 27 6 19 28 22 30 7 12 24 29 14 21 20 2 10 13

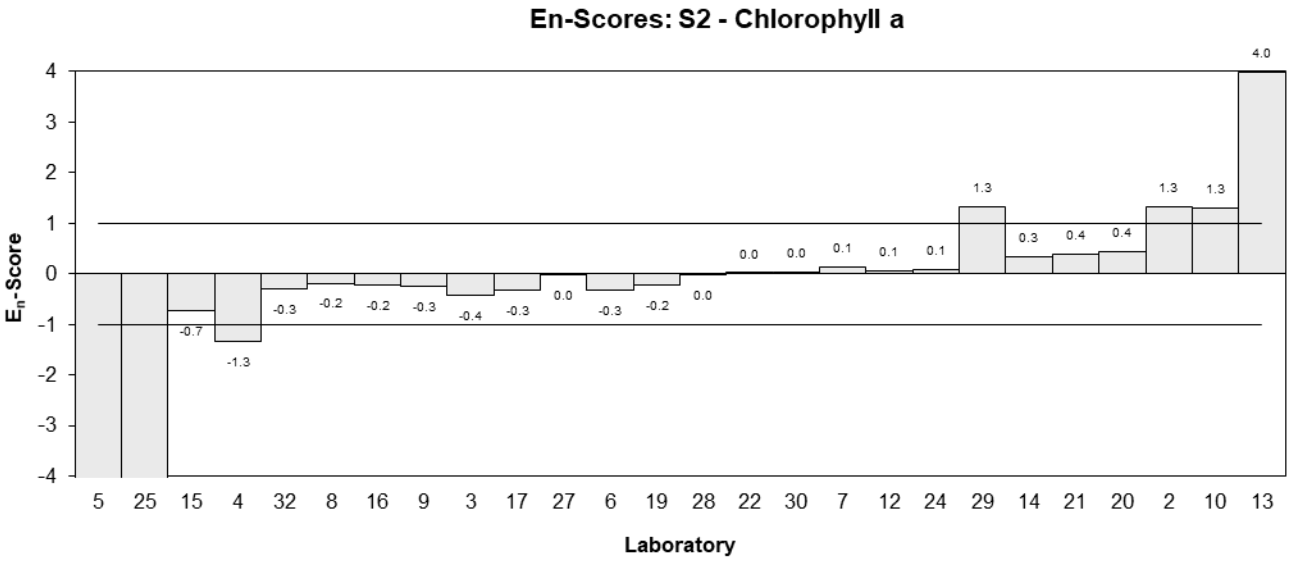

En-Scores: S2 - Chlorophyll a
En-Score
Laboratory
5 25 15 4 32 8 16 9 3 17 27 6 19 28 22 30 7 12 24 29 14 21 20 2 10 13

Figure 4

Table 9

Sample Details

Sample No.	S2
Matrix.	Water
Analyte.	Pheophytin a
Units	ug/L

Participant Results

Lab Code	Result	Uncertainty
1	NT	NT
2	<1	0.2
3	NR	NR
4	3.6	0.58
5	<0.004	NR
6	<2.0	0.26
7	NT	NT
8	2.60	1.17
9	<1	NR
10	13.8	NR
11	NT	NT
12	NR	NR
13	<2	NR
14	<0.5	NR
15	NR	10.58
16	3.54	1.14
17	<2.0	0.26
18	NT	NT
19	NR	NR
20	NR	NR
21	NR	NR
22	<1	NR
23	NT	NT
24	NT	NT
25	3.0	0.33
26	NT	NT
27	<1	NR
28	NR	NR
29	1.4	NR
30	1.1	0.11
31	NT	NT
32	5.45	1.09

Statistics

Assigned Value	Not Set	
Spike	Not Spiked	
Robust Average	2.7	1.9
Median	2.8	1.6
Mean	3.45	
N	8	
Max.	13.8	
Min.	1.1	
Robust SD	2.4	
Robust CV	90%	

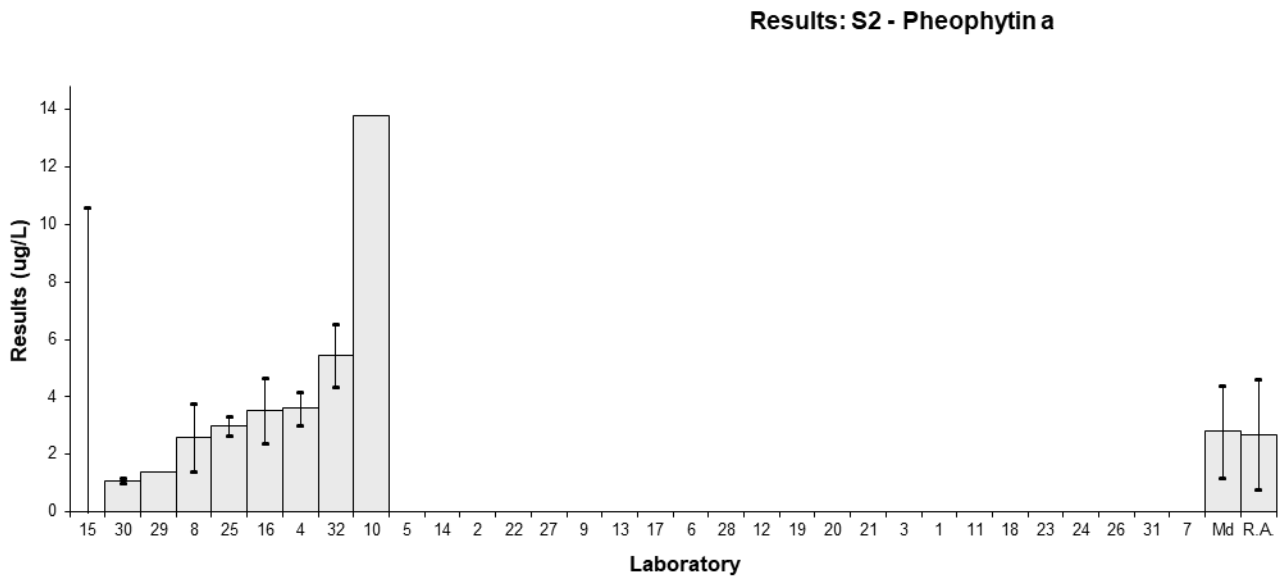

Results: S2 - Pheophytin a
Results (ug/L)
0
2
4
6
8
10
12
14
15 30 29 8 25 16 4 32 10 5 14 2 22 27 9 13 17 6 28 12 19 20 21 3 1 11 18 23 24 26 31 7 Md R.A.
Laboratory

Figure 5

7 DISCUSSION OF RESULTS

7.1 Assigned Value

Assigned values for chlorophyll a in the study samples were the robust averages of participants' results. The robust averages and their associated expanded uncertainties were calculated using the procedures described in ISO 13528:2015(E). Results less than 50% and more than 150% of the robust average were removed before calculation of the assigned value.[5] Appendix 2 sets out the calculation for the assigned value of chlorophyll a in Samples S2 and its associated uncertainty.

No assigned value was set for pheophytin a in water. This analyte was introduced only as a measure of chlorophyll a degradation.

Traceability The assigned value is not traceable to any external reference; it is traceable to the consensus of participants' results deriving from a variety of measurement methods and (presumably) a variety of calibrators. So although expressed in SI units, the metrological traceability of the assigned values has not been established.

7.2 Measurement Uncertainty Reported by Participants

Participants were asked to report an estimate of the expanded measurement uncertainty associated with their results. All but 10 numerical results were reported with an expanded measurement uncertainty, indicating that most laboratories have addressed this requirement of ISO 17025.[7] The participants used a wide variety of procedures to estimate the expanded measurement uncertainty. These are presented in Table 3.

Approaches to estimating measurement uncertainty include: standard deviation of replicate analysis, Horwitz formula, long term reproducibility, professional judgement, bottom up approach, top down approach using precision and estimates of method and laboratory bias, and top down approach using only the reproducibility from inter-laboratory comparisons studies.[9 – 14]

Proficiency tests allow a check of participants' uncertainty estimates. Results and the expanded measurement uncertainties are presented in the bar charts for each analyte (Figure 2 to 5). In this study, in some cases, the reported expanded measurement uncertainty has been over-estimated (e.g. laboratories 12 and 27 for chlorophyll a in S1) or under-estimated (e.g. laboratory 23 for chlorophyll a in S1). As a simple rule of thumb, when the uncertainty estimate is smaller than the uncertainty of the assigned value or larger than the uncertainty of the assigned value plus twice the target standard deviation then this should be reviewed as suspect.

Laboratories 2, 6, 17, 25, 31 and 32 attached estimates of the expanded measurement uncertainty for results reported as less than their limit of detection. An estimate of uncertainty expressed as a value cannot be attached to a result expressed as a range.[8]

In some cases the results were reported with an inappropriate number of significant figures. The recommended format is to write uncertainty to no more than two significant figures and then to write the result with the corresponding number of decimal places. For example, instead of 1.3 ± 0.559 µg/L, it is better to report 1.30 ± 0.56 µg/L or instead of 3.085 ± 0.5 µg/L, it is better to report 3.1 ± 0.5 µg/L.[8]

7.3 z-Score

The z-score compares the participant's deviation from the assigned value with the target standard deviation set for proficiency assessment.

The target standard deviation defines satisfactory performance in a proficiency test. Target standard deviation equivalent to 20% PCV were used to calculate z-scores. Unlike the standard deviation based on between laboratories CV, setting the target standard deviation as a realistic, set value enables z-scores to be used as fixed reference value points for assessment of laboratory performance, independent of group performance.

The between laboratory coefficient of variation predicted by the Thompson equation[6] and the between laboratory coefficient of variation resulted in this study are presented for comparison in Table 10.

Table 10 Between Laboratory CV of this Study, Thompson CV and Set Target CV

Sample	Analyte	Assigned value (µg/L)	Between Laboratories CV*	Thompson CV	Target SD (as PCV)
S1	chlorophyll a	4.58	18%	22%	20%
S2	chlorophyll a	32.3	5.5%	22%	20%

*Robust between Laboratories CV with outliers removed

The dispersal of participants' z-scores is presented in Figure 6. Of 55 results for which z-scores were calculated, 49 (89%) returned a satisfactory score of $|z| \leq 2.0$ and 1 (2%) were questionable of $2.0 < |z| \leq 3.0$.

Participants with both z-scores larger than 2 or smaller than -2 should check for laboratory bias.

7.4 E_n-Score

E_n-score should be interpreted only in conjunction with z-scores. The E_n-score indicates how closely a result agrees with the assigned value taking into account the respective uncertainties. An unsatisfactory E_n score for an analyte can either be caused by an inappropriate measurement, an inappropriate estimation of measurement uncertainty, or both.

The dispersal of participants' E_n-scores is graphically presented in Figure 7. Where a laboratory did not report an expanded uncertainty with a result, an expanded uncertainty of zero (0) was used to calculate the E_n-score. Of 55 results for which E_n-scores were calculated, 34 (62%) returned a satisfactory score of $|E_n| \leq 1.0$ indicating agreement of the participants' results with the assigned values within their respective expanded measurement uncertainties.

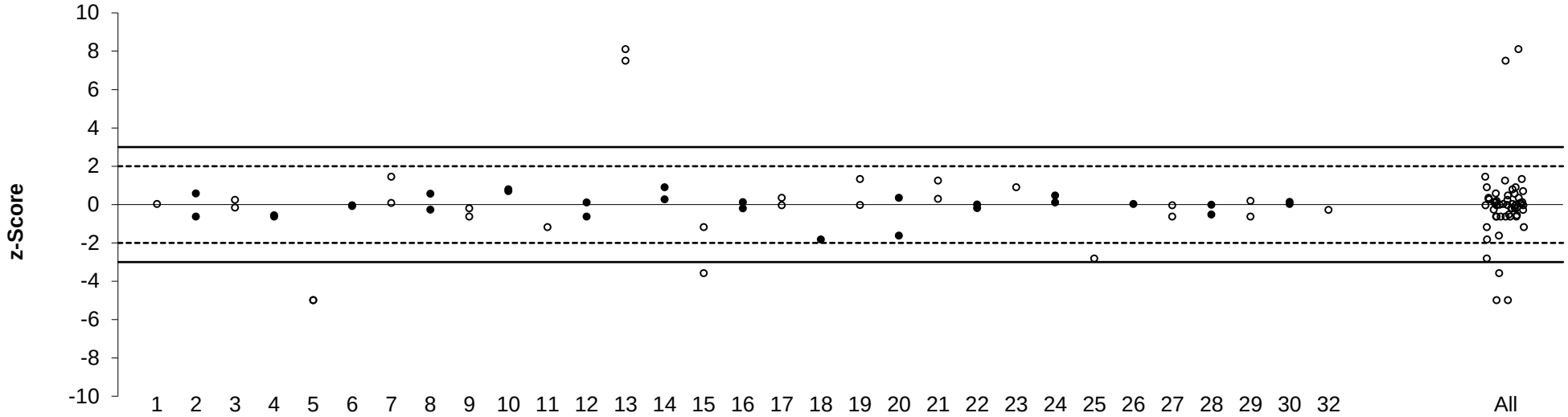
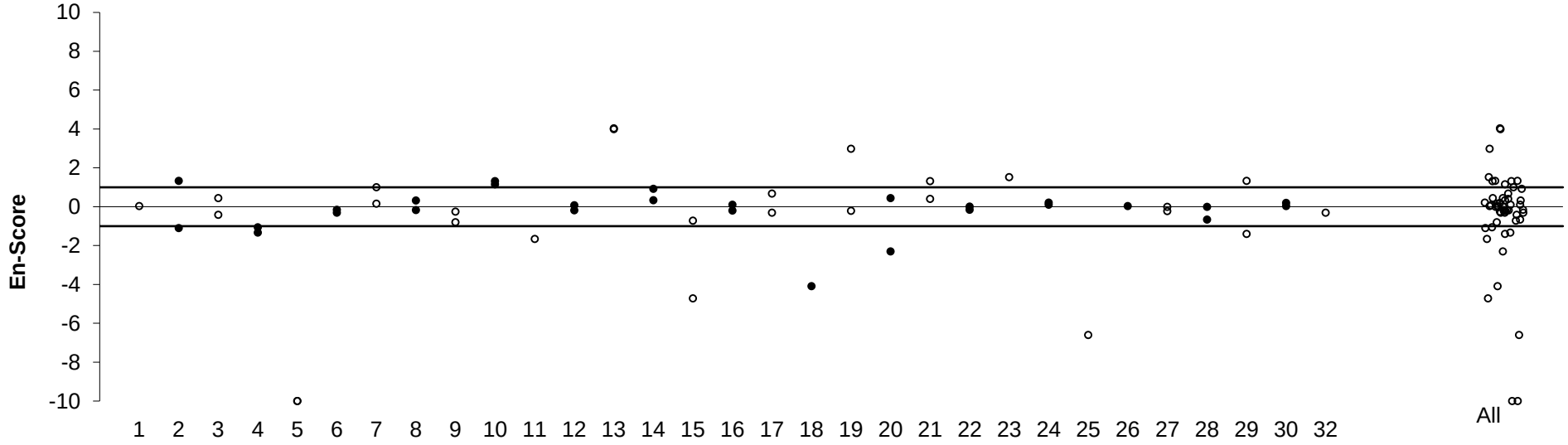


Figure 6 z-Score Dispersal by Laboratory



Scores of <-10 have been plotted as -10.

Figure 7 E_n-Score Dispersal by Laboratory

Table 11 Summary of Participants' Results and of Their Performance

Lab. Code	S1-Chlorophyll a (µg/L)	S2-Chlorophyll a (µg/L)
A.V.	4.58	32.3
H.V.	5.03	31.3
1	4.6	NT
2	4	36
3	4.8	31.2
4	4.0	28.6
5	0.00396	0.027
6	4.5	32
7	5.9	32.8
8	5.09	30.56
9	4	31
10	5.3	36.8
11	3.5	NT
12	4	33
13	12	80.7
14	5.4	34
15	1.3	24.6
16	4.69	30.93
17	4.9	32
18	2.9	NT
19	5.8	32.1
20	3.085	34.5
21	5.72	34.16
22	4.41	32.31
23	5.4	NT
24	5	33
25	<3.0	14.1
26	4.6	NT
27	4	32
28	4.09	32.2
29	4	33.5
30	4.7	32.4
31	<2	NT
32	<5	30.4

Shaded cells are results which returned a questionable or unsatisfactory z-score. A.V. = Assigned Value, H.V. = Homogeneity Value.

7.5 Participants' Results and Analytical Methods

A summary of participants' results and performance in the two study samples is presented in Table 11 and Figures 6 and 8.

Pheophytin a results were too variable and no assigned value could be set for this test. The quantitative conversion of chlorophyll a in pheonhytin a depends on many different factors such as: pigment concentrations and composition of the sample, acidic concentration, reaction time and rate. The end point of this conversion reaction is not defined and variations in

analytical procedure used by participants may explain the variation between the reported results for this test.[15]

Chlorophyll a Lab 5 results were 1000 times lower than the assigned value, which may be due to reporting results in the wrong units. The results from this laboratory were not included in the analysis of the extraction methods and instrumental techniques employed by participants.

Laboratory 13 may need to check their sample preparation, dilution factors and/or standard preparation procedure. Their reported results were higher than the assigned value by almost the same factor, approximatively 2.5.

Chlorophyll a concentration in Sample S1 was six times lower than in Sample S2. The results reported for Chlorophyll a in S1 were three times more variable than in S2 (Table 10 and Figure 8).

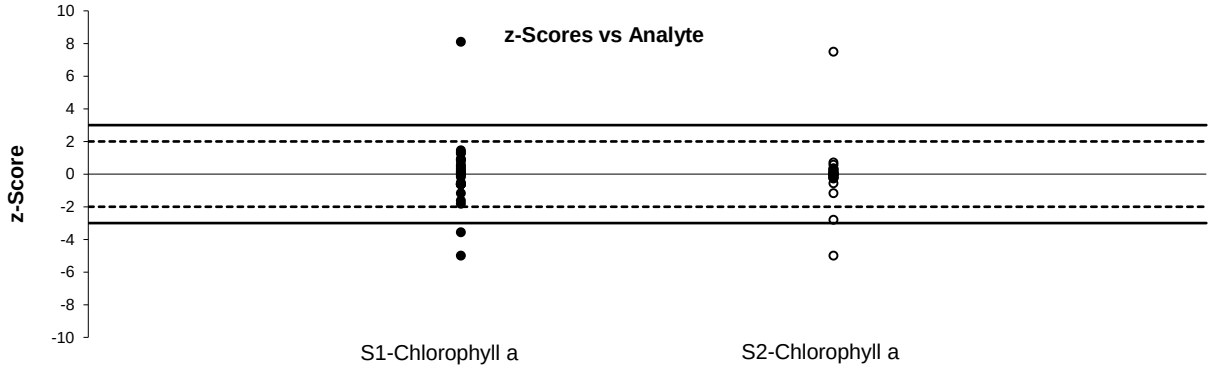


Figure 8 z-Scores Dispersal by Analyte

The methods used by participants for chlorophyll a analysis in the present study are presented in Tables 1 and 2 while the measurement techniques used are presented in Appendix 6.

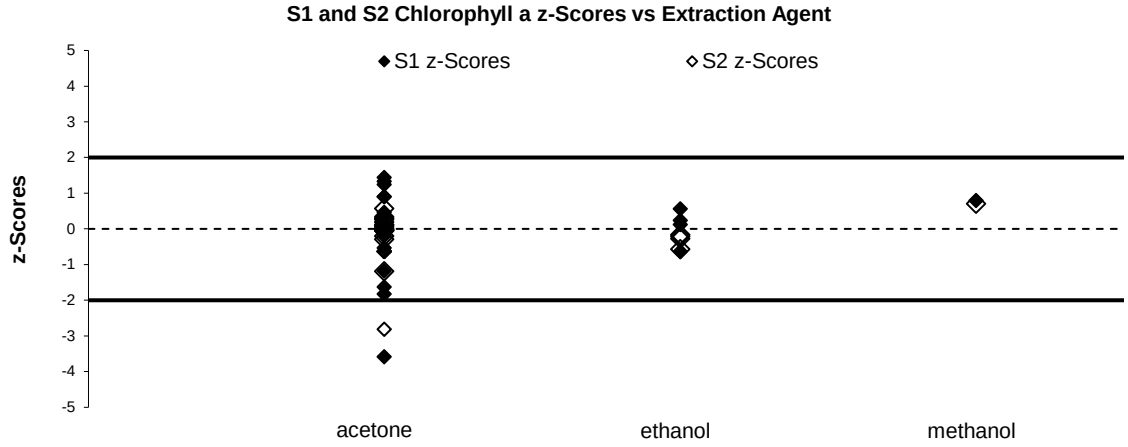
Extraction Agent

Measurement of chlorophyll a in water is an empirical measurement, where the method of extraction defines the measurand. With testing laboratories each using different extraction reagents (acetone, ethanol, methanol or acetone-dimethyl sulphoxide mixture) at different concentrations and in different combinations, each could be considered to be measuring a different measurand that is their version of chlorophyll a in water. This lack of uniformity in the procedures can make it difficult to compare participants' results. In the present study, participants were requested to analyse the samples using their normal test method but with a specified extraction solution of 90% (v/v) acetone.

All but five participants used 90% (v/v) acetone as instructed. Four laboratories used 90% or 96% ethanol and one used methanol.

One participant reported using a mix of 90% acetone and saturated $MgCO_3$ solution. The addition of a small quantity of $MgCO_3$ is often recommended to prevent acidity which would cause the breakdown of chlorophyll a to pheophytin a. In addition a more effective retention of the algae on the filter was reported, however previous studies have found a decrease in chlorophyll a values when filters containing $MgCO_3$ were stored. This was attributed to the formation of aggregates of algae and $MgCO_3$ which are difficult to dissolve. Thus the benefits of the addition of $MgCO_3$ appear to be outweighed by the problems with its use, particularly when chlorophyll degradation products are to be measured.[15]

Plots of participants' results versus extraction agent are presented in Figure 9. There is a relatively good agreement between the results produced by acetone extraction, ethanol extraction and methanol extraction.



z-Scores from laboratory 5 were excluded (extreme outlier).

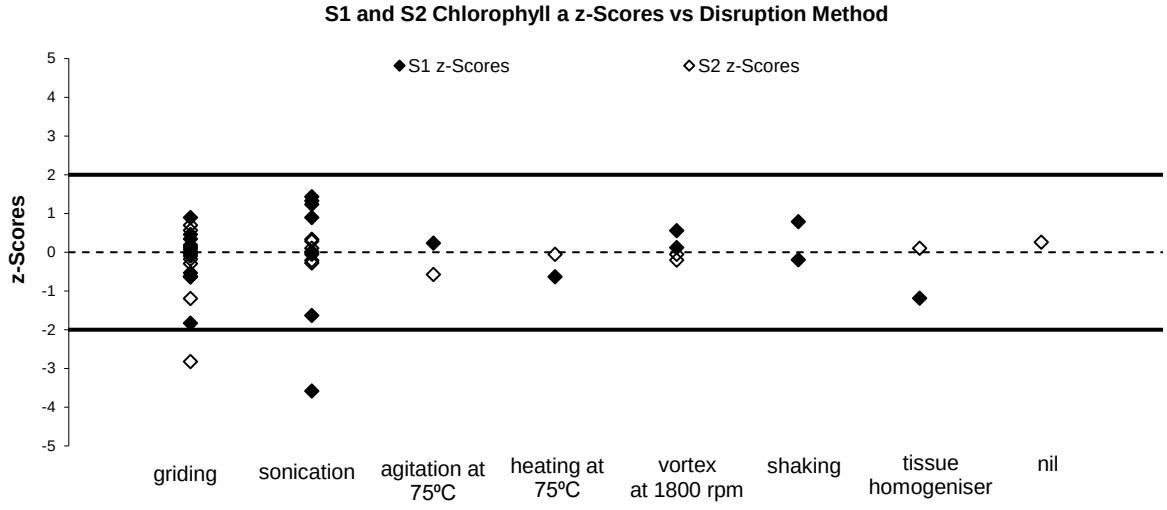
Figure 9 z-Scores vs. Extraction Reagent

Disruption methods

Extraction was generally aided by either grinding or sonication; one laboratory did not use a disruption method for chlorophyll a extraction.

Two laboratories used heating as the disruption method.

Figure 10 presents plots of participants' results versus disruption method.



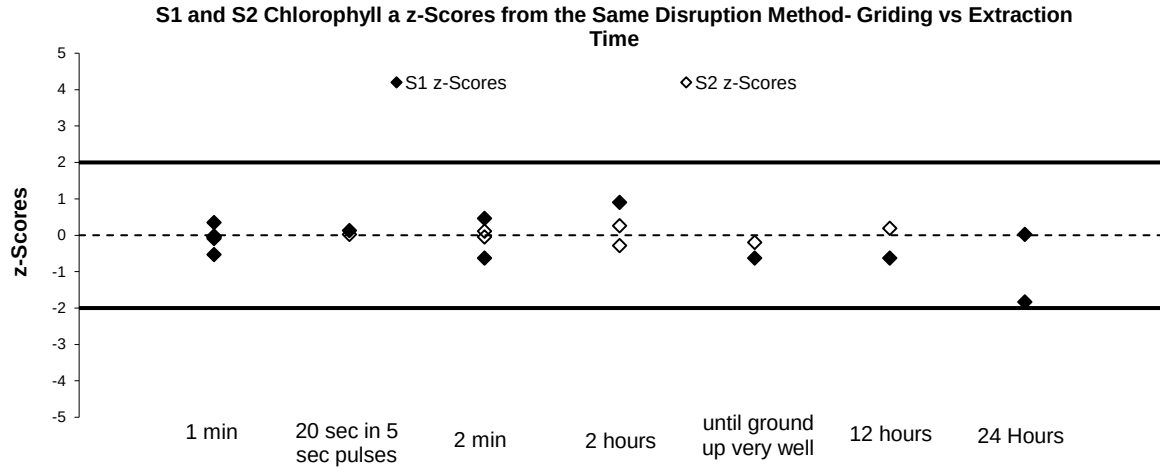
z-Scores from laboratory 5 were excluded (extreme outlier).

Figure 10 z-Scores vs. Disruption Method

Caution should be exercised during the disruption process; although improved extraction has been reported with sonication and mechanical grinding, both disruption procedures have also been found to increase the risk of chlorophyll a degradation. [15]

Extraction Time

Participants reported using various extraction times ranging from 1 minute to 24 hours. Plots of participants' results from the same extraction reagent/disruption method versus extraction time are presented in Figures 11 to 13.

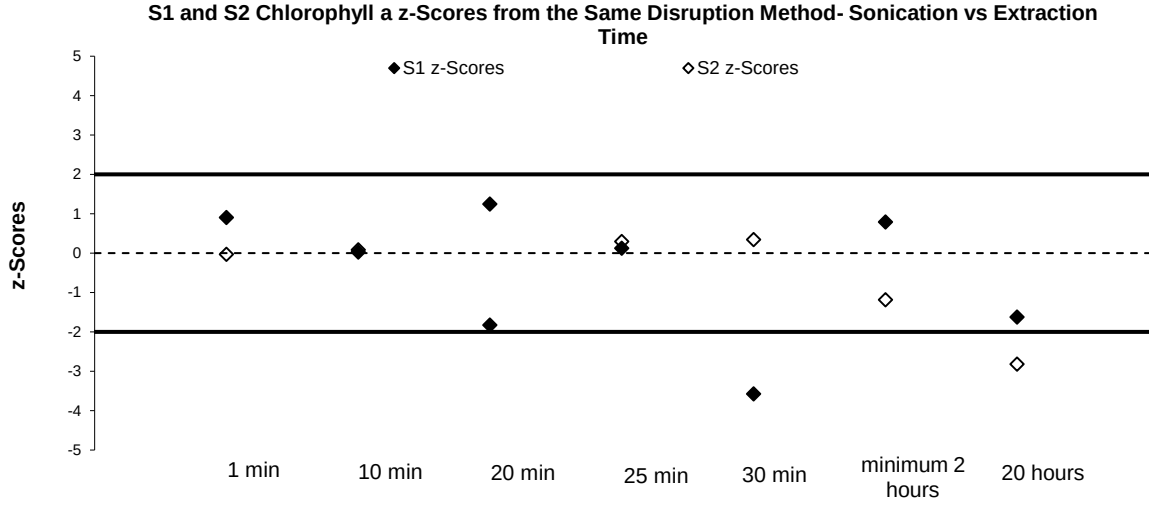


z-Scores from laboratory 5 were excluded (extreme outlier).

Figure 11 Chlorophyll a z-Scores from Acetone Extraction Aided by Grinding vs. Extraction Time

All laboratories that reported using grinding as disruption method also used acetone as extraction agent but various extraction time (Figure 11).

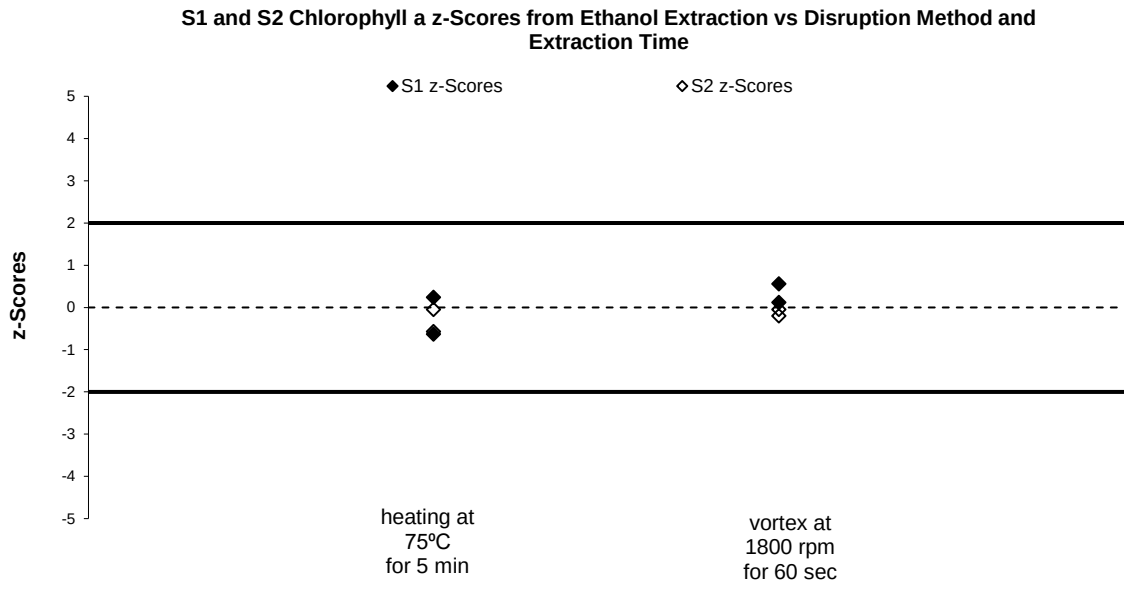
Participants who used sonication as disruption method, used acetone as extraction agent and reported various extraction time ranging from 1 minute to 20 hours (Figure 12).



z-Scores from laboratory 5 were excluded (extreme outlier).

Figure 12 Chlorophyll a z-Scores from Acetone Extraction Aided by Sonication vs. Extraction Time

Four participants reported using ethanol for extraction, two used heating at 75ºC for 5 minutes and two vortexed it for 1 minute and then left it overnight for extraction (Figure 13).

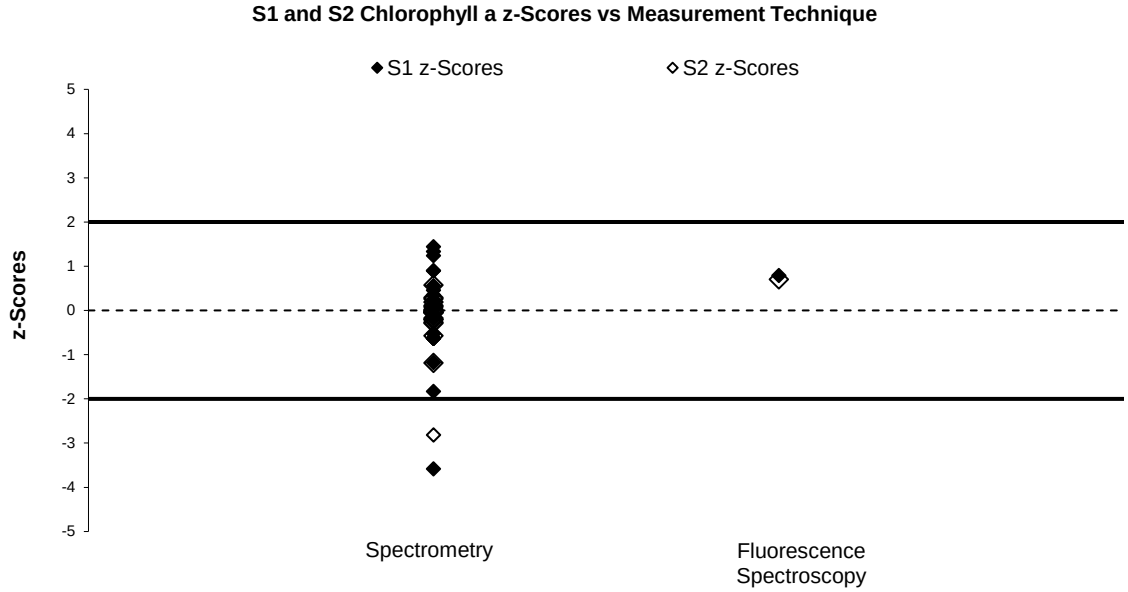


z-Scores from laboratory 5 were excluded (extreme outlier).

Figure 13 Chlorophyll a z-Scores from Ethanol Extraction vs. Disruption Methods and Extraction Time

Measurement Technique

Twenty-nine laboratories reported using a spectrophotometric method for chlorophyll a measurements and one used fluorescence spectroscopy. Laboratory 20 used both a fluorometer and UV-Vis spectrometer. A plot of chlorophyll a results versus measurement technique is presented in Figure 14.



z-Scores from laboratory 5 were excluded (extreme outlier)..

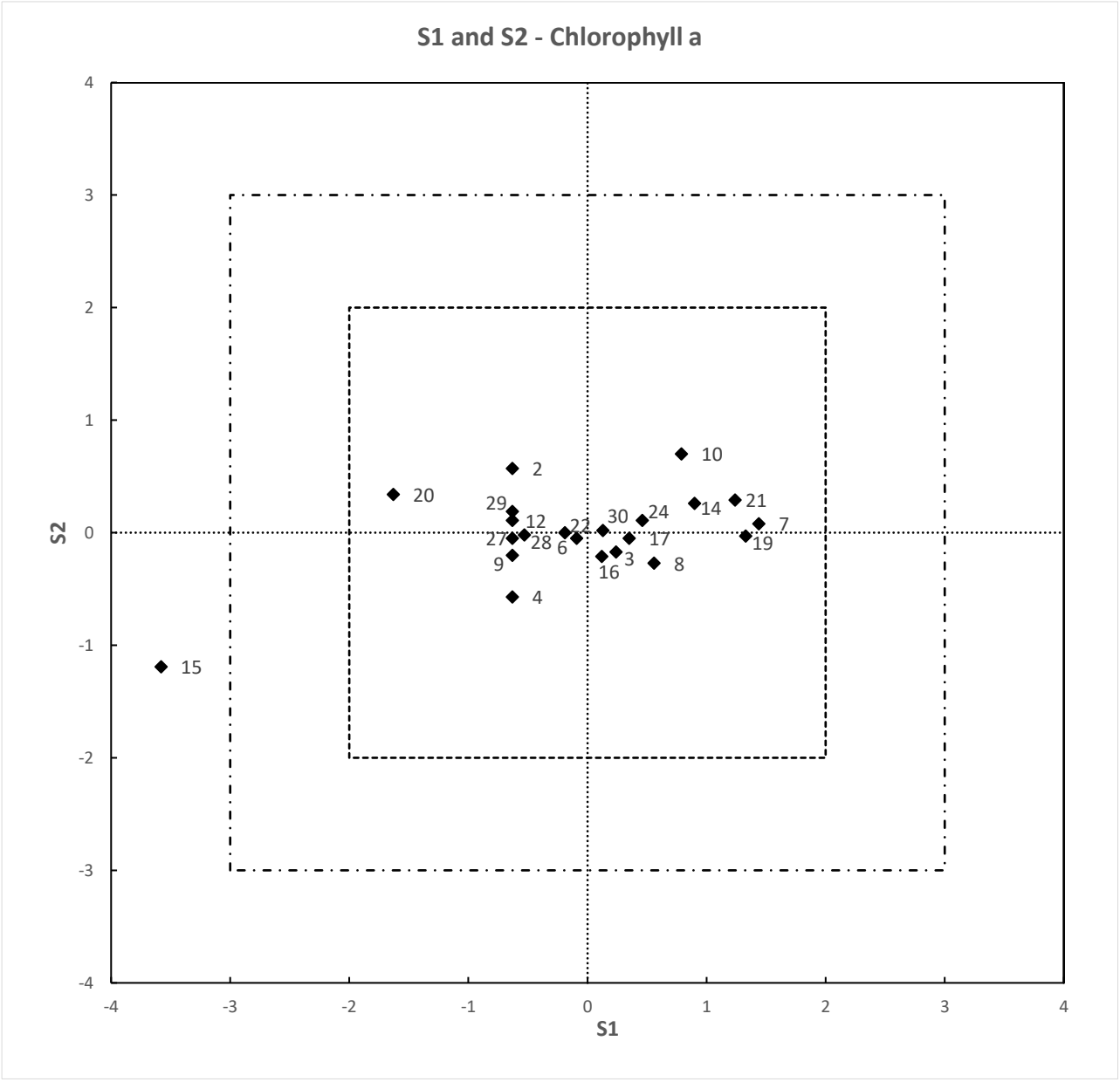
Figure 14 Chlorophyll a Results vs. Measurement Technique

Laboratory 10 reported: “S1 filter had more loosen cellulose fibres than S2 filter that could contribute to S1 background to be higher for spectrometry analysis, S1 was centrifuged little longer to avoid this interference. . .”

7.6 Participants' Within – Laboratory Repeatability

The same target standard deviation was used to calculate z-scores for Chlorophyll a in both samples. This allowed evaluation of participants' within laboratory repeatability.

Scatter plots of z-scores for S1 and S2 are presented in Figure 15. Points close to the diagonal axis represent excellent repeatability and points close to zero represent excellent accuracy and repeatability.



Laboratories 5 and 13 are off the scale

Figure 15 z-Score Scatter Plots for Chlorophyll a in S1 and S2

Chlorophyll a measurement is challenging, as it is sensitive to light and oxygen, and to avoid oxidative and photochemical destruction the samples should not be exposed to bright light or air during analysis.[15] Most laboratories were plotted in the inner quadrant indicating that they have successfully overcome these problems.

7.7 Comparison with Previous NMI Proficiency Studies of Chlorophyll a in Water

AQA 21-05 is the fourth NMI proficiency test of Chlorophyll a in water. Despite a lower concentration of chlorophyll a in the test samples, on average participants' performance has improved over time (Figure 16).

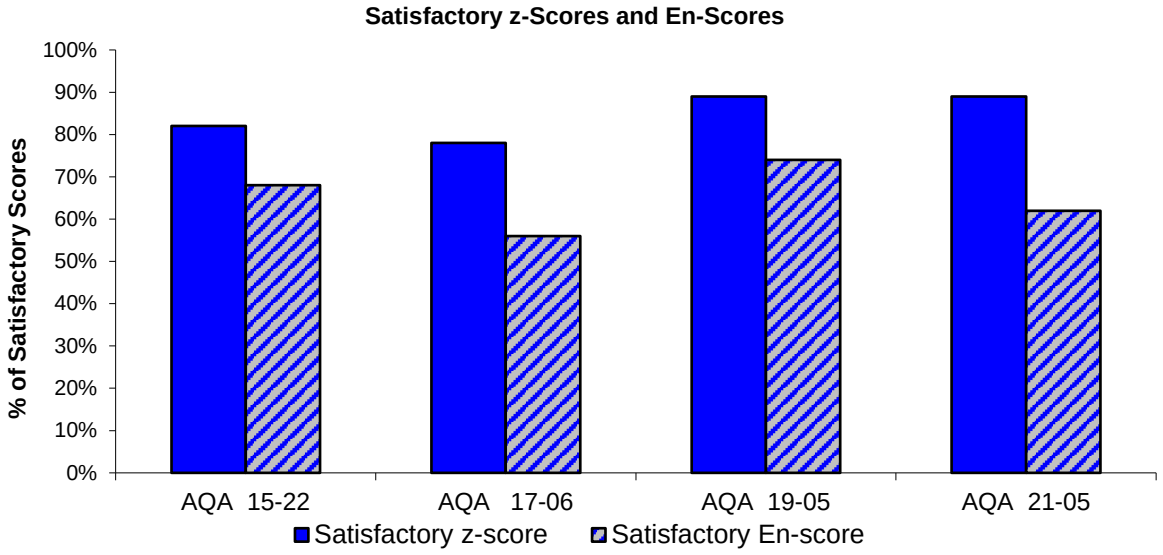


Figure 16 z-Score Scatter Plots for Chlorophyll a in S1 and S2

Individual performance history reports are emailed to each participant at the end of the study; the consideration of z-scores for an analyte over time provides much more useful information than a single z-score.

7.8 Reference Materials and Certified Reference Materials

Participants reported whether control samples (spiked samples, certified reference materials-CRMs or matrix specific reference materials-RMs) had been used (Table 12).

The Chlorophyll a PT samples are homogeneous and well characterised, both by in-house testing and from the results of the proficiency round. A stability study conducted over two years found no significant changes in Chlorophyll a level in PT study samples over time if stored frozen. These samples can be used for quality control, method development and method validation. Surplus test samples from this study are available for sale.

Table 12 Control Samples Used by Participants

Lab. Code	Description of Control Samples
1	RM - Inhouse
3	NMI PT from last round (now have run out)
5	Copper sulfate solution
6	RM
7	SS – Inhouse & Sigma-Aldrich standard
8	Ultrapure water
9	RM – Sigma 1mg Chlorophyll-a
10	Ultra pure water: results 0.00ppb/L
11	CRM – Blank
12	RM

15	RM – Sigma Aldrich Chlorophyll a
16	Ultrapure water
17	RM
18	RM – Sigma Aldrich 1mg Chlorophyll-a Standard
19	Sigma pure chlorophyll a
20	RM – Chlorophyll Sigma Aldrich
21	Sigma Aldrich chlorophyll “a” from spinach
22	ROP water as Blank
25	De-ionised Water Blank
26	RM - CRM
27	SS – Sigma Aldrich C5753 – Chlorophyll a from spinach
30	CRM
32	CRM

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APPENDIX 1 - SAMPLE PREPARATION, ANALYSIS AND HOMOGENEITY TESTING

A1.1 Sample Preparation

Samples S1 and S2, each consisted of one glass fibre filter. A chlorophyll a standard was diluted to an appropriate concentration (50mg/L) in 90% (v/v) acetone solution. 0.1 mL of this standard solution was then used to spike each S1 filter sample and 0.6 mL of the same standard solution was used to spike each S2 filter sample. All preparation was conducted under subdued light.

A1.2 Sample Analysis and Homogeneity Testing

Sample Analysis for Chlorophyll a

Measurements for chlorophyll a for homogeneity testing were subcontracted to ChemCentre which holds third party (NATA) accreditation to ISO 17025 for this test. In brief the method used involves grinding the sample in 90% (v/v) acetone followed by extracting at 4^{o}C for 2 hours. The resulting solution is filtered and analysed using UV-Vis at the varying wavelengths. All measurements were carried out using a 2 cm cuvette.

Homogeneity Testing

The same preparation procedure was followed for the preparation as in previous NMI PT studies however a full homogeneity test was still conducted for both samples. Homogeneity testing was based on that described in the International Protocol. Seven samples (each consisting of one filter) were analysed in random order by ChemCentre. The average of the results was reported as the homogeneity value for chlorophyll a. [4, 5]

Since the entire sample was used in each analysis, it was not possible to apply analysis of variance (ANOVA) to determine if samples were sufficiently homogeneous. When it is not possible to conduct replicate measurements, the standard deviation of the results (sd) will be compared with the target standard deviation of the PT (σ) calculated as described in Section 5.5. The proficiency test samples may be considered sufficiently homogeneous if: $sd \leq 0.3\ \sigma$.[5]

Data from the homogeneity testing is presented in Table 13 and Table 14. The between sample sd as CV was 6 % less than 30% of the target standard deviation as PCV set for this study (20%).[5]

The samples were found to be sufficiently homogeneous for participants' performance assessment.

Table 13 S1 Chlorophyll a Homogeneity Data

Sample number	Result (ug/L)
S1-44	4.9
S1-69	3.8*
S1-22	5.1
S1-41	5.6
S1-2	4.8
S1-53	5.0
S1-23	4.8
Overall Average	**5.03**
CV	**5.98%**

*outlier was due to analytical variation and were not included in the calculation[4, 5]

	Value	Critical (<30% of Target PCV)	Result
CV	5.98%	6%	**Pass**

Table 14 S2 Chlorophyll a Homogeneity Data

Sample number	**Result (ug/L)**
S2-51	30.5
S2-68	33.9
S2-25	31.1
S2-2	29.6
S2-38	31.9
S2-12	31.1
S2-42	31.2
Overall Average	**31.3**
CV	**4.3%**

	Value	Critical (<30% of Target CV)	Result
CV	4.3%	6%	**Pass**

APPENDIX 2 - ASSIGNED VALUE, Z-SCORE AND EN SCORE CALCULATION

Assigned value

The assigned value was calculated as the robust average using the procedure described in 'ISO13258:2015(E), Statistical methods for use in proficiency testing by interlaboratory comparisons – Annex C[5]; the uncertainty was estimated as:

$$u_{rob\ av} = 1.25 * S_{rob\ av} / \sqrt{p} \qquad \text{Equation 3}$$

where:

$u_{rob\ av}$ robust average standard uncertainty
$S_{rob\ mean}$ robust average standard deviation
p number of results

The expanded uncertainty ($U_{rob\ av}$) is the standard uncertainty multiplied by a coverage factor of 2 at approximately 95% confidence level.

A worked example is set out below in Table 15.

Table 15 Uncertainty of Assigned Value for Chlorophyll a in Sample S1

No. results (p)	26
Assigned Value*	4.58 ug/L
$S_{rob\ av}$*	0.83 ug/L
$u_{rob\ av}$	0.20 ug/L
k	2
$U_{rob\ av}$	0.41 ug/L

*Results from Laboratories 13 and 15 were excluded from assigned value and $S_{rob\ av}$ calculation.

The assigned value for **Chlorophyll a** in Sample S1 is **4.58 ± 0.41 ug/L**.

z-Score and E_n-Score

For each participant's result a z-score and E_n-score are calculated according to Equation 2 and Equation 3 respectively (see page 14).

A worked example is set out below in Table 16.

Table 16 z-Score and E_n-score for Chlorophyll a Result Reported by Laboratory 1 in S1

Chlorophyll a Result ug/L	Assigned Value ug/L	Set Target Standard Deviation	z-Score	E_n-Score
4.6 ± 0.5	4.58 ± 0.41	20% as PCV or 0.2 x 4.58 = 0.916 ug/L	$z = \frac{(4.6 - 4.58)}{0.916}$ z = 0.02	$En = \frac{(4.6 - 4.58)}{\sqrt{0.5^2 + 0.41^2}}$ E_n = 0.03

APPENDIX 3 - STABILITY STUDY

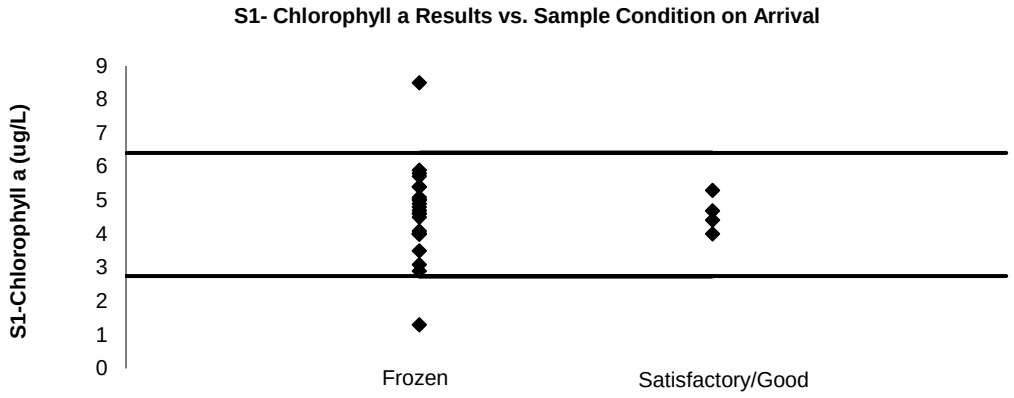
Participants were advised to store the samples frozen if analysis could not be commenced on the day of receipt. Additionally subdued light conditions were advised for all procedures. A summary of the date and condition of samples upon receipt, along with the date of analysis, is presented in Table 17 below.

Table 17 Sample Condition on Receipt and the Date When the Sample was Received and Analysed

Lab Code	Received Date	Arrival Condition	Analysis Date
1	28/04/2021	Frozen	29/04/2021
2*	28/04/2021	Frozen	06/05/2021
3	28/04/2021	Frozen	29/04/2021
4	28/04/2021	Frozen	29/04/2021
5**	27/04/2021	Frozen	27/04/2021
6	28/04/2021	Frozen	04/05/2021
7*	28/04/2021	Good	29/04/2021
8	28/04/2021	Good	04/05/2021
9	28/04/2021	Satisfactory	29/04/2021
10	28/04/2021	Intact	29/04/2021
11	28/04/2021	Frozen	29/04/2021
12	28/04/2021	Good	29/04/2021
13	28/04/2021	Frozen	29/04/2021
14*	28/04/2021	Frozen	04/05/2021
15	28/04/2021	Frozen	06/05/2021
16	28/04/2021	Satisfactory	06/05/2021
17	28/04/2021	Frozen	04/05/2021
18	28/04/2021	Frozen	28/04/2021
19	28/04/2021	Frozen	29/04/2021
20	30/04/2021	Frozen	05/05/2021
21	28/04/2021	Good	29/04/2021
22*	28/04/2021	Good	05/05/2021
23	28/04/2021	Frozen	28/04/2021
24	28/04/2021	Good	07/05/2021
25**	27/04/2021	Frozen	27/04/2021
26	28/04/2021	Frozen	03/05/2021
27	28/04/2021	Frozen	30/04/2021
28**	28/04/2021	Acceptable	30/04/2021
29	28/04/2021	Good	30/04/2021
30	28/04/2021	Frozen	04/05/2021
31*	05/05/2021	Frozen	06/05/2021
32	28/04/2021	Frozen	05/05/2021
Homogeneity Testing (T0)	28/04/2021	Frozen	30/04/2021
Stability Testing (T32)***	26/05/2021	Frozen	28/05/2021

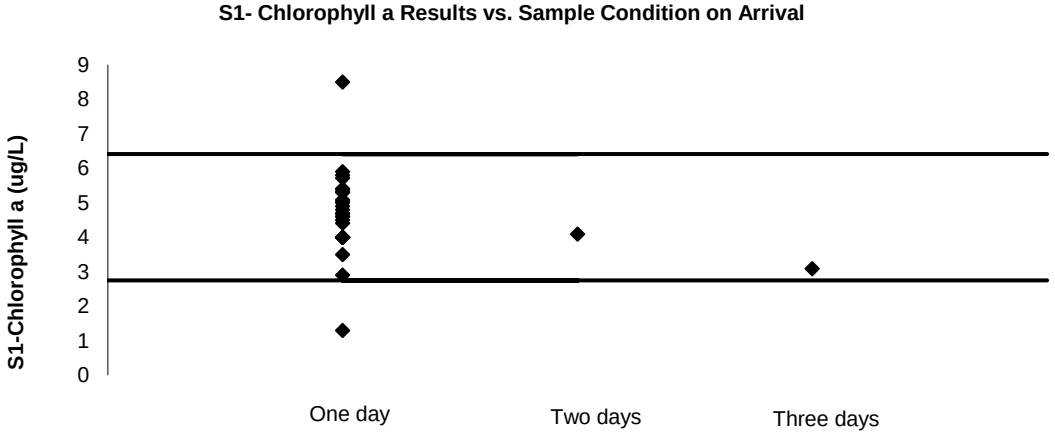
*As per courier delivery notification **Samples were dispatched on 26/04/2021***Stability samples were dispatched on 25/05/2021

No correlation was observed between chlorophyll a results and the number of days that the sample spent on the road, nor between results and analysis date or sample condition on arrival (Figures 17 to19).



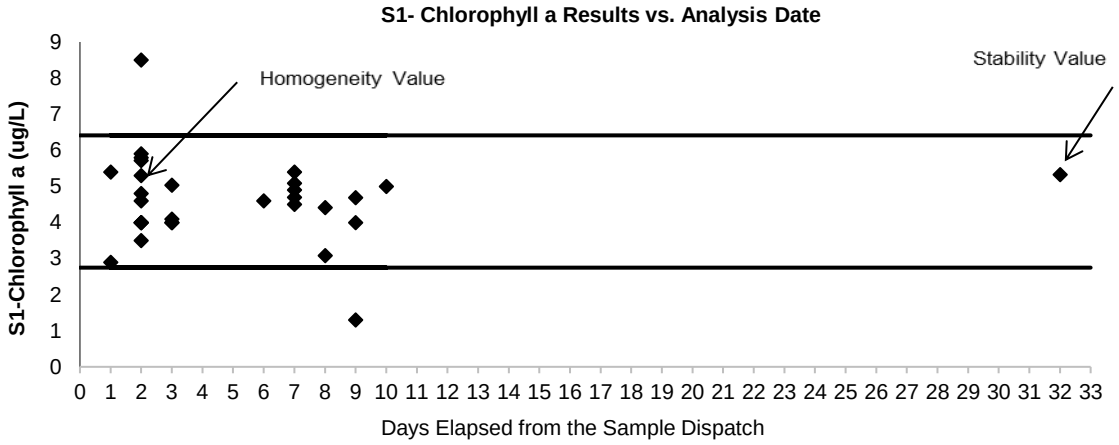
Horizontal lines on the above chart correspond to z-scores of 2 and -2. Laboratory 13's result has been plotted as 8.5 ug/L. Laboratory 5's result was not included (extreme outlier).

Figure 17: Chlorophyll a Concentration in S1 vs. Condition on Arrival



Horizontal lines on the above chart correspond to z-scores of 2 and -2. Laboratory 13's result has been plotted as 8.5 ug/L. Laboratory 5's result was not included (extreme outlier).

Figure 18: Chlorophyll a Concentration in S1 vs. Days on the Road



Horizontal lines on the above chart correspond to z-scores of 2 and -2. Laboratory 13's result has been plotted as 8.5 ug/L. Laboratory 5's result was not included (extreme outlier).

Figure 19: Chlorophyll a Concentration in S1 vs. Analysis Date

Stability Study

Previous PT studies in chlorophyll a, found no significant changes in short term stability studies. A long term stability study (over two years) similarly found no significant changes in the level of Chlorophyll a overtime, if stored frozen (Appendix 4).

However, a stability study was still conducted in the present study. The analyses were carried out by ChemCentre over the entire period of study: when the study started (T0) and at its end, 32 days later (T32).

A Student t-test was used to compare the two sets of results. No significant change in chlorophyll a concentration over the elapsed time was evident (p=0.18).

The chlorophyll a results at T0 and T32 were also in good agreement with the assigned value (A.V.) and spike value (S.V.) within their stated uncertainties (Figure 20).

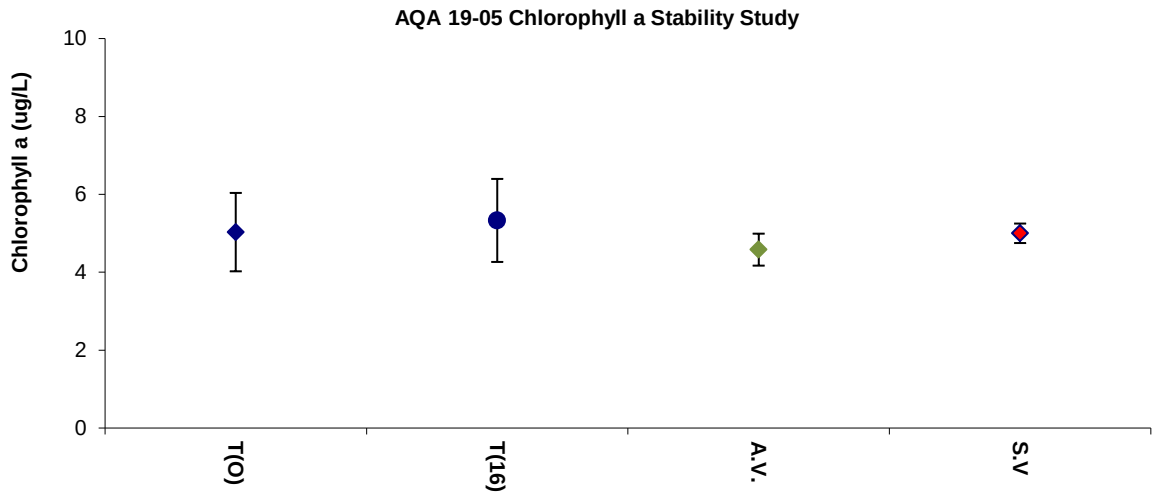


Figure 20: Chlorophyll a Stability Results

APPENDIX 4– LONG TERM STABILITY STUDY

A long term stability study was conducted for chlorophyll a in water.

The sample was a prepared in March 2019 as a blind duplicate sample of PT study AQA 19-05. The analyses for stability were carried out on monthly basis by ChemCentre, one year after sample preparation and homogeneity analysis, from February 2020 until February 2021. Results are presented in Table 18.

Table 18: Long Term Stability Results

Sample	Date of Analysis	Chlorophyll a µg/L
Spike Value		9.38
Homogeneity Value	02/04/2019	9.0
Short Term Stability Value	10/04/2019	9.51
Bottle No 1	11/02/2020	8.79
Bottle No 22	18/03/2020	9.4
Bottle No 21	08/04/2020	9.5
Bottle No 31	20/05/2020	9.2
Bottle No 17	01/07/2020	9.03
Bottle No 14	12/08/2020	9.33
Bottle No 50	09/09/2020	9.4
Bottle No 6	07/10/2020	9
Bottle No 24	04/11/2020	8.67
Bottle No 21	02/12/2020	8.67
Bottle No 9	20/01/2021	8.77
Bottle No 8	10/02/2021	9.27

Linear regression was performed to check for significant trends indicating possible degradation of the material. The concentration was fitted against time with day 0 being the day of measurement of the homogeneity value. The observed slope was tested for significance using a Student t-test, with $t_{\alpha\ df}$ being the critical t-value (two-tailed) for a significance level of α=0.05 (95% confidence interval). Results are presented in Table 18 and Figure 21.

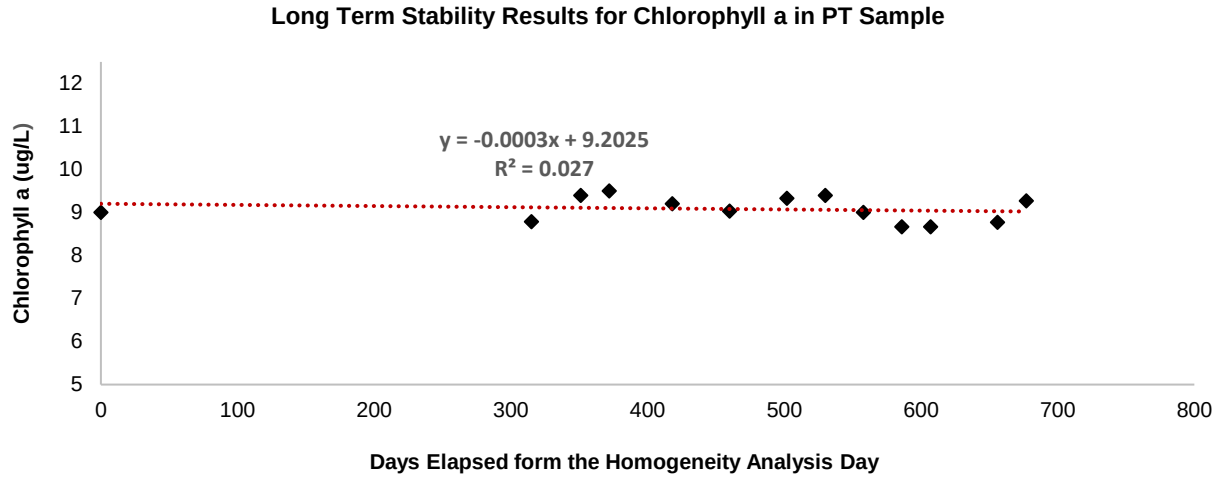


Figure 21 Chlorophyll a Stability Results

Table 19 Long Term Stability Study Results

Analyte	t-test	$t_{cr(95,df-2)}$	Is the slope significantly different from 0 at a 95% confidence interval (t-test >tcr $_{(95, df-2)}$)?
Chlorophyll a	-0.553	2.21	**Not significant**

There are no statistically significant changes in the level of chlorophyll a in the frozen PT sample over time.

APPENDIX 5 - ACRONYMS AND ABBREVIATIONS

HV	Homogeneity Value
Max	Maximum value in a set of results
Md	Median
Min	Minimum value in a set of results
NMI	National Measurement Institute (of Australia)
NR	Not Reported
NT	Not Tested
PT	Proficiency Test
PCV	Performance Coefficient of Variation
RA	Robust Average
RM	Reference Material
Robust CV	Robust Coefficient of Variation
Robust SD	Robust Standard Deviation
S	Spiked or formulated concentration of a PT sample
SI	The International System of Units
s^2_{sam}	Sampling variance
s_a/σ	Analytical standard deviation divided by the target standard deviation
SRM	Standard Reference Material (Trademark of NIST)
Target SD	Target standard deviation
σ	Target standard deviation

APPENDIX 6 – MEASUREMENT TECHNIQUE

Table 20 Measurement Technique for Chlorophyll a and Pheophytin a

Lab. Code	Measurement Technique
1	Spectrophotometric
2	Spectrophotometric
3	Spectrophotometer in 10mm quartz cell
4	Spectrophotometric
5	Spectrophotometric
6	Spectrophotometric
7	Spectrophotometric
8	Spectrophotometric
9	Spectrophotometric
10	Fluorometric; Pheophytin a: Spectrophotometer
11	Spectrophotometric
12	
13	Spectrophotometric
14	Spectrophotometric
15	Spectrophotometric
16	Spectrophotometric
17	Spectrophotometric
18	Spectrophotometric
19	Spectrophotometric
20	Fluorometer and UV-VIS Spectrometer
21	Spectrophotometric
22	Spectrophotometric
23	Spectrophotometric
24	Spectrophotometric
25	Spectrophotometric
26	Spectrophotometric
27	Spectrophotometric
28	Spectrophotometric
29	Spectrophotometric
30	Spectrophotometric
31	Spectrophotometric
32	Spectrophotometric

END OF REPORT