Centre for Environment Fisheries & Aquaculture Science

Cefas

FAO Reference Centre for Bivalve Mollusc Sanitation

Norovirus (Genogroup I and II) and Hepatitis A virus Proficiency Testing (PT 89)

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Date: September 2022

This publication is available at www.gov.uk/government/publications

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Cefas Document Control

Submitted to:	PT 89 participants
Date submitted:	15.09.22
Project Manager:	C. Kent
Report compiled by:	L. Stockley
Quality control by:	Dr J. Lowther
Approved by and date:	Sam Course 27.09.22
Version:	Draft V2
Recommended citation for this report:	N/A

Version control history

Version	Author	Date	Comment
Draft V1	L. Stockley	15.09.22	Sent to JL and Charlotte King for review
Draft V2	L. Stockley	27.09.22	Sent to CK (PM) for review
Final V1	L. Stockley	18.10.22	No changes to be made

Contents

1. Preparation of sample material

1.1. Sample and virus strain origin

Materials dispatched consisted of whole Pacific oysters (*C. gigas*), blended digestive glands from the same species and dsDNA control solutions for quantification (1 x 10^5 copies/µl) for each target virus. The origin of the viruses used for preparing the samples are given in Table 1. All samples were held at <-15°C until required for quality control testing, dispatch and/or reference analysis.

Table 1 - Origin and strain/genotype of viruses used for shellfish contamination

Description	Source	Strain ID / genotype
Hepatitis A virus	Cell culture supernatant	HM175/43c
Norovirus genogroup I	Faecal material	GI.7 (based on capsid sequence)
Norovirus genogroup II	Faecal material	GII.4 (based on capsid sequence)

1.2. Preparation of negative digestive glands

A single batch of approximately 1000 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area in September 2021. The shellfish were shucked, and the digestive glands removed and blended before being pooled together to form a homogenous mixture. The single batch was tested to demonstrate the absence of norovirus (NoV) genogroups I and II (GI and GII) and hepatitis A virus (HAV) prior to being used to prepare different samples depending on availability.

1.3. Preparation of highly contaminated digestive gland blends

Approximately 1000 Pacific oysters (*C. gigas*) were collected from a UK commercial harvesting area and a representative sample was tested to demonstrate the absence of NoV GI, NoV GII and HAV. The remaining live shellfish were placed in trays and immersed in 500 litres of re-circulating natural seawater at 16±1 °C. The shellfish were left to acclimatise for approximately 24 hours and checked for filtering behaviour before 50 ml of commercial shellfish food mix containing high levels of NoV GII from human faeces (provided by UK HSA) was added to the

tank. The shellfish were left to feed for approximately 16 hours to allow bioaccumulation to occur after which they were removed from the tank, shucked and the digestive glands removed. The digestive glands were pooled together before being blended to form a homogenous mixture. In a separate tank this procedure was repeated with HAV cell culture supernatant instead of NoV GII to produce two highly contaminated blends, each contaminated with a single target virus.

1.4. Sample preparation

1.4.1. Sample 1

Pacific oysters (*C. gigas*) collected from a UK commercial harvesting area in September 2021 were initial tested to demonstrate the absence of all 3 target viruses. The shellfish were then placed in a large sterile container and thoroughly mixed before subsamples of 10 oysters were randomly selected and placed in sample bags and stored at <-15°C.

1.4.2. Sample 2

Blended negative glands (see above) were mixed with NoV GI positive faecal material and the highly contaminated digestive gland blends containing NoV GII and HAV (as described above) to obtain the desired target levels (see Table 2 for target levels) before being split into 2 g aliquots. The samples were held at <-15 °C until required for quality control testing, dispatch and/or reference analysis.

1.4.3. Sample 3

Blended negative glands (see above) were mixed with NoV GI positive faecal material and the highly contaminated digestive gland blend containing HAV (as described above) to obtain the desired target levels (see Table 2 for target levels) before being split into 2 g aliquots. The samples were held at <-15 °C until required for quality control testing, dispatch and/or reference analysis.

1.4.4. Sample 4

Blended negative glands (see above) were mixed with the highly contaminated digestive gland blends containing NoV GII and HAV (as described above) to obtain the desired target levels (see Table 2 for target levels) before being split into 2 g aliquots. The samples were held at

<-15 °C until required for quality control testing, dispatch and/or reference analysis. These samples were used for both samples 4.

2. Sample distribution

A total of 14 laboratories requested to participate in PT 89. Samples were dispatched on dry ice in accordance with IATA packing instructions 650 for UN3373 'Diagnostic Specimens' on 18th July 2022 to 8 participating laboratories. Due to delays in receiving the required documentation to transport the material, dispatch of packages for the remaining 6 laboratories was delayed until the following week (25th July 2022). On arrival participants were given a month to analyse the test samples using their routine method. For a single laboratory (169), delivery of the package was significantly delayed due to customs processes, meaning that the laboratory was unable to complete testing before the deadline for result submission.

Those laboratories using quantitative real-time RT-PCR were requested to calculate the quantity of target virus in each sample using both their own standard material and using the dsDNA control solutions provided with this PT distribution.

3. Results

3.1. Reference results

Reference analyses were performed by the FAO Reference Centre (FAO RC) for Bivalve Mollusc Sanitation on samples stored at <-15˚C. Six randomly selected samples from each sample type were extracted in duplicate and qRT-PCR (TaqMan™) was carried out using duplicate PCR reactions for each RNA extract and each target. Reference results for each sample are shown in Table 2, with box and whisker plots included in Appendix 1.

Table 2 - Reference results for PT 89 Proficiency testing material

Sample	Norovirus		HAV
	GI	GII	
Sample 1 (Whole animal)	-	-	-
Sample 2 (Digestive gland)	+ ($1.78 \times 10^3 – 2.45 \times 10^3$)	+ ($1.43 \times 10^4 – 2.61 \times 10^4$)	+ ($5.90 \times 10^3 – 1.17 \times 10^4$)
Sample 3 (Digestive gland)	+ ($2.99 \times 10^3 – 4.81 \times 10^3$)	-	+ ($3.61 \times 10^4 – 8.99 \times 10^4$)
Sample 4 (Digestive gland)	-	+ ($1.45 \times 10^4 – 2.26 \times 10^4$)	+ ($3.40 \times 10^3 – 1.42 \times 10^4$)

Quantities in copies/g.

Note: Ranges based on a 95% confidence limit determined as 2 geometric standard deviations above and below the geometric mean.

3.2. Participants' results

Participant's results are tabulated in Appendices 2, 3 and 4 and quantitative results are shown in graphical form alongside the reference values in Appendix 5.

3.3. Performance scoring

3.3.1. Presence / absence

Performance scoring was undertaken on each participant's presence/absence results. A single score for each sample and each target virus (NoV GI, NoV GII and HAV) was assigned as follows: Correct = 2 points, Incorrect = 0 points. For each laboratory an overall score is provided for each target virus, taking into account the results of all 4 samples (Table 4).

3.3.2. Quantification

For those laboratories submitting quantitative results, an additional performance scoring for quantification was undertaken following the median absolute deviation from the median (MAD) approach described in ISO/TS 22117 Microbiology of food and animal feeding stuffs – specific requirements and guidance for proficiency testing by interlaboratory comparison (ISO, 2019). The MAD approach is recommended for assessment of PT data where less than 50 participants

return quantitative results and/or for new proficiency assessment. Where laboratories submitted quantitative results determined using both their own quantification standards, and those provided by the FAO RC, only the results using their own standards were considered for performance scoring; however, where laboratories submitted quantitative results using the FAO RC standards only, these were considered.

For each sample/target virus combination where the intended result was positive, a statistically robust acceptability range was determined by calculation of the median absolute deviation (MAD) of each participant's result from the median of all participants' results. This figure was then multiplied by a constant (1.4826) to obtain a robust estimate of the standard deviation (σ_{MAD}; Table 3). For each individual result, its absolute deviation from the participants' median was compared with the calculated σ_{MAD} to determine its acceptability and score as follows:-

- Difference between result and participants' median <2 σ_{MAD} = satisfactory (2 points)
- Difference between result and participants' median >2 σ_{MAD} and <3 σ_{MAD} = questionable (1 point)
- Difference between result and participants' median >3 σ_{MAD} = unsatisfactory (0 points)
- Result reported as negative = unsatisfactory (0 points)

Where laboratories reported quantities for some samples as below or above a specific limit, these samples were not considered for quantitative performance scoring. The differences between individual participants' results and the participants' median, expressed in terms of σ_{MAD} are shown in Appendix 4, and the graphs in Appendix 5 include lines showing the boundaries of the satisfactory and questionable ranges for each sample/target matrix combination.

For each sample/target virus combination where the intended result was negative, its acceptability and score was determined as follows:-

- Result reported as negative = satisfactory (2 points)
- Result reported as positive = unsatisfactory (0 points)

Table 3 - Dataset characteristics for quantitative results

Characteristic	Sample 2			Sample 3		Sample 4	
	GI	GII	HAV	GI	HAV	GII	HAV
MEDIAN	3.291	4.223	4.041	3.633	4.756	4.572	4.275
MAD	0.210	0.197	0.163	0.155	0.342	0.046	0.219
σMAD	0.312	0.292	0.241	0.229	0.506	0.068	0.325

Values in $\log_{10}$ copies/g

For each laboratory an overall score (usually out of 8) is provided for each target virus, taking into account the results of all 4 samples (Table 4).

Table 4 - Performance scoring

Lab ID	Presence / absence			Quantification		
	NoV		HAV	NoV		HAV
	GI	GII		GI	GII	
2	8	8	8	NQ	NQ	NQ
3	8	8	8	8	8	NQ
9	8	8	8	8	8	8
10	8	8	8	8	8	8
20	8	8	8	NQ	NQ	NQ
24	8	8	8	5	8	8
57	8	8	8	7	6	8
96	8	8	8	8	6	8
113	4	2	NE	2	2	NE
169 *	-	-	-	-	-	-
190	8	8	8	8	8	4**
237	8	8	8	7	6	8
246	NE	NE	8	NE	NE	NQ
324	8	8	8	8	8	7

Key: NE = Target virus not examined; NQ = Target virus not quantified; labs that scored less than full marks (normally 8) are highlighted in yellow. * = Laboratory was unable to submit results within the reporting deadline due to delays caused by customs processes. ** = Score is out of 6 due to exclusion of one sample with quantitative result reported as <LOQ

4. Discussion

Thirteen laboratories received samples. Laboratory 113 only analysed the samples for GI and GII and laboratory 246 only analysed the samples for HAV. Methods used by participants to analyse the test samples are shown in Appendix 6, while brief details of the types of materials used as quantification standards are included as Appendix 7.

4.1. Material dispatch

Eight laboratories received material within 4 days of dispatch. A number of participants experienced delays at customs due to paperwork issues. The FAO RC recommends those laboratories who experience problems to review the documentation requested by customs and/or broker and ensure the documents are available prior to dispatch in future PT schemes.

4.2. Presence / absence determination

Twelve laboratories out of 13 that returned results (92%) obtained the intended presence/absence result (as determined by the FAO RC) for all samples and all target viruses tested (See Appendix 2). Overall sensitivity, specificity and accuracy levels were 96%, 97% and 97% respectively.

For NoV, 11 out of 12 labs that tested for NoV (92%) obtained the intended presence/absence result (as determined by the FAO RC) for all samples and both genogroups. A total of 3 false negative results (1 for GI, 2 for GII) were reported by laboratory 113, resulting in an overall sensitivity for NoV of 94%. A total of 2 false positive results (1 each for GI and GII) were reported by laboratory 113, resulting in an overall specificity for NoV of 96%. The overall accuracy for norovirus results was 95%.

For HAV, all 12 laboratories that tested samples for HAV obtained the intended presence/absence result (as determined by the FAO RC) for all samples. Overall sensitivity, specificity and accuracy levels were all 100%.

4.3. Quantification

A total of 10 laboratories (77%) reported quantitative data for at least one sample/virus combination. Of the 10 laboratories reporting quantitative data, 3 (30%) reported all results in the satisfactory range, scoring full marks for quantification for all target viruses for which they

reported quantitative data. Seven laboratories (70%) reported at least one questionable or unsatisfactory result.

The FAO RC recommends any laboratory with unsatisfactory results for either presence / absence or quantification refers to the trouble shooting guide available on the FAO RC website Troubleshooting guidance for virus PT (cefas.co.uk)

5. References

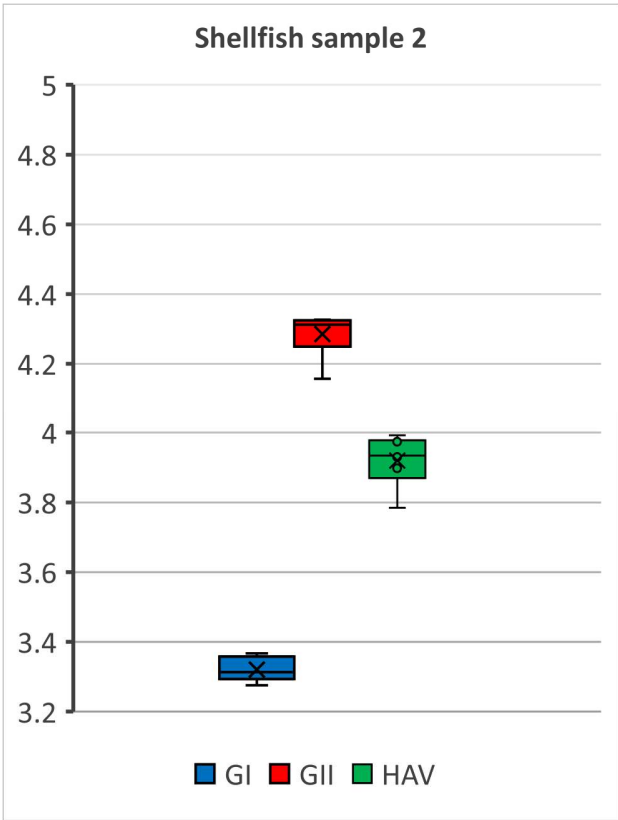
Codd AA, Richardson IR, Andrews N. 1998. Lenticules for the control of quantitative methods in food microbiology. J Appl Microbiol. 85(5):913–7.

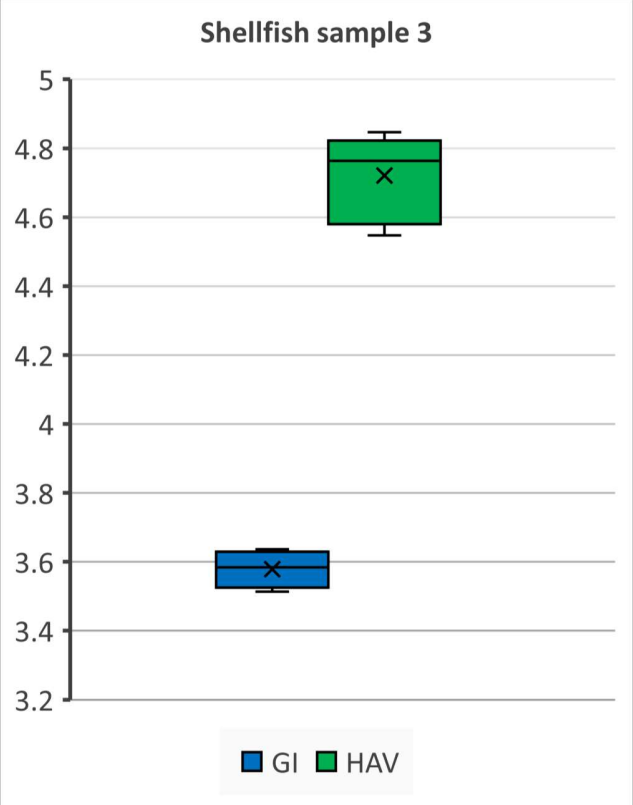
Anon 2017. ISO 15216-1:2017 Microbiology of the food chain -- Horizontal method for determination of hepatitis A virus and norovirus using real-time RT-PCR -- Part 1: Method for quantification.

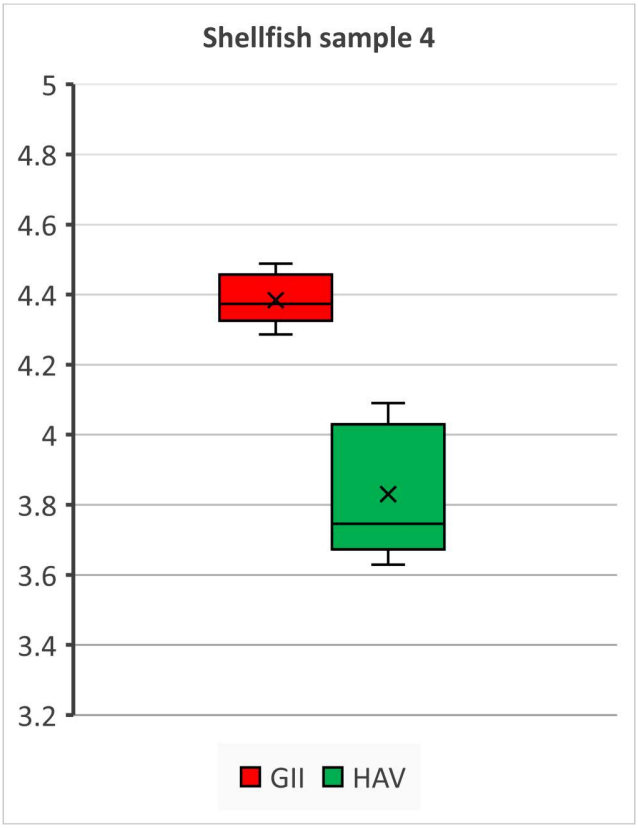
Anon 2019. ISO 22117:2019 Microbiology of the food chain – Specific requirements and guidance for proficiency testing by interlaboratory comparison.

6. Appendices

Appendix 1 - FAO Reference Centre results displayed as box and whisker plots of log_{10} detectable genome copies per gram.







Key: Box and whisker plots (log scale) showing FAO Reference Centre results of log_{10} detectable genome copies per gram.

Appendix 2 - Participants' results and C_t values

Lab ID	Shellfish sample 1						Shellfish sample 2						Shellfish sample 3						Shellfish sample 4					
	GI		GII		HAV		GI		GII		HAV		GI		GII		HAV		GI		GII		HAV	
	-	Ct	-	Ct	-	Ct	+	Ct	+	Ct	+	Ct	+	Ct	-	Ct	+	Ct	-	Ct	+	Ct	+	Ct
2	-		-		-		+	33.382	+	34.351	+	29.791	+	33.169	-		+	27.583	-		+	34.11	+	28.982
3	-		-		-		+	38.97 / 38.54	+	33.86 / 34.22	+	35.71 / 35.39	+	38.45 / 37.52	-		+	33.46 / 33.67	-		+	33.85 / 33.99	+	35.68 / 35.40
9	-		-		-		+	35.9	+	33.8	+	34.5	+	34.8	-		+	30.3	-		+	32.3	+	32
10	-		-		-		+	36.75	+	33.59	+	35.61	+	32.92	-		+	29.98	-		+	33.35	+	34.85
20	-		-		-		+	32.32	+	29.29	+	31.49	+	31.79	-	-	+	28.27	-	-	+	29.64	+	31.14
24	-		-		-		+	35.6	+	29.3	+	29.5	+	34.8	-		+	28.6	-		+	28.5	+	28.99
57	-		-		-		+	34.51	+	29.97	+	33.21	+	34014	-		+	30.26	-		+	29.57	+	31.77
96	-		-		-		+	34.63	+	31.06	+	30.54	+	34.03	-		+	26.36	-		+	30.8	+	29.49
113	-		-		NE		+	37.68	-		NE		-		+	35.79	NE		+	36.24	-		NE	
169																								
190	-		-		-		+	34.32	+	32.68	+	38.71	+	34.51	-		+	33.29	-		+	31.73	+	36.78
237	-		-		-		+	35.97	+	32.33	+	34.06	+	35.96	-		+	31.94	-		+	32.32	+	33.37
246	NE		NE		-		NE		NE		+	32.61 / 33.15	NE		NE		+	33.99 / 33.57	NE		NE		+	33.50 / 33.71
324	-		-		-		+	35.51	+	33.36	+	31.93	+	34.54	-		+	29.23	-		+	31.41	+	30.67

Key: NE= sample/target virus combination not examined. Yellow shading denotes false negative results. Red shading denotes a false positive.

Appendix 3 - Participants reported quantities for each target (copies/g) for positive sample/target combinations

Lab ID	Shellfish sample 2						Shellfish sample 3				Shellfish sample 4			
	GI		GII		HAV		GI		HAV		GII		HAV	
	A	B	A	B	A	B	A	B	A	B	A	B	A	B
3	2.66E+03	2.90E+03	3.34E+04	5.05E+04			4.64E+03	5.05E+03			3.73E+04	5.63E+04		
9		1.10E+03		1.30E+04		1.10E+04		2.30E+03		1.20E+05		3.90E+04		4.40E+04
10	2.10E+03		2.71E+04		1.26E+04		4.72E+03		7.28E+04		3.36E+04		2.21E+04	
20														
24	3.80E+02		2.55E+04	1.80E+04	1.35E+04	2.04E+04	6.30E+02		2.49E+04	3.73E+04	4.55E+04	2.94E+04	1.94E+04	2.91E+04
57	1.32E+03	1.13E+03	1.67E+04	1.91E+04	6.40E+03	6.13E+03	1.29E+03	1.75E+03	4.47E+04	4.37E+04	2.18E+04	2.38E+04	1.52E+04	1.68E+04
96	2.52E+03	6.12E+02	5.34E+03	8.31E+02	7.56E+03	4.95E+03	4.30E+03	1.05E+03	1.81E+05	1.16E+05	7.09E+03	1.10E+04	1.83E+04	1.19E+04
113	2.49E+01	5.74E+01	ND	ND			ND	ND			ND	ND		
169														
190	7.05E+03	1.28E+03	1.82E+04	3.64E+03	<560	<280	6.14E+03	1.12E+03	1.03E+04	4.65E+03	3.93E+04	7.32E+03	9.67E+02	4.04E+02
237	8.58E+03	1.29E+03	1.02E+04	6.54E+03	2.17E+04	3.49E+03	9.89E+03	1.49E+03	1.13E+05	1.67E+04	1.17E+04	7.54E+03	4.17E+04	6.53E+03
246														
324	1.82E+03	<LOQ	1.06E+04	<LOQ		3.22E+03	3.61E+03	<LOQ		2.35E+04	3.91E+04	4.55E+03		8.30E+03

Key: ND = Not detected. A = Results obtained with lab's own quantification standards; B = Results obtained with FAO Reference Centre quantification standards; results reported as <x or >x are included for information and are presented as reported by the relevant participant.

Appendix 4 - Differences between participants' results and the participants' median, expressed in terms of σ_{MAD} for positive sample/target combinations

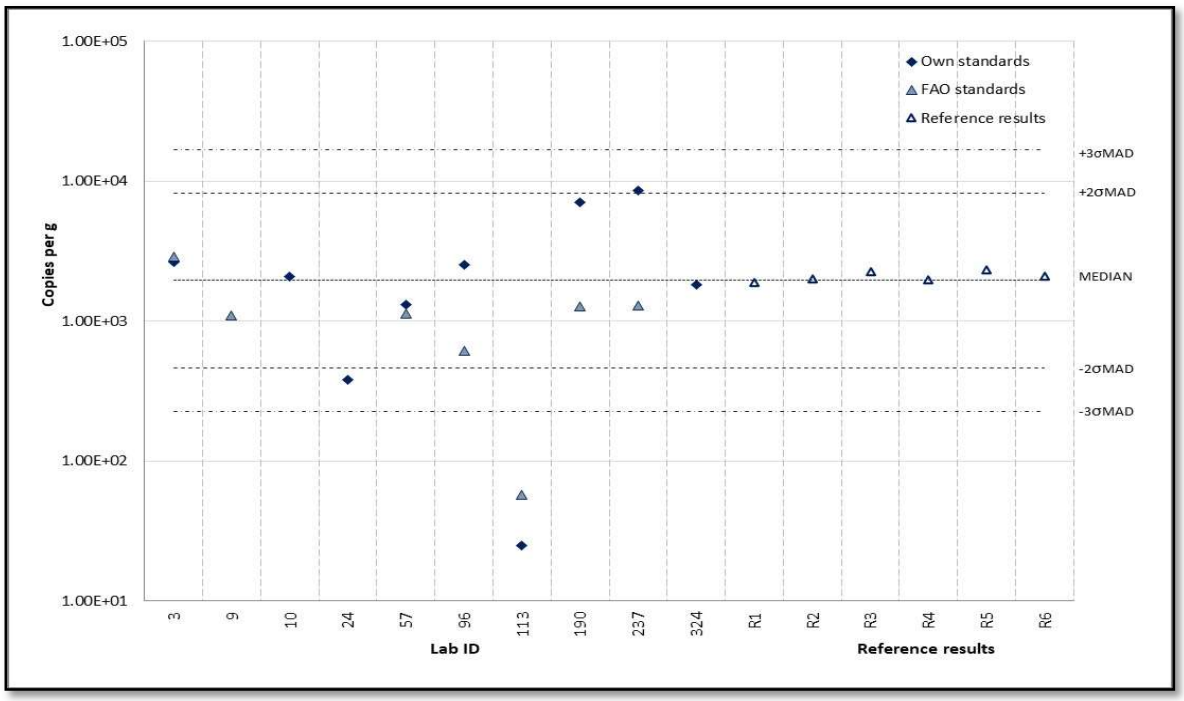
Lab ID	Shellfish sample 2			Shellfish sample 3		Shellfish sample 4	
	GI	GII	HAV	GI	HAV	GII	HAV
3	0.43	1.03	NQ	0.14	NQ	0.00	NQ
9	-0.80	-0.37	0.00	-1.18	0.64	0.28	1.13
10	0.10	0.72	0.24	0.18	0.21	-0.67	0.21
24	-2.28	0.63	0.37	-3.64	-0.71	1.27	0.04
57	-0.55	0.00	-0.97	-2.28	-0.21	-3.44	-0.29
96	0.35	-1.69	-0.67	0.00	0.99	-10.64	-0.04
113	-6.08	ND	NE	ND	NE	ND	NE
190	1.78	0.13	<LOQ	0.67	-1.47	0.33	-3.97
237	2.06	-0.74	1.22	1.58	0.59	-7.42	1.06
324	-0.10	-0.67	-2.21	-0.33	-0.76	0.30	-1.10

Key: Red shading = Unsatisfactory results (false negative, or magnitude of difference between result and participants' median >3 σ_{MAD}); Orange shading = Questionable results (magnitude of difference between result and participants' median >2 σ_{MAD} and <3 σ_{MAD}); NQ = quantitative results not reported, excluded from scoring; NE = Not examined; ND = Not detected (false negative); <LOQ = results reported as below limit of quantification, excluded from scoring.

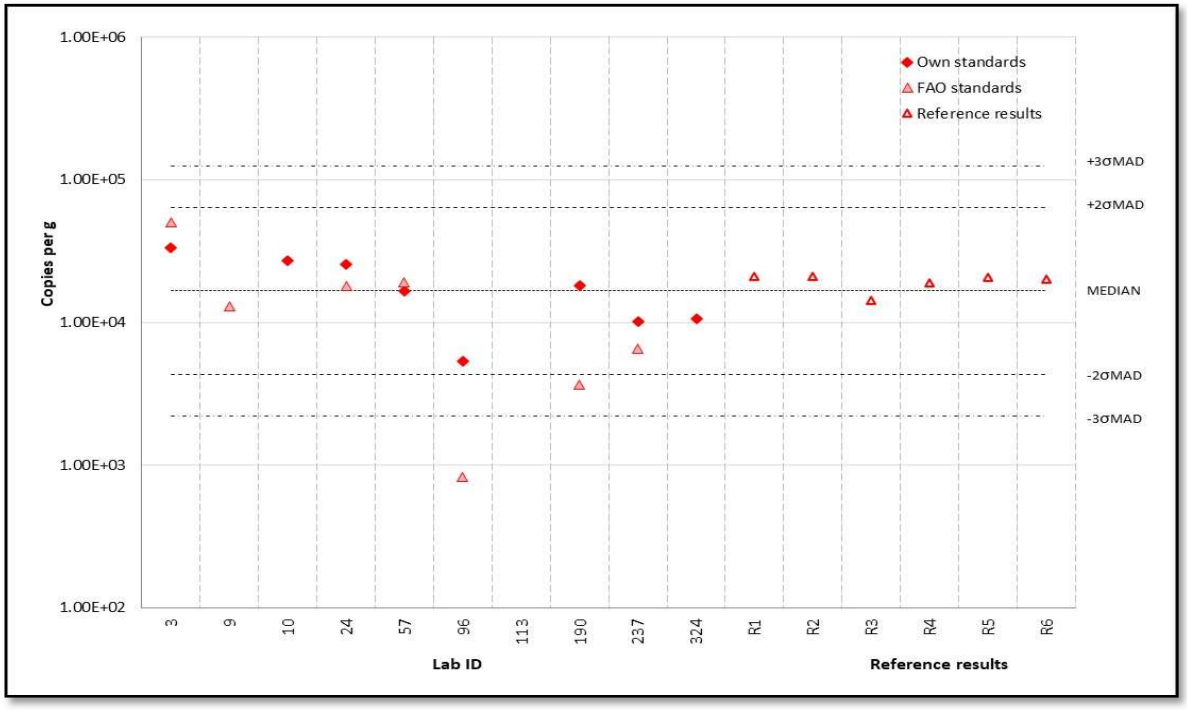
Appendix 5 - Participants' and reference quantities for each sample.

Note: Where quantities were reported using both the laboratory's own quantification standards and those provided by the FAO Reference Centre, only those using the lab's own standards are considered for performance scoring.

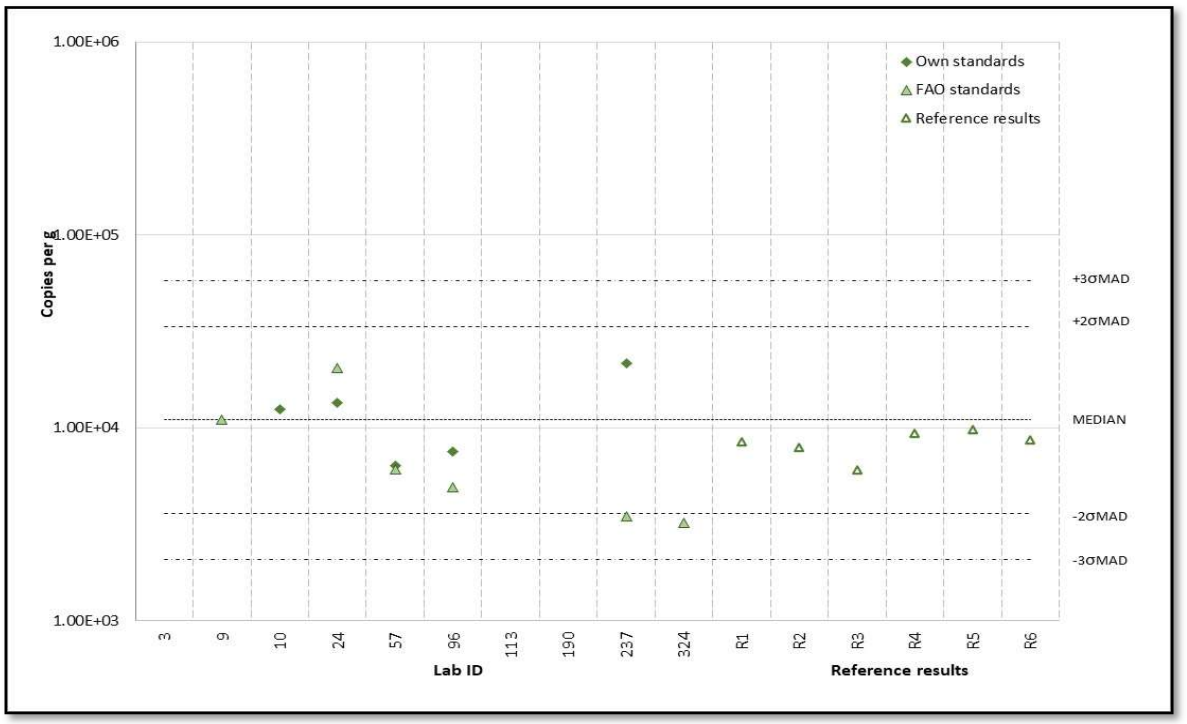
Shellfish sample 2 – GI



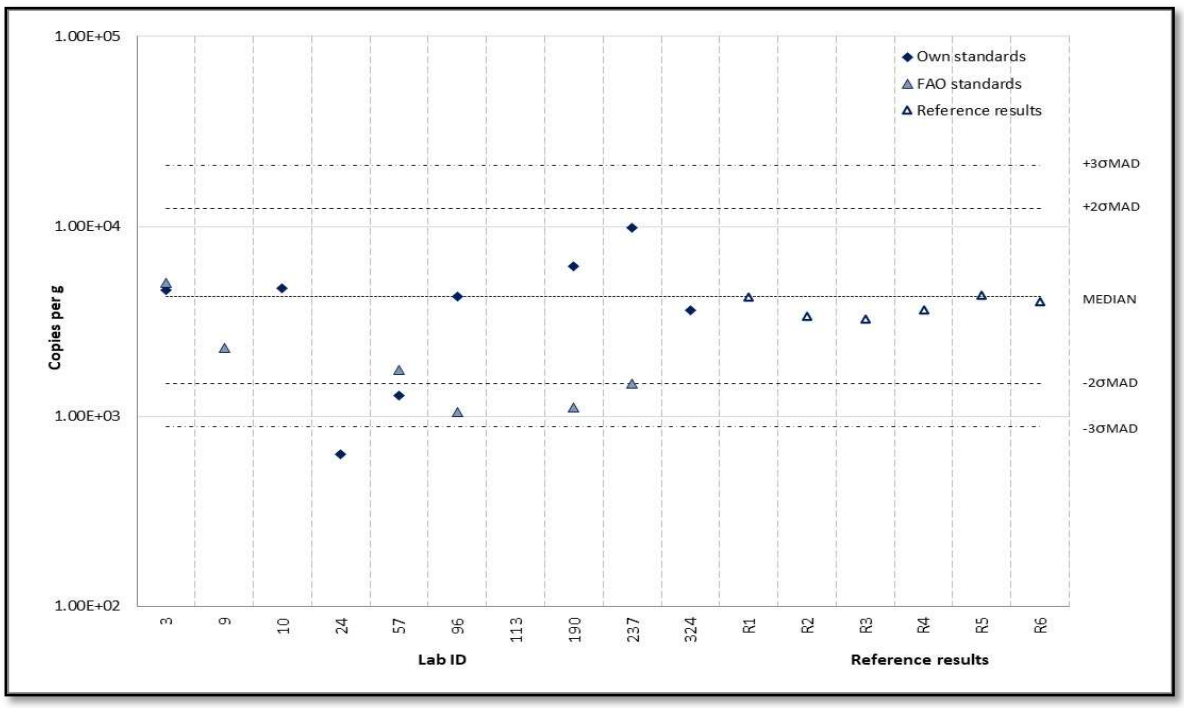
Shellfish sample 2 – GII



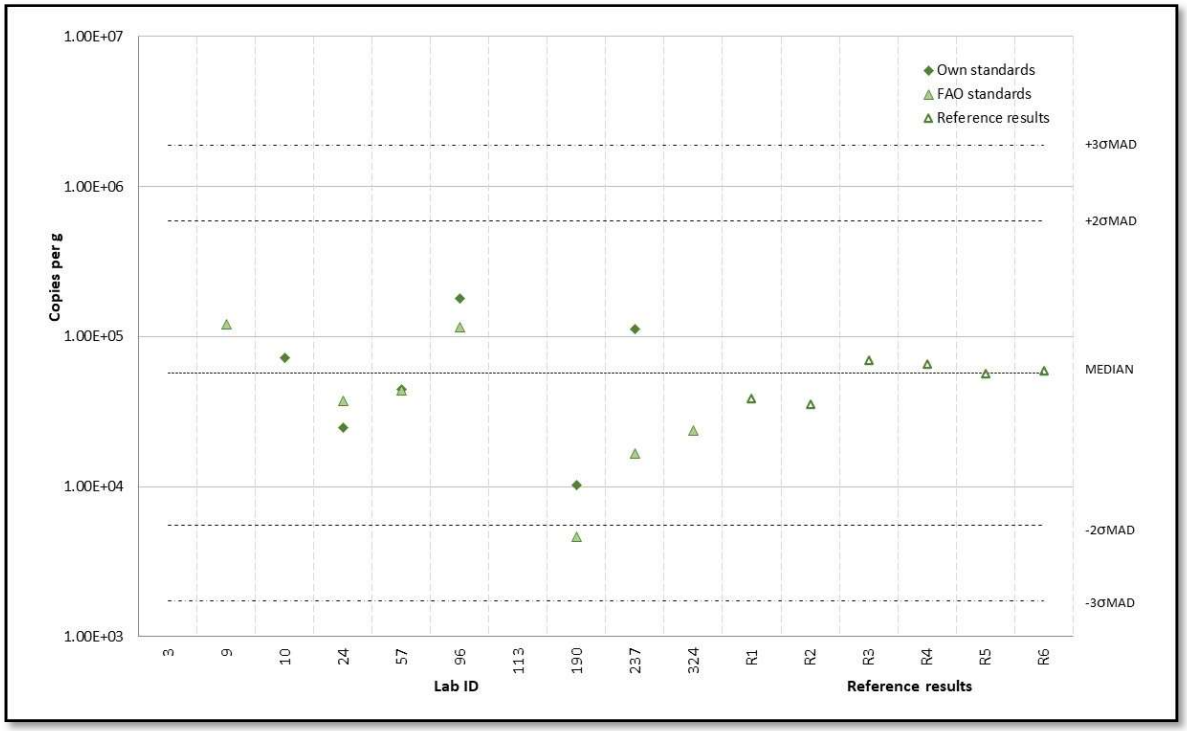
Shellfish sample 2 – HAV



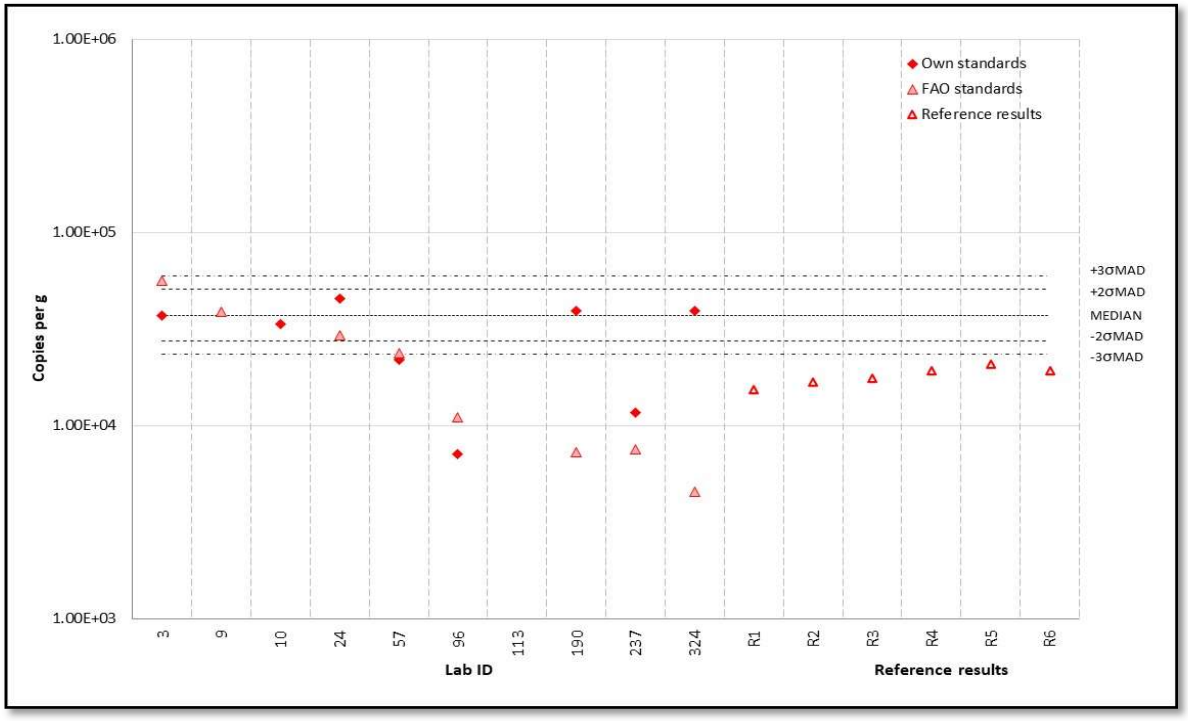
Shellfish sample 3 – GI



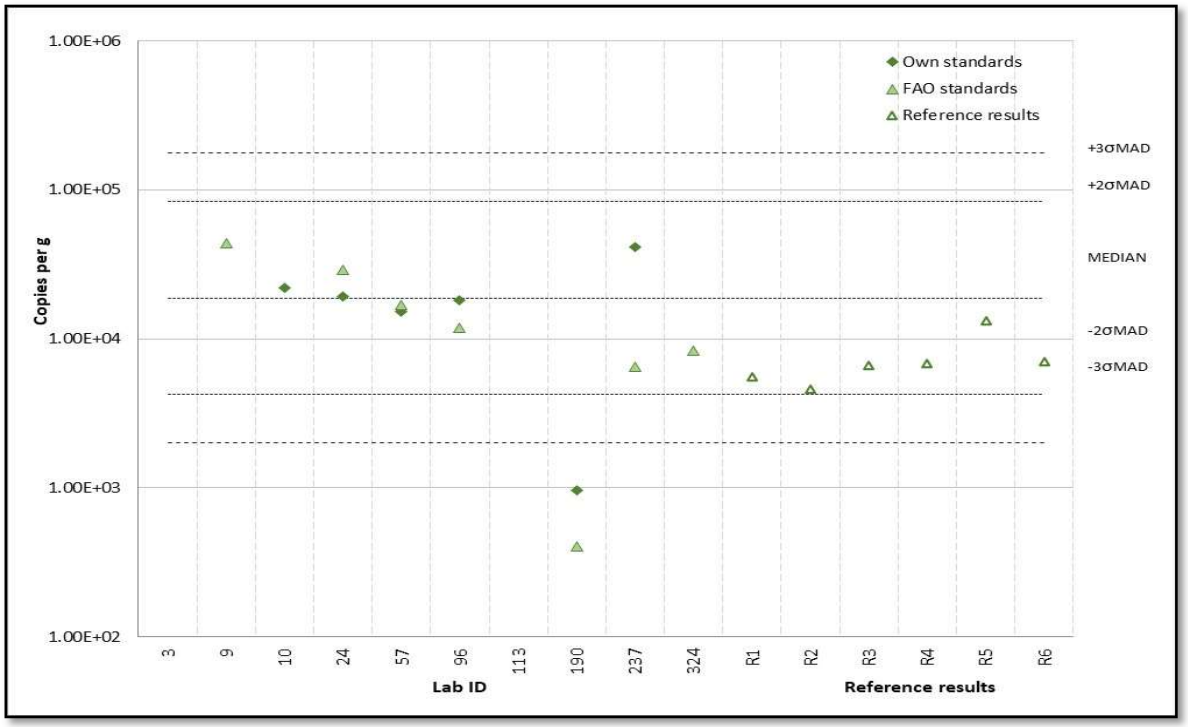
Shellfish sample 3 – HAV



Shellfish sample 4 – GII



Shellfish sample 4 – HAV



Appendix 6 - Results and methods used.

Lab ID	Shellfish sample 1			Shellfish sample 2			Shellfish sample 3			Shellfish sample 4			Extraction		RT PCR		Primers		
	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	GI	GII	HAV	Virus	RNA	Method	Reagents	GI	GII	HAV
2	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	F	L-1	L	P
3	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-1	L	L
9	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-1	L	L
10	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-1	L	L
20	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	H	M	M	M
24	-	-	-	+	+	+	+	-	+	-	+	+	A	C	E	J	N	O	L
57	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-2	L	L
96	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-2	L	L
113	-	-	NE	+	-	NE	-	+	NE	+	-	NE	A	B	D	K	L-2	L	-
190	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	H	L-2	L	L
237	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	G	L-2	L	L
246	NE	NE	-	NE	NE	+	NE	NE	+	NE	NE	+	A	B	D	G	-	-	L
324	-	-	-	+	+	+	+	-	+	-	+	+	A	B	D	H	M	M	M

(For key to method codes see page 24)

Key: NE = target virus not examined, yellow shading = denotes false negative results, red shading = false positive results, grey shading = method elements as described in the main body and informative annexes of ISO 15216-1.

Key to method codes

Virus extraction methods	
A	Proteinase K digestion
RNA extraction methods	
B	NucliSens Magnetic extraction reagents (BioMerieux)
C	Roche High Pure Viral Nucleic Acid Kit
RT-PCR methods	
D	Real-time (quantitative) PCR - one-step
E	Real-time (quantitative) PCR - two-step
RT-PCR reagents	
F	Norovirus GI and HAV: TaqMan® Fast Virus 1-Step Master Mix (Applied Biosystems). Norovirus GII: RNA Ultrasense (Invitrogen)
G	RNA Ultrasense (Invitrogen)
H	Ceeram Tools
J	RT: Invitrogen Superscript III ; PCR: Invitrogen Platinum® qPCR
K	qRT PCR LC 480 Master Hydrolysis Probes (Roche)
Primers/probes	
L	ISO 15216-1; 1) with TM9 probe for NoV GI; 2) with NVGG1p probe for NoV GI
M	Ceeram Tools (sequences as L-2)
N	Wolf et al, 2010
O	Kageyama et al, 2003
P	OPFLP-07

Appendix 7 - Details of laboratory's own quantification standards (preparation and quantification)

LAB ID	
3	Linearised ISO 15216-1 plasmid DNA, quantified using fluorimetry
10	PCR product amplified from ISO 15216-1 plasmid, quantified using A260 spectrophotometry
24	Linearised plasmid DNA
57	cDNA from a commercial supplier
96	Linearised ISO 15216-1 plasmid DNA, quantified using A260 spectrophotometry and fluorimetry
113	Standards provided in Ceeram Tools kit
190	Standards provided in Ceeram Tools kit
237	Commercially produced linear dsDNA (quantified by supplier)
324	Standards provided in Ceeram Tools kit

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We are the government's marine and freshwater science experts. We help keep our seas, oceans and rivers healthy and productive and our seafood safe and sustainable by providing data and advice to the UK Government and our overseas partners. We are passionate about what we do because our work helps tackle the serious global problems of climate change, marine litter, over-fishing and pollution in support of the UK's commitments to a better future (for example the UN Sustainable Development Goals and Defra's 25 year Environment Plan).

We work in partnership with our colleagues in Defra and across UK government, and with international governments, business, maritime and fishing industry, non-governmental organisations, research institutes, universities, civil society and schools to collate and share knowledge. Together we can understand and value our seas to secure a sustainable blue future for us all, and help create a greater place for living.

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