National Reference laboratory
for monitoring bacteriological and viral
contamination of bivalve molluscs

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The effect of time and temperature on the *Escherichia coli* content of live bivalve molluscs

Introduction

In the EU, the hygiene status of a shellfish harvesting area, and thus the degree of post-harvest treatment required before sale for consumption, is based on a time series assessment of the faecal indicator bacterium *Escherichia coli* in the shellfish in the area. It is important that the *E. coli* concentration in the shellfish received by the testing laboratory reflect those that were extant at the time of sampling. Sampling protocols in use in the UK specify both a temperature range and a maximum transport period in order to achieve this. The UK National Reference Laboratory currently recommends a temperature of less than 8°C (without freezing) and a maximum time between sampling and commencement of analysis of 24 hours.

Cook and Ruple (1989) reported that no changes were seen in either faecal coliforms or *E. coli* concentrations in oysters (*Crassostrea virginica*) stored at 10°C while storage at 22°C yielded an increase in both indicators by 3 days (no testing was done at 2 days).

Initial experiments undertaken in the UK in support of definition of protocols for the sampling, sample transport and testing of bivalve mollusc harvesting areas for *Escherichia coli* showed no significant effect of sample storage at 4°C, 10°C or 13°C for up to 72 hours (Lart & Hudson 1993). The effect of storage at 19-22°C differed between three experiments. One (mussels) showed no change, one (mussels) showed significant growth by 48 hours and one (Pacific oysters) showed some decline.

Subsequent experiments undertaken at FRS Aberdeen showed that there was a general tendency for *E. coli* levels to decline with storage at 2-8°C for up to 72 hours. In some experiments a significant effect was noted at 24 hours.

Changes in *E. coli* concentration in bivalve molluscs during transit of samples to the laboratory could affect the classification status assigned to harvesting areas and therefore could affect the level of public health protection given to consumers.

The present work was proposed in order to add to the data available on the effect of storage/transport at low temperature on the *E. coli* concentrations in bivalve molluscs in order to inform a review of recommended practices in the UK. Initial trials were undertaken during 2004 and 2005. Following a review of the results, further trials were

undertaken in 2006 and 2007 in order to obtain supplementary data. In particular, the range of temperatures was modified for the latter trials, primarily because significant mortalities in stored shellfish were observed at 25°C, but also to give better resolution at the lower temperatures.

Materials and Methods

2004 – 2005 Trials

Four separate trials were conducted, two using Pacific oysters (*Crassostrea gigas*) and two using mussels (*Mytilus edulis*). For the first trial oysters were obtained from an experimental site subject to contamination by secondary-treated sewage. The second trial used oysters from a Class B harvesting area. Both mussel trials used batches collected from a Class C harvesting area.

Between 300-1000 shellfish were harvested for each of the trials and transported under refrigerated conditions to the laboratory at Cefas. The first oyster trial was initiated on the day of harvesting. Shellfish for the second oyster trial (taken from a different location) were stored overnight at 4°C and the trial initiated the following day. Both mussel trials were begun on the day of harvest.

On arrival at the laboratory the shellfish were cleaned and placed into plastic bags of between 30 and 50 shellfish each. A minimum of three bags was refrigerated in sample transport boxes at each of the trial temperatures of 4°C, 15°C or 25°C. This procedure was undertaken in order to provide storage conditions that were as similar as possible to those used for sample transport. Replicate samples were also tested at the time of commencement of each trial to determine the *E. coli* levels at time zero.

2006 – 2007 Trials

A second set of trials was conducted using four different species: cockles (*Cerastoderma edule*), manila clams (*Tapes philippinarum*), mussels (*Mytilus edulis*) and pacific oysters (*Crassostrea gigas*). All of the shellfish were collected from a class B harvesting site. Between 300 and 1000 shellfish were harvested for each of the trials and transported at ambient temperature to the laboratory at Cefas within 4 hours. The trials were initiated on the day of harvesting.

On arrival at the laboratory the shellfish were cleaned and placed into plastic bags of between 30 and 70 shellfish each. Three of the plastic bags were placed in storage sample boxes at each of the following temperatures: 4°C, 10°C, 15°C and 20°C. Replicate samples were tested at the time of commencement of each trial to determine the levels of *E. coli* at time zero.

Shellfish analysis

One bag was removed from each box at each temperature and used for subsequent analysis at time 24, 48 and 72 hours. The shellfish in each bag were split into three or four sub-samples prior to analysis (see Results section). The samples were cleaned and prepared for analysis as described in the Appendix to Donovan *et al*. 1998. Each subsample was tested for *E. coli* by the method given in the Appendix to Donovan, *et*

al. 1998 for the 2004/5 trials and the method given in ISO 16649-3:2005 for the 2006/7 samples (these methods are essentially equivalent). As all of the trials were undertaken prior to the publication of ISO 7218:2007, the *E. coli* Most Probable Number (MPN) per 1OOg was determined from the tables given in the Appendix to Donovan *et al*. 1998.

Analysis carried out on the shellfish was conducted as previously stated in the first experiment under shellfish analysis.

Statistical analyses

Statistical analyses for both experiments were undertaken using Minitab v14. For each trial, ratios (together with 95% confidence intervals) were determined for the geometric mean *E. coli* concentrations per 100g at each time/temperature combination and the geometric mean *E. coli* concentrations per 100g at time 0. One-way Analysis of Variance (ANOVA) was conducted separately for each trial/temperature combination using on $\log_{10}$-transformed *E. coli* concentrations as the response variable and time as the factor. In addition, for the second series of trials, two-way ANOVA was conducted on the log-transformed *E. coli* results for each trial using storage temperature and storage time as the two factors.

Results

2004 – 2005 trials

Oyster Trial 1

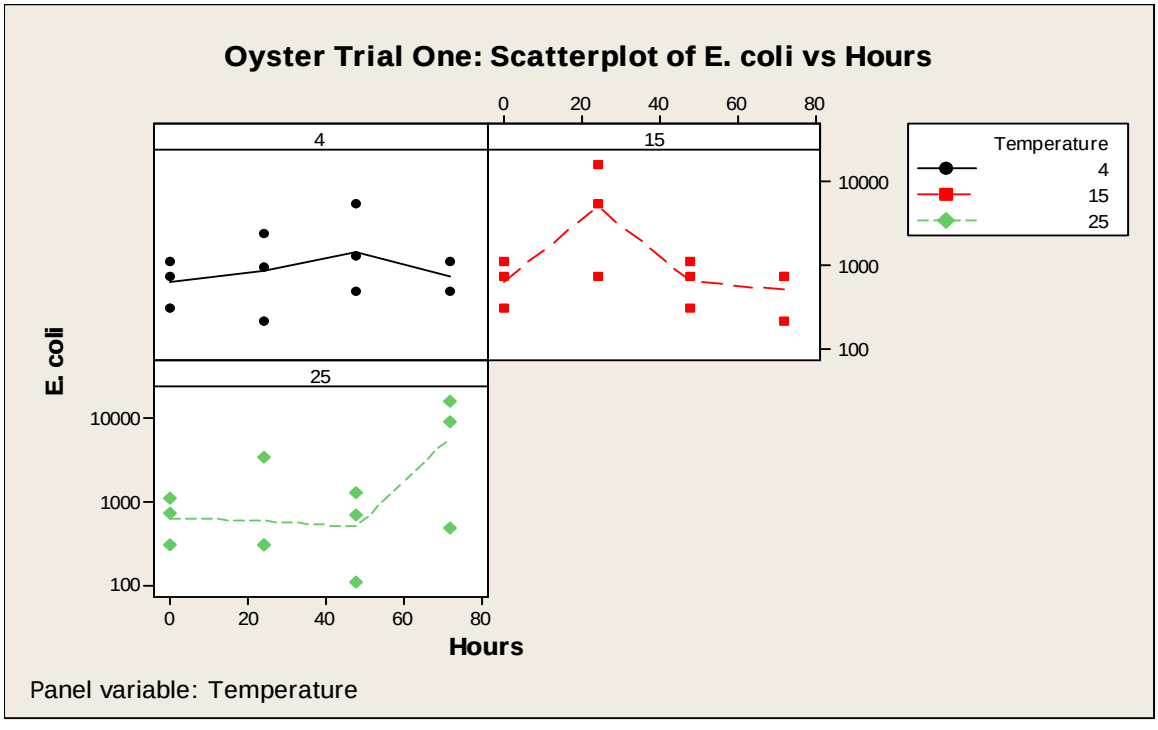
Oysters were harvested from the experimental site on 2nd June 2004 and transported to the laboratory at Cefas, Weymouth. Samples analysed at time 0 were tested within four hours of receipt in the laboratory. The results are presented in Table 1 and Figure 1.

Table 1: Results of *E. coli* analysis for oyster trial 1

Temp of storage °C	Hours in storage	Replicate *E. coli* results/100g			Geometric mean *E. coli* /100g	Ratio relative to time 0 (95% CI)
		1	2	3		
N/A	0	750	1100	310	640	
4	24	2400	950	220	800	1.3 (0.10, 15)
	48	5400	1300	500	1520	2.4 (0.20, 29)
	72	500	1100	NT	740	1.2 (0.11, 12)
15	24	16000	750	5400	4020	6.3 (0.10, 413)
	48	1100	750	310	640	1.0 (0.23, 4.4)
	72	750	220	750	500	0.79 (0.13, 4.6)
25	24	310	310	3500	700	1.1 (0.02, 51)
	48	110	1300	700	460	0.73 (0.02, 26)
	72	16000	500	9100	4180	6.6 (0.05, 880)

NT = not tested

Figure 1. Oyster trial 1: effect of time and temperature on *E. coli*



Oyster trial 2

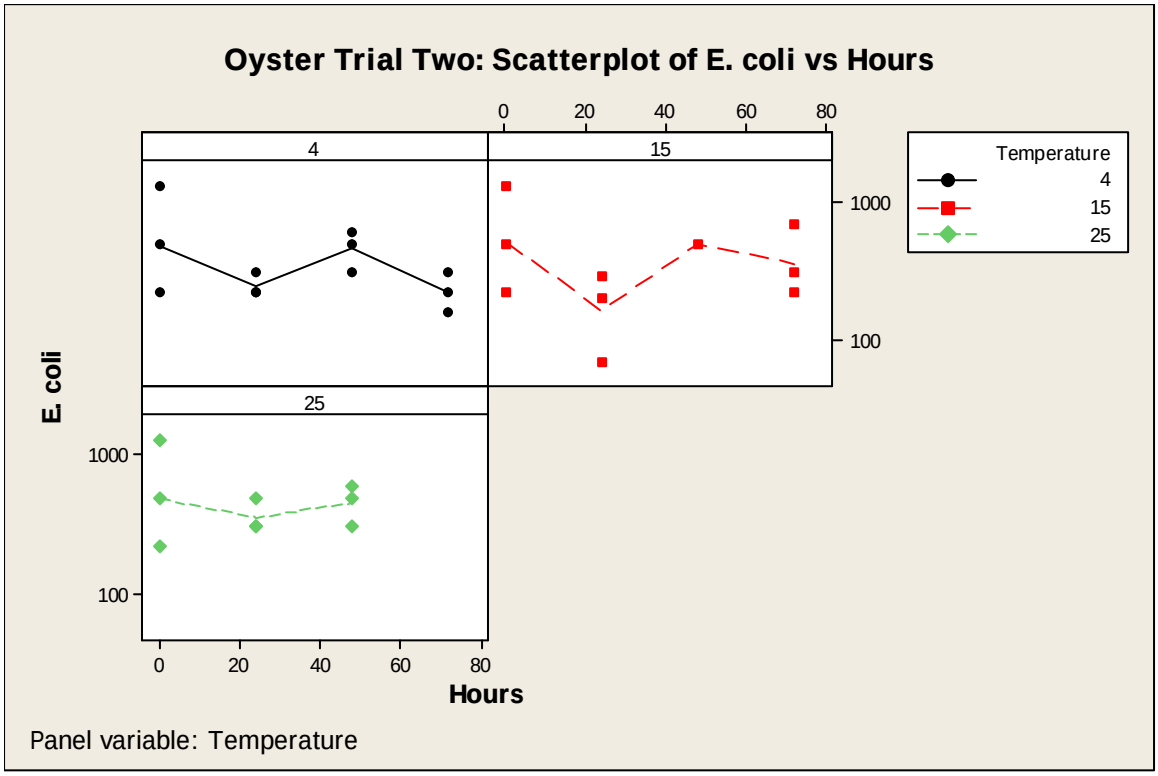
Oysters were harvested from a shellfishery and transported to a holding company on 25th August 2004. They were refrigerated overnight and transported to Cefas under non-refrigerated conditions for one hour. The samples analysed at time 0 were tested within five hours of receipt in the laboratory. The results are presented in Table 2 and Figure 2.

Table 2: Results of *E. coli* analysis for oyster trial 2

Temp of storage °C	Hours in storage	Replicate *E. coli* results/100g			Geometric mean *E. coli* /100g	Ratio relative to time 0 (95% CI)
		1	2	3		
N/A	0	220	500	1300	520	
4	24	220	220	310	250	0.47 (0.05, 4.5)
	48	310	500	600	450	0.87 (0.08, 9.2)
	72	160	220	310	190	0.42 (0.04, 4.5)
15	24	70	200	290	160	0.31 (0.04, 2.5)
	48	310	310	1300	500	0.96 (0.10, 8.9)
	72	220	310	700	360	0.69 (0.10, 5.0)
25	24	310	310	500	360	0.70 (0.07, 7.0)
	48	500	INS	INS	500	-
	72	INS	INS	INS	-	-

INS = insufficient live oysters remaining

Figure 2. Oyster trial 2: effect of time and temperature on *E. coli*



Mussel Trial 1

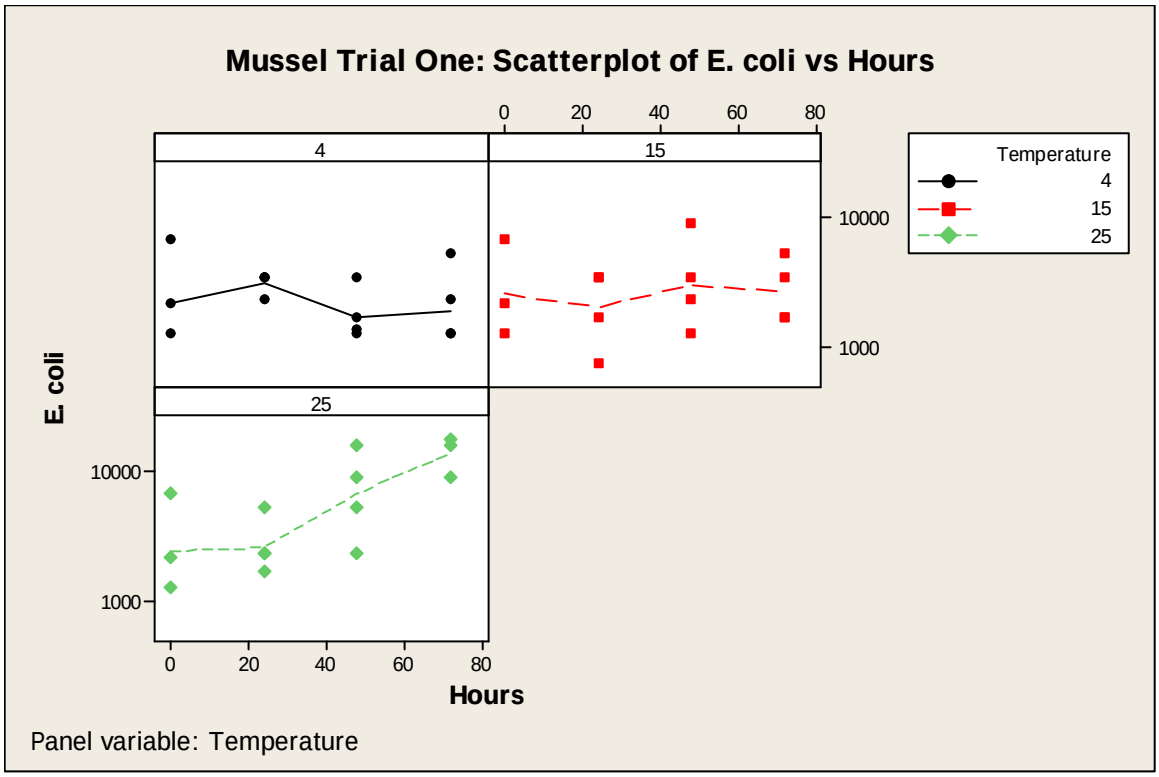
Mussels were harvested from a shellfishery on 24th January 2005 and transported to the laboratory at Cefas, Weymouth. Samples analysed at time 0 were tested within two hours of receipt in the laboratory. The results are presented in Table 3 and Figure 3.

Table 3: Results of *E. coli* analysis for mussel trial 1

Temp of storage °C	Hours in storage	Replicate *E. coli* results/100g				Geometric mean *E.coli* /100g	Ratio relative to time 0 (95%CI)
		1	2	3	4		
N/A	0	7000	2200	1300	NT	2720	
4	24	3500	3500	2400	3500	3190	1.2 (0.13, 10)
	48	1300	1700	3500	1400	1810	0.67 (0.06, 7.0)
	72	1300	2400	5400	1300	2160	0.80 (0.12, 5.4)
15	24	3500	750	3500	1700	1990	0.73 (0.10, 5.2)
	48	9100	1300	3500	2400	3160	1.16 (0.20, 6.9)
	72	1700	5400	1700	3500	2720	1.00 (0.16, 6.2)
25	24	5400	2400	2400	1700	2700	0.99 (0.09, 11)
	48	5400	9100	16000	2400	6590	2.4 (0.41, 14)
	72	18000	16000	9100	INS	13800	5.1 (0.50, 52)

INS = insufficient live mussels remaining

Figure 3. Mussel trial 1: effect of time and temperature on *E. coli*



Mussel Trial 2

Mussels were harvested from a shellfishery on 21st February 2005 and transported to the laboratory at Cefas, Weymouth. Samples analysed at time 0 were tested within two hours of receipt in the laboratory. The results are presented in Table 4 and Figure 4.

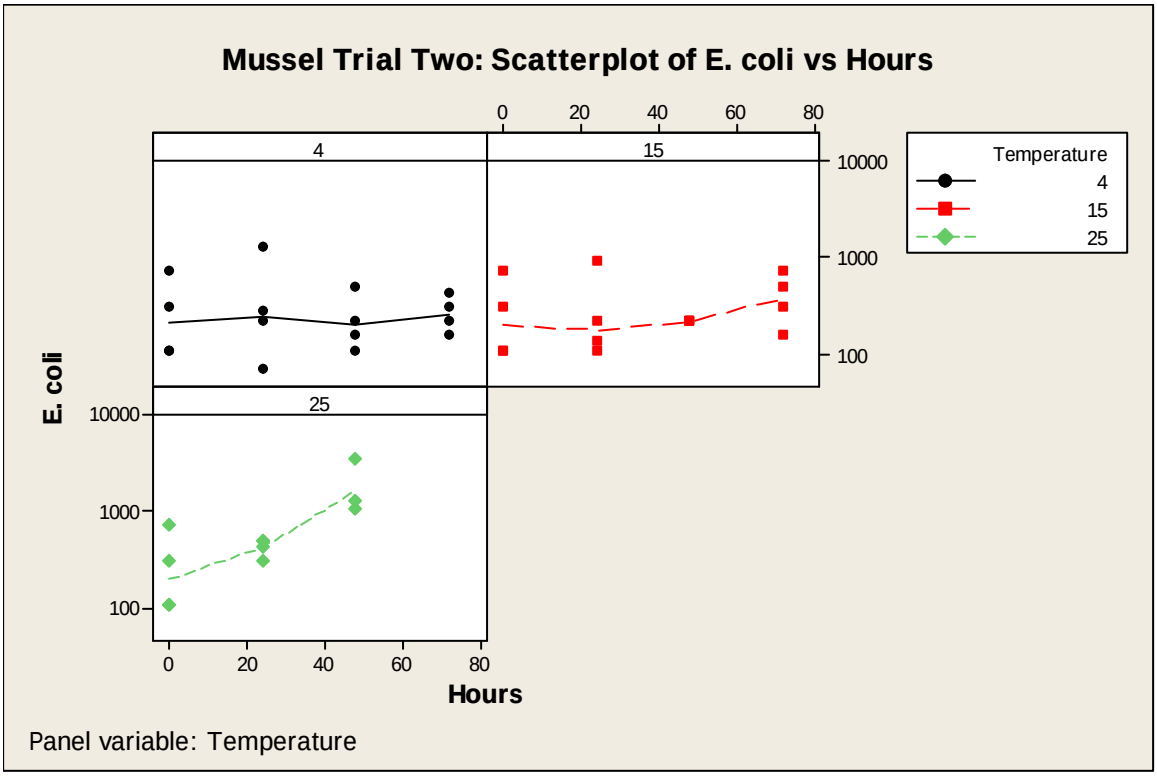
Table 4: Results of *E. coli* analysis for mussel trial 2

Temp of storage °C	Hours in storage	Replicate *E. coli* results/100g				Geometric mean *E.coli* /100g	Ratio relative to time 0 (95%CI)
		1	2	3	4		
N/A	0	750	110	110	310	210	
4	24	220	290	1300	70	280	1.2 (0.17, 8.4)
	48	500	160	220	110	210	0.91 (0.21, 3.9)
	72	310	160	220	430	260	1.1 (0.28, 4.7)
15	24	950	220	140	110	240	1.0 (0.19, 5.8)
	48	220	220	220	220	220	1.1*
	72	310	750	500	160	370	1.6 (0.37, 6.9)
25	24	430	500	500	310	430	1.9 (0.41, 8.5)
	48	3500	1100	1300	INS	1710	7.4 (1.5, 38)
	72	INS	INS	INS	INS	-	-

INS = insufficient live mussels remaining

- All values for this time/temperature combination identical

Figure 4. Mussel trial 2: effect of time and temperature on *E. coli*



*Statistical Analyses (*2004 *– 2006 Trials)*

Ratios of the geometric mean *E. coli* concentration after storage versus that at time zero ranged from 0.3 (implying die-off) to 6.6 (implying multiplication). However, the 95% confidence intervals for these values included 1 (no change) in most cases.

One-way ANOVA was conducted separately on the results of each trial at a single temperature, effectively comparing the effect of time of storage at a single temperature on the *E. coli* concentration. For all trials except one undertaken at temperatures of 4°C and 15°C, the p values were significantly greater than 0.1, indicating that the effect of storage time at these temperatures was not significant. For the single trial at 15°C showing a p value <0.1 (Oyster trial 1; p=0.095), the weakly significant effect of time was due to two of the three replicate results at 24 hours being higher than all other results at 0, 48 and 72 hours. This did not therefore represent a consistent effect with time and may have been due to unknown factors or random effects in the allocation of sub-samples, subsequent sample treatment or bacteriological analysis. However, it could represent a real increase, with subsequent inhibition of *E. coli* detection by concomitant proliferation of non-target bacteria.

The one-way ANOVAs for the two oyster trials at 25°C also yielded p values significantly greater than 0.1. The first mussel trial gave a p value of 0.029 for the results at this temperature, indicating a significant effect of storage time. The graph shows that by 48 hours the *E. coli* concentrations had started to increase over those obtained at 0 hours, and that the *E. coli* concentrations were markedly higher by 72 hours. There was far weaker evidence for this in the results of the second mussel trial, where the p value was only 0.12. However, this was undoubtedly affected by the fact that all of the shellfish were dead by 72 hours and therefore no results were available for this time.

2006 – 2007 Trials

Samples were taken from the class B shellfishery on the following dates:

Cockles: 30.01.2006
Mussels: 08.01.2007
Oysters: 16.01.2007
Clams: 30.01.2007

The results for the four trials undertaken in 2006 and 2007 are presented in Tables 5 to 8 and Figures 5 to 8 for cockles, mussels, oysters and clams respectively.

Statistical Analyses (2006-2007 trials)

Ratios of the geometric mean *E. coli* concentration after storage versus that at time zero ranged from 0.2 (implying die-off) to 4.4 (implying multiplication). However, the 95% confidence intervals for these values included 1 (no change) in most cases.

Two-way ANOVA was conducted on each trial to look at the individual effects the temperature and the hours in storage had on the concentrations of *E. coli.* The oyster and mussel trials gave p values for the temperature effect below 0.05 (p=0.036 and 0.033 respectively). For both species, the overall geometric mean *E. coli* concentrations were lower at 10°C and 15°C than at 4°C and 20°C. The oyster and clam trials showed p values below 0.05 for the effect of time in storage (P= 0.011 and p=0.001 respectively). For both species, the overall geometric mean *E. coli* concentrations decreased with time of storage, with the concentration after 72 hours being markedly lower than that at time 0. A similar pattern was seen with the mussels, although the effect was only weakly significant (p=0.058). With these three species, the overall geometric mean *E. coli* concentrations at 24 and 48 hours were similar. The interaction between storage temperature and time in storage on the concentration of *E. coli* was either only weakly significant, or not significant, with all p values being greater that 0.08.

One-way ANOVA was conducted separately on the results of each trial at a single temperature, effectively comparing the effect of time of storage at a single temperature on the *E. coli* concentration. Trials carried out with clams at 4°C and 10°C and cockles at 4°C gave p values of less than 0.05 (the values were 0.028, 0.003 and 0.001 respectively), indicating a significant change of *E. coli* with time at these temperatures. The trial with mussels at 10°C showed a weakly significant effect of time of storage (p=0.06). In the case of these clam and mussel trials, *E. coli* declined with time. The significant effect at 4°C in the cockle trial was related to the fact that the results at 24 hours were markedly higher than at 0, 48 and 72 hours. This effect cannot be explained on the basis of current information but is similar to that seen in Oyster Trial 1 in the 2004/2005 series. For all other trials, the p values were greater than 0.10 indicating that the effect of storage time at these temperatures was not significant.

Table 5: Results of *E. coli* analysis for Cockles

Temp of storage (°C)	Hours in storage	MPN *E. coli* results/100g				Geometric mean *E.coli*/100g	Ratio relative to time 0 (95% CI)
		Sample 1		Sample 2			
		Replicate 1	Replicate 2	Replicate 1	Replicate 2		
N/A	0	40	200	90	160	104	
4°C	24	220	500	750	500	451	4.35 (1.40, 13.5)
	48	70	110	50	70	72	0.70 (0.23, 2.08)
	72	110	110	40	40	66	0.64 (0.19, 2.10)
10°C	24	110	250	110	160	148	1.43 (0.46, 4.45)
	48	110	160	110	220	144	1.39 (0.46, 4.16)
	72	200	500	40	70	129	1.25 (0.23, 6.91)
15°C	24	200	110	160	110	140	1.35 (0.39, 4.66)
	48	90	160	160	40	98	0.95 (0.27, 3.30)
	72	200	200	70	40	103	0.99 (0.25, 3.96)
20°C	24	140	70	160	290	146	1.41 0.43, 4.63)
	48	950	110	140	310	260	2.50 (0.53, 11.8)
	72	130	220	160	200	174	1.68 (0.50, 5.59)

Figure 5: *E. coli* analysis for Cockles

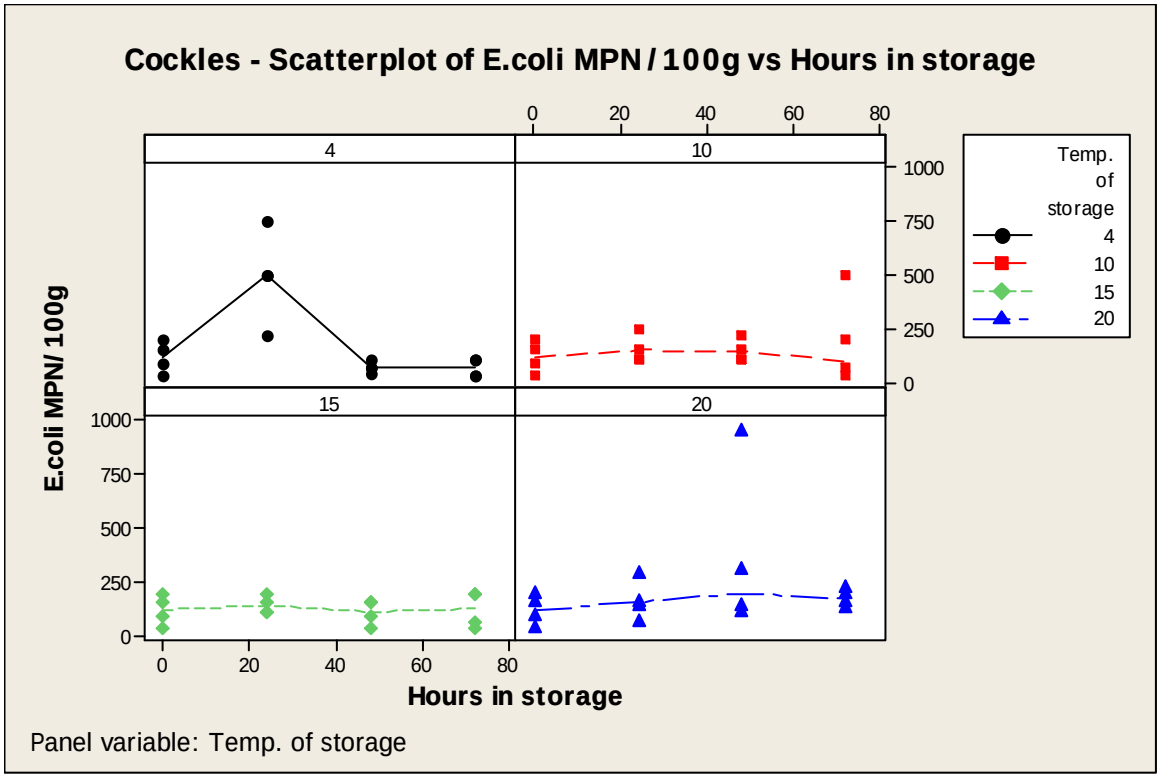


Table 6: Results of *E. coli* analysis for mussels

Temp of storage (°C)	Hours in storage	MPN *E. coli* results/100g				Geometric mean *E.coli*/100g	Ratio relative to time 0 (95% CI)
		Sample 1		Sample 2			
		Replicate 1	Replicate 2	Replicate 1	Replicate 2		
N/A	0	11000	28000	22000	91000	28000	
4°C	24	35000	17000	24000	16000	21900	0.78 (0.17, 3.54)
	48	17000	35000	22000	24000	23700	0.84 (0.19, 3.70)
	72	35000	22000	5400	5400	12200	0.44 (0.08, 2.34)
10°C	24	16000	9100	16000	16000	13900	0.50 (0.11, 2.16)
	48	9100	24000	16000	16000	15400	0.55 (0.14, 2.10)
	72	9100	17000	2400	5400	6690	0.24 (0.05, 1.13)
15°C	24	17000	24000	16000	35000	21900	0.78 (0.17, 3.54)
	48	11000	16000	11000	24000	14700	1.91 (0.14, 1.97)
	72	11000	17000	13000	3100	9320	0.33 (0.07, 1.48)
20°C	24	5400	35000	17000	54000	20400	0.73 (0.13, 4.06)
	48	35000	14000	35000	16000	22900	0.82 (0.20, 3.31)
	72	91000	35000	35000	70000	52900	1.89 (0.47, 7.62)

Figure 6: *E. coli* analysis for mussels

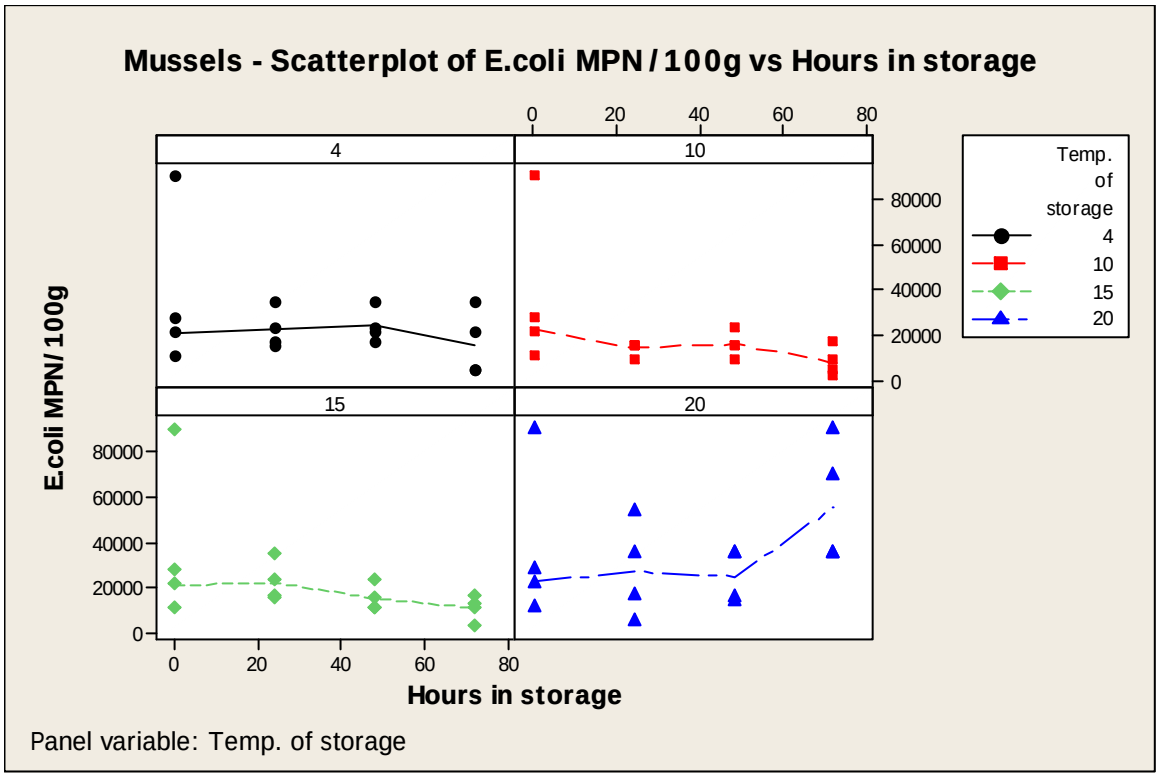


Table 7: Results of *E. coli* analysis for oysters

		MPN *E. coli* results/100g					
Temp of storage (°C)	Hours in storage	Sample 1		Sample 2		Geometric mean *E.coli*/100g	Ratio relative to time 0 (95% CI)
		Replicate 1	Replicate 2	Replicate 1	Replicate 2		
N/A	0	950	500	500	2400	869	
4°C	24	500	430	750	410	507	0.58 (0.17, 2.05)
	48	750	1300	1700	750	1060	1.22 (0.37, 3.94)
	72	500	750	500	500	553	0.64 (0.19, 2.17)
10°C	24	310	500	500	430	427	0.49 (0.14, 1.69)
	48	750	1300	220	310	508	0.58 (0.14, 2.40)
	72	220	200	750	290	313	0.36 (0.11, 1.23)
15°C	24	500	500	310	310	394	0.45 (0.13, 1.60)
	48	500	310	310	310	349	0.40 (0.12, 1.39)
	72	310	750	750	160	409	0.47 (0.12, 1.83)
20°C	24	1300	2400	750	1300	1320	1.52 (0.49, 4.72)
	48	500	700	310	750	534	0.61 (0.19, 1.99)
	72	310	430	1100	500	520	0.60 (0.18, 1.94)

Figure 7: *E. coli* analysis for oysters

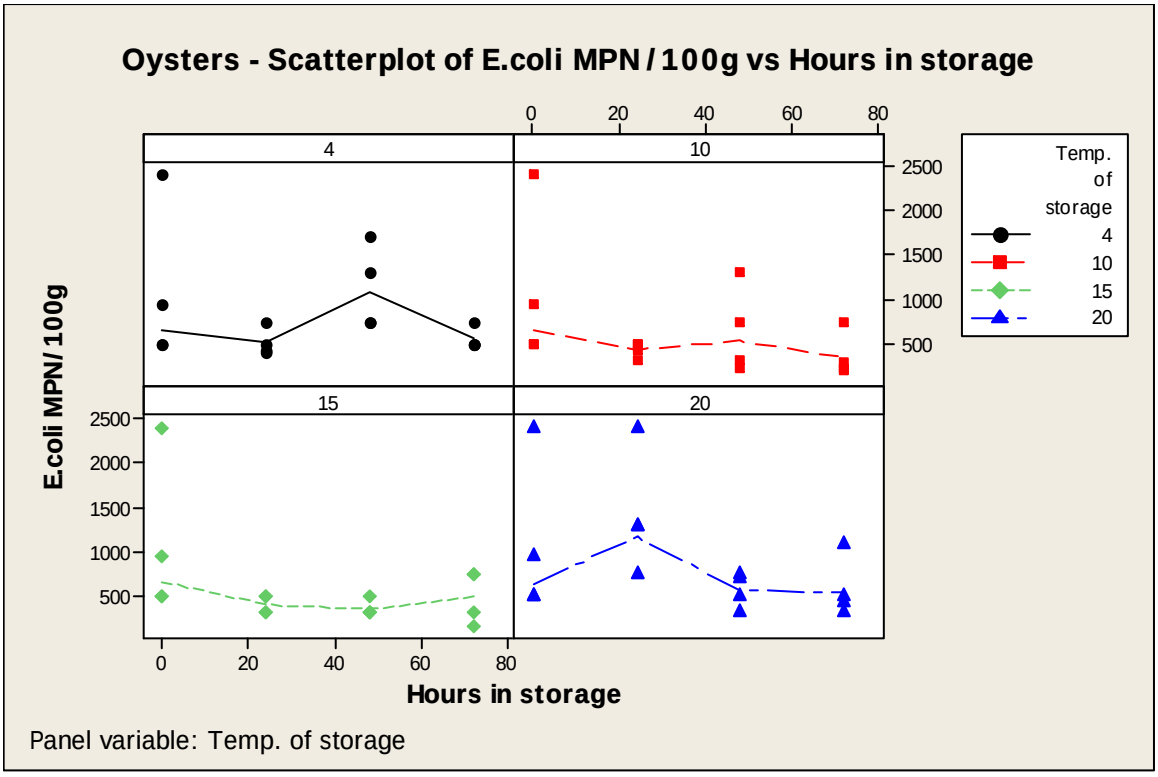
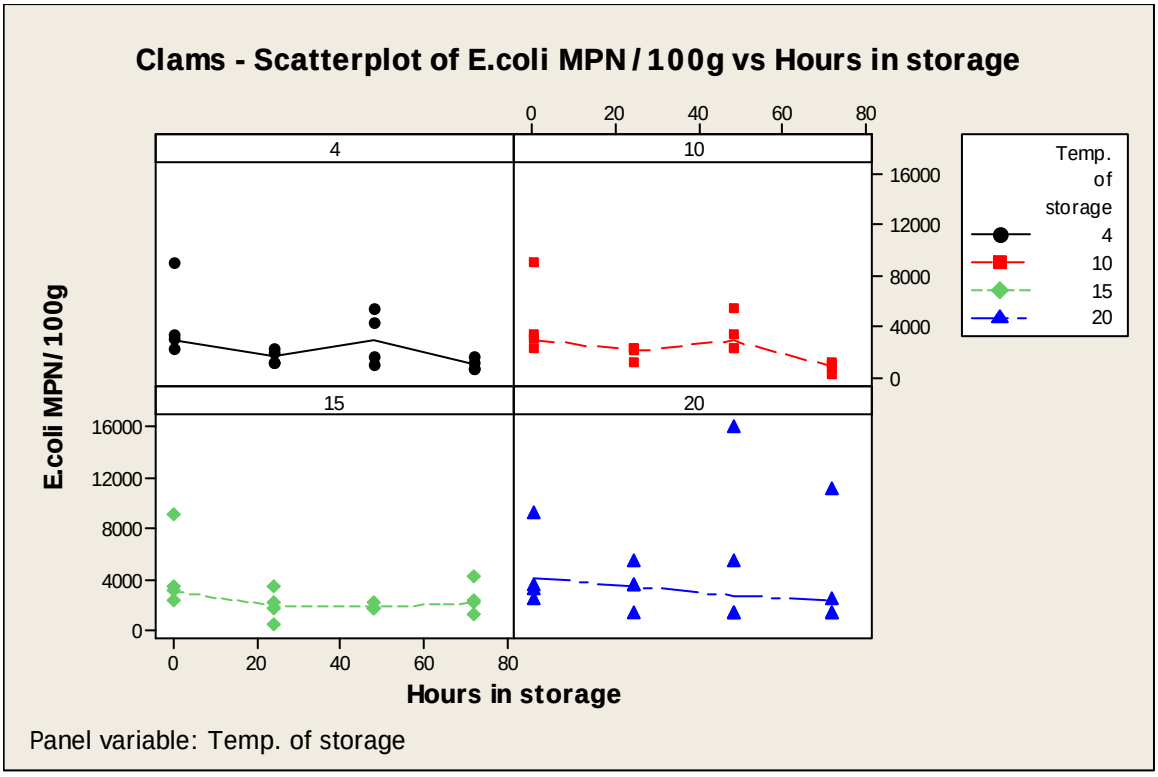


Table 8: Results of *E. coli* analysis for Manila Clams

Temp of storage (°C)	Hours in storage	MPN *E. coli* results/100g				Geometric mean *E.coli*/100g	Ratio relative to time 0 (95% CI)
		Sample 1		Sample 2			
		Replicate 1	Replicate 2	Replicate 1	Replicate 2		
N/A	0	9100	3100	2400	3500	3923	
4°C	24	1300	2000	2400	1300	1688	0.43 (0.17, 1.08)
	48	5400	1700	4300	1100	2567	0.65 (0.19, 2.23)
	72	1700	750	1300	750	1056	0.27 (0.11, 0.67)
10°C	24	2200	2400	2400	1300	2015	0.51 (0.21, 1.27)
	48	2400	2400	3500	5400	3230	0.82 (0.34, 2.02)
	72	1100	1300	750	290	747	0.19 (0.06, 0.60)
15°C	24	1700	500	2200	3500	1599	0.41 (0.11, 1.50)
	48	1700	1700	1700	2200	1813	0.46 (0.18, 1.19)
	72	4300	2400	2200	1300	2331	0.59 (0.22, 1.58)
20°C	24	5400	3500	1300	3500	3045	0.78 (0.26, 2.28)
	48	5400	16000	1300	1300	3476	0.89 (0.14, 5.78)
	72	1300	1300	11000	2400	2584	0.66 (0.13, 3.32)

Figure 8: *E. coli* analysis for Manila Clams



Discussion

The key outcome from the 2004/5 series of trials was that a significant increase of *E. coli* was seen in mussels at 25°C and this reinforced the need to control the temperature of samples during transport. Figure 6 shows a similar pattern in mussels at 20°C although the effect was not as marked as previously seen at 25°C.

The overall effect of storage temperature seen in the 2006/7 oyster and clam trials, with lower *E. coli* results at 10°C and 15°C than at 4°C and 20°C, is difficult to explain but may be due to differences in survival and multiplication of *E. coli* and competitor bacteria at the different temperatures. These results would emphasize that the temperature should be maintained below 10°C during sample transport and storage. This is in agreement with both current NRL advice (below 8°C) and ISO 7218:2007 (1 to 8°C).

In general, in the 2006/7 trials, ratios of the geometric mean results at 24, 48 and 72 hours were less than one, indicating a general trend to lower results with time, although the confidence intervals for the ratios included 1 in most cases, indicating that the difference was not significant. Where significant effects were seen with time of storage at individual temperatures for a species, these related to a decline in *E. coli* concentration with storage, apart from an anomalous result in the cockles held at 4°C.

The overall effect of time of storage seen with oysters, clams and mussels in the 2006/7 trials emphasizes the need for the *E. coli* test to commence as soon as is practically possible after sampling. The tendency towards stability between 24 and 48 hours supports the use of the latter limit in exceptional cases. The marked differences seen by 72 hours means that the results of samples tested more than 48 hours after sampling should not be accepted as valid.

Recommendations

The sample transport temperature range of 1°C to 8°C recommended in ISO 7218:2007 for “other products not stable at ambient temperature” be adopted for the official control *E. coli* testing of live bivalve molluscs sampled for official control purposes in the UK.

Analysis to be undertaken as soon as practically possible after sampling with a normal limit of 24 hours after sampling and an absolute limit of 48 hours after sampling in exceptional cases.

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