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

ESCHERICHIA COLI CONTENT OF LIVE BIVALVE MOLLUSCS: THE EFFECT OF RINSING SAMPLES BEFORE TRANSPORTATION TO THE LABORATORY

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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 from the area. It is important that the *E. coli* concentration in the shellfish received by the testing laboratory reflects that which was extant at the time of sampling. The UK NRL advice on sampling currently contains the following with respect to preparation of samples:

"Any mud and sediment adhering to the shellfish should be removed. This is best achieved by rinsing/scrubbing with clean seawater or fresh water of potable quality. If these are unavailable the seawater from the immediate area of sampling may be used instead. Do not totally re-immerse the shellfish in water as this may cause them to open. Allow to drain before placing in a food grade plastic bag. The container/bag should be labelled with the sender's reference number and any other relevant information (e.g. species)."

It has been identified that, in some areas, samplers may have difficulties undertaking this rinsing operation and that a proportion of samples may reach the laboratory still covered with a significant amount of sediment. Such sediment could be the source of additional contamination of the bivalves and, if so, the measured *E. coli* concentration would not relate to that in the bivalves as sampled. It had been proposed that laboratories should reject samples if they were received still covered with sediment but there was an unwillingness to do so unless the problem had been explicitly confirmed by a study. However, in some areas members of the shellfish industry have tried to contest the results of classification samples taken by local authorities on the basis that the outside of the shells were not essentially sterile prior to bagging.

The present work was therefore undertaken to determine the effect of rinsing/not rinsing following sampling but prior to transport on the *E. coli* content of bivalve molluscs. Cockles and mussels were chosen as the species for initial study as these had been identified as the ones most often received in a potentially unsatisfactory state. In a second study, this was extended to incorporate oysters as it was identified by the shellfish industry that these were more difficult to clean in the field. This further work also included additional elements to look at the potential effect of residual fluid in the sample bags.

Materials and Methods

Experimental procedure - Study 1

Two class B harvesting areas were identified in the UK (Areas 1 and 2). In Area 1, both mussels (Site A) and cockles (Site B) were available and samples of each species were obtained on 4 separate dates. In Area 2, only mussels were available but samples were obtained on 5 separate dates. Sediment samples were taken at the same time from the upper 1cm layer in the immediate vicinity of the shellfish. No sediment samples were received for the last two sampling occasions in Area 2.

Approximately half of the sample collected from each site was rinsed and drained at the site of collection while the rest of the shellfish were left with sediment residue on their shells. Each was placed in a separate food grade bag and placed in a cool box with frozen gel packs. Sediment samples were taken into sterile screw-topped plastic containers. Shellfish and sediment samples were sent to the Cefas Weymouth laboratory by TNT next day delivery.

Experimental procedure - Study 2

Mussels, cockles and oysters were collected from Poole harbour a class B harvesting site and samples of each species were taken on 3 separate dates. Three sediment samples were taken at the same time from the upper 1cm layer in the immediate vicinity of the shellfish. At the point of harvesting the batch of shellfish were divided into two. One half of the batch were rinsed and the other half were left unrinsed. Both the rinsed and unrinsed batches were divided into three with sufficient shellfish for two samples in each of the test portions. Each of these test portions were then prepared for transportation and placed either in a plastic bag with the drain water, in a plastic bag without the drain water or in a net bag. The bags and sediment samples were placed in cool boxes with frozen gel packs and transported to the Cefas laboratory, Weymouth within 4 hours of harvest.

Shellfish

On arrival in the laboratory the samples were cleaned and prepared for analysis as described in the Appendix to Donovan *et al*. 1998. Each sample was tested in duplicate for *E. coli* by the method given in ISO 16649-3:2005. As the studies were undertaken prior to the publication of ISO 7218:2007, the *E. coli* Most Probable Number (MPN) per 100g was determined from the tables given in the Appendix to Donovan *et al*. 1998.

Sediment

E. coli analysis was carried out on each sediment sample by resuspending 10g of sediment into 40ml 0.1% w/v peptone water. The resuspended sediment was ultrasonicated twice at an output of 20kHz for a 1 second duration. A further 50ml of 0.1% w/v peptone water was added, mixed and left for 10 minutes. Duplicate copies of both 10ml and 1ml volumes of each sediment sample were tested by membrane filtration using a 0.45µm pore size filter. Each membrane was placed onto the surface of a Tryptone-Soy agar (Oxoid CM131) supplemented with 0.1% (w/v) yeast extract (Oxoid L21) plate and incubated for 180±10minutes at 37±0.5˚C. After incubation the membranes were transferred to mFC agar (Difco 0677-01-0) without rosolic acid and incubated for 18±1hour at 44±0.5˚C. After incubation the number of blue colonies were recorded as presumptive *E. coli*: of these up to 10 colonies were subcultured onto Tryptone bile glucuronide agar (TBGA) for confirmation of *E. coli.* The proportion of subcultures yielding blue/green colonies was used to adjust the count obtained on the membranes to give the confirmed *E. coli* concentration per g of sediment.

Statistical analyses

Statistical analyses were undertaken on $\log_{10}$-transformed *E. coli* concentrations using Minitab v14.

For the results of study 1, the ratio of the geometric mean *E. coli* results for the unrinsed and rinsed samples were determined for each area, species and sampling date combination, together with the 95% confidence intervals for these ratios. For each area and species, a two-way Analysis of Variance (ANOVA) was undertaken on $\log_{10}$ transformed *E. coli* MPN values using sampling date as one factor and the unrinsed/rinsed state as the other.

For the results of study 2, the ratio of the geometric mean *E. coli* results for the unrinsed and rinsed samples were determined for each species, drain status and bag type combination, together with the 95% confidence intervals for these ratios.

For each area and species, simple linear regression was undertaken of $\log_{10}$ transformed *E. coli* concentrations of the unrinsed shellfish samples against the $\log_{10}$ transformed *E. coli* concentrations of the sediments.

Results

Study 1

The results of the 4 separate samplings for each site/species combination are presented in Tables 1-3 and Figures 1-3.

Table 1*:* Area 1 (Site A)*: E. coli* concentrations in mussel and sediment samples

Date collected	Shellfish				Sediment		
	Status	Replicate	*E. coli* MPN/100g	Ratio Unrinsed/ Rinsed (95% CI)	Volume tested (ml)	Replicate	*E. coli* cfu/g
07/06/04	Unrinsed	1	310	4.20 (0.09, 190)	10	1	4
	Unrinsed	2	500		10	2	4
	Rinsed	1	40				
	Rinsed	2	220				
05/07/04	Unrinsed	1	750	0.45 (0.14, 1.4)	10	1	24
	Unrinsed	2	1100		10	2	27
	Rinsed	1	1700				
	Rinsed	2	2400				
18/10/04	Unrinsed	1	220	1.8 (1.3, 2.6)	10	1	22
	Unrinsed	2	220		10	2	20
	Rinsed	1	130				
	Rinsed	2	110				
15/02/05	Unrinsed	1	1300	1.7 (0.10, 27)	10	1	14
	Unrinsed	2	500		10	2	12
	Rinsed	1	750				
	Rinsed	2	310				

Figure 1: Area 1 (Site A): *E. coli* concentrations in mussel samples

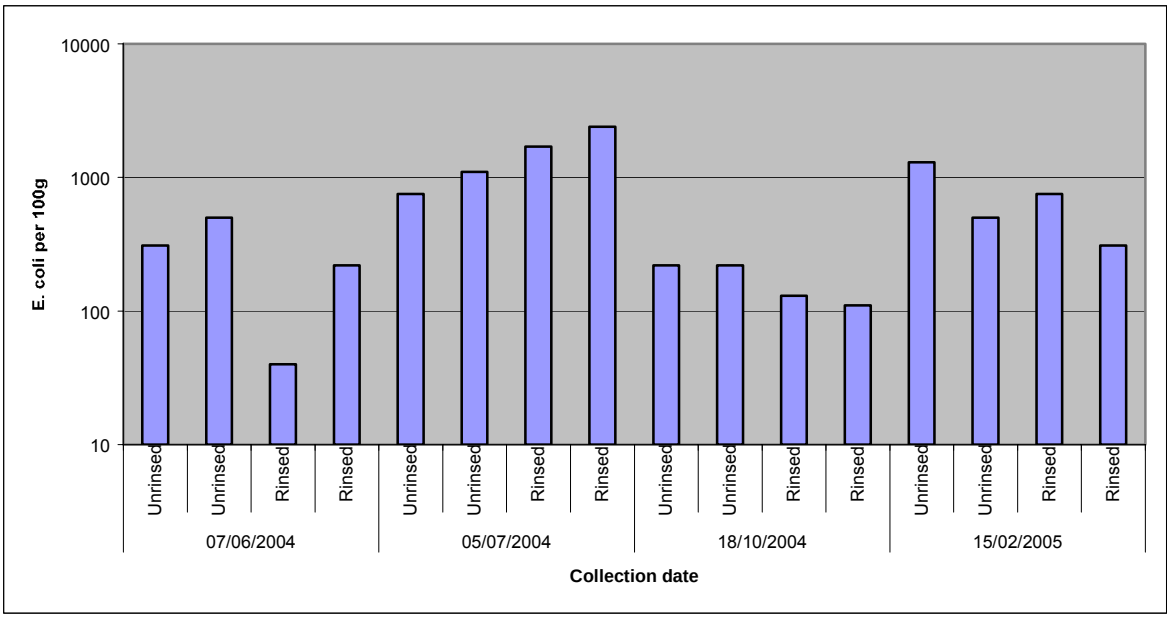


Table 2*:* Area 1 (Site B): *E. coli* concentrations in cockle and sediment samples

Date collected	Shellfish				Sediment		
	Status	Replicate	*E. coli* MPN/100g	Ratio Unrinsed/ Rinsed (95% CI)	Volume tested (ml)	Replicate	*E. coli* cfu/g
07/06/04	Unrinsed	1	90	0.17 (0.00, 43)	10	1	<2
	Unrinsed	2	70		10	2	<2
	Rinsed	1	130				
	Rinsed	2	1700				
05/07/04	Unrinsed	1	9100	1.3 (0.42, 4.0)	10	1	2
	Unrinsed	2	9100		10	2	3
	Rinsed	1	5400				
	Rinsed	2	9100				
18/10/04	Unrinsed	1	2400	0.88 (0.41, 1.9)	10	1	18
	Unrinsed	2	1700		10	2	20
	Rinsed	1	2400				
	Rinsed	2	2200				
15/02/05	Unrinsed	1	1300	0.58 (0.03, 9.7)	10	1	<2
	Unrinsed	2	2400		10	2	<2
	Rinsed	1	1700				
	Rinsed	2	5400				

Figure 2: Area 1 (Site B): *E. coli* concentrations in cockle samples

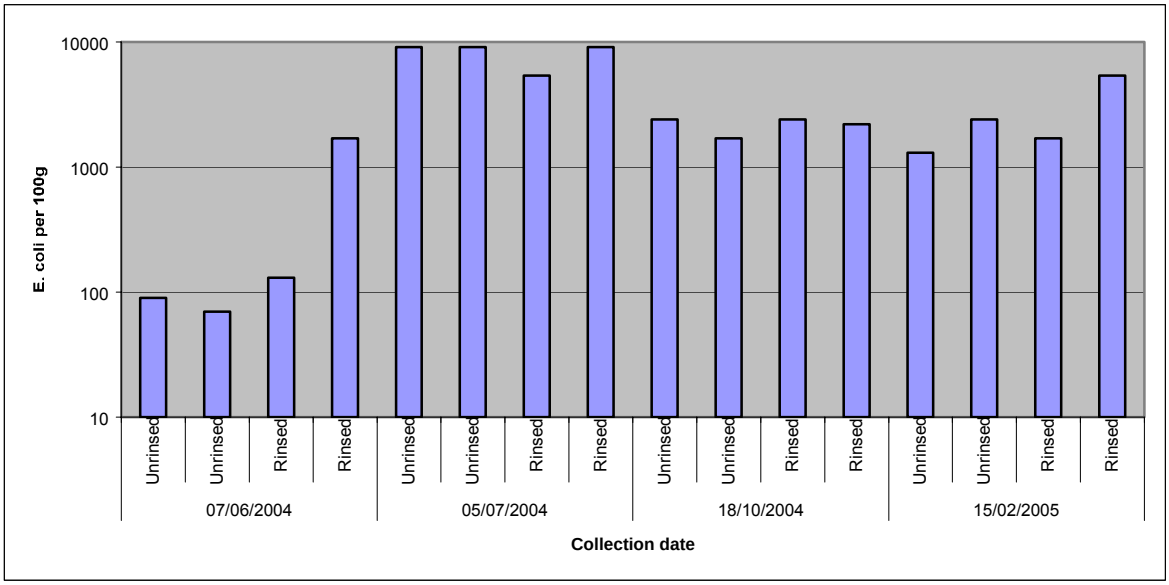
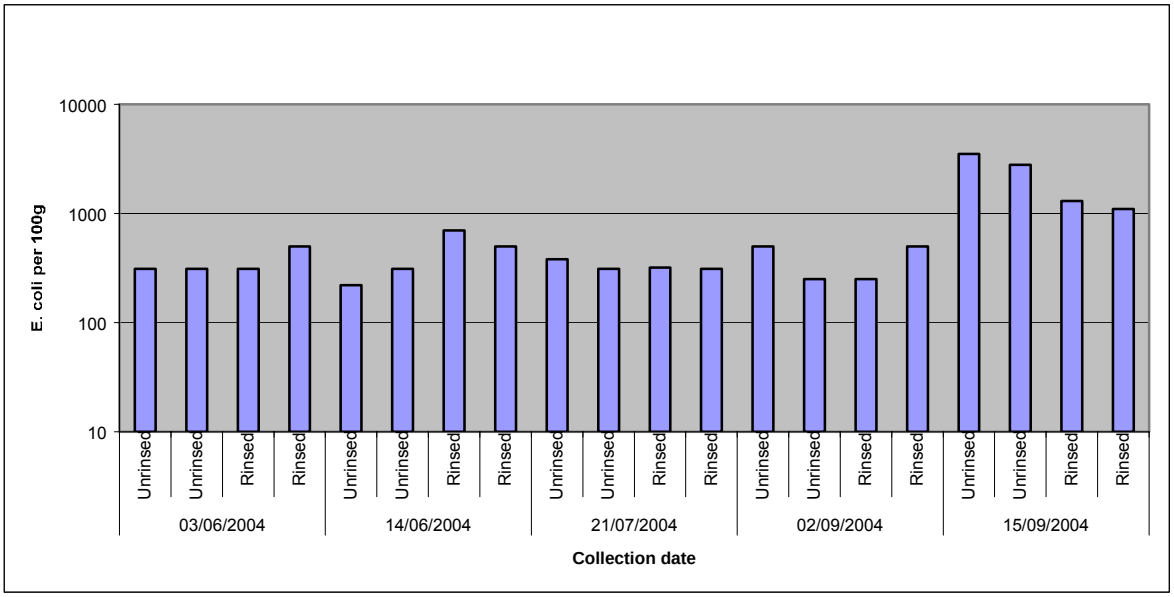


Table 3: Area 2: *E. coli* concentrations in mussel and sediment samples

Date collected	Shellfish				Sediment		
	Status	Replicate	*E. coli* MPN/100g	Ratio Unrinsed/ Rinsed (95% CI)	Volume tested (ml)	Replicate	*E. coli* cfu/g
03/06/04	Unrinsed	1	310	0.79 (0.28, 2.2)	10	1	<2
	Unrinsed	2	310		10	2	<2
	Rinsed	1	310				
	Rinsed	2	500				
14/06/04	Unrinsed	1	220	0.44 (0.16, 1.2)	10	1	5
	Unrinsed	2	310		10	2	<2
	Rinsed	1	700				
	Rinsed	2	500				
21/07/04	Unrinsed	1	380	1.1 (1.1, 1.7)	10	1	13
	Unrinsed	2	310		10	2	13
	Rinsed	1	320				
	Rinsed	2	310				
02/09/04	Unrinsed	1	500	1.0 (0.12, 8.2)	10	1	nt
	Unrinsed	2	250		10	2	nt
	Rinsed	1	250				
	Rinsed	2	500				
15/09/04	Unrinsed	1	3500	2.6 (1.4, 4.8)	10	1	nt
	Unrinsed	2	2800		10	2	nt
	Rinsed	1	1300				
	Rinsed	2	1100				

Figure 3: Area 2: *E. coli* concentrations in mussel samples



Study 2

The results obtained for study 2 are presented in Tables 4-6 and Figures 4-6.

Table 4: *E. coli* concentrations in mussel and sediment samples (collected 03/07/07)

Shellfish					
Drained status	Rinsed Status	Replicate	Sample 1	Sample 2	Ratio unrinsed/ rinsed (95% CI)
with drain water	Unrinsed	1	160	310	0.36 (0.19, 0.68)
		2	220	310	
	Rinsed	1	500	500	
		2	750	1100	
without drain water	Unrinsed	1	310	310	1.00 (0.29, 3.39)
		2	1300	750	
	Rinsed	1	310	750	
		2	310	1300	
in net bag	Unrinsed	1	700	310	1.26 (0.23, 6.88)
		2	750	500	
	Rinsed	1	220	1700	
		2	200	430	

Sediment		
Volume tested (ml)	Replicate	*E.coli* cfu/g
10	1	<0.5
10	2	1

Figure 4: *E. coli* concentrations in mussel samples

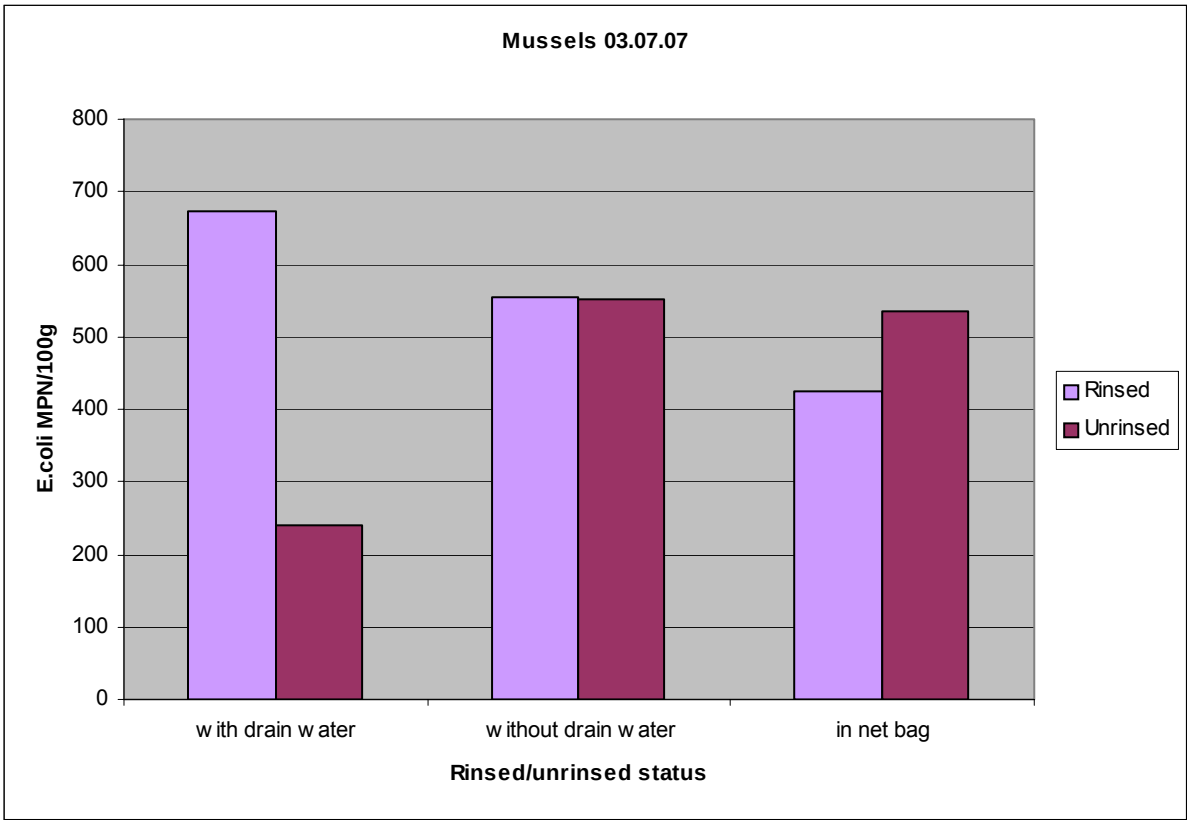


Table 5: *E. coli* concentrations in cockle and sediment samples (collected 22/05/07)

Shellfish					
Drained status	Rinsed Status	Replicate	Sample 1	Sample 2	Ratio unrinsed/ rinsed (95% CI)
with drain water	Unrinsed	1	700	2400	0.84 (0.23, 3.09)
		2	1700	1100	
	Rinsed	1	1100	750	
		2	1400	5400	
without drain water	Unrinsed	1	2800	1300	1.81 (0.42, 7.85)
		2	1100	1700	
	Rinsed	1	1700	2400	
		2	500	310	
in net bag	Unrinsed	1	1300	750	0.82 (0.21, 3.18)
		2	2200	3500	
	Rinsed	1	5400	1700	
		2	2400	750	

Sediment		
Volume tested (ml)	Replicate	*E.coli* cfu/g
10	1	1
10	2	<0.5

Figure 5: *E. coli* concentrations in cockle samples

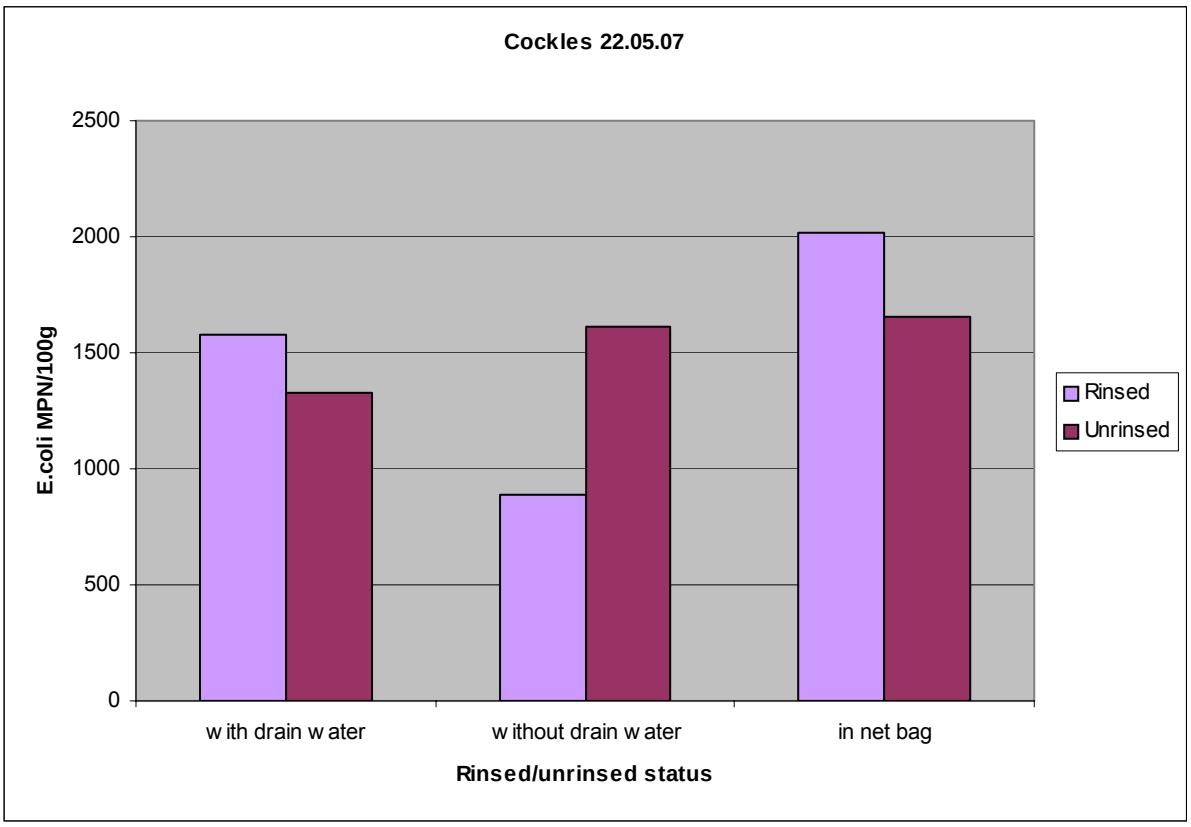


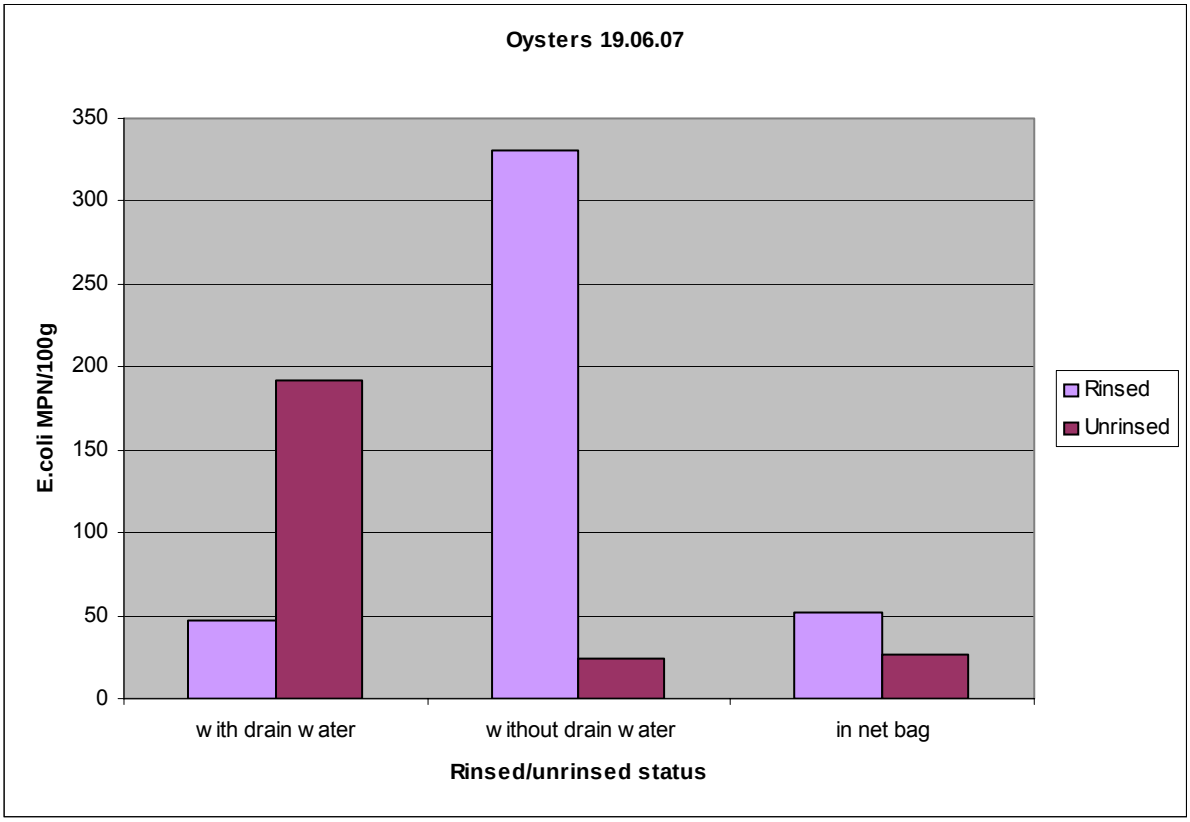
Table 6: *E. coli* concentrations in oyster and sediment samples (collected 19/06/07)

Shellfish					
Drained status	Rinsed Status	Replicate	Sample 1	Sample 2	Ratio unrinsed/ rinsed (95% CI)
with drain water	Unrinsed	1	220	NR	4.04 (1.37, 11.9)
		2	160	200	
	Rinsed	1	70	40	
		2	90	20	
without drain water	Unrinsed	1	20	40	0.07 (<0.01, 3.05)
		2	20	20	
	Rinsed	1	40	3500	
		2	50	1700	
in net bag	Unrinsed	1	20	70	0.53 (0.19, 0.68)
		2	20	20	
	Rinsed	1	40	90	
		2	50	40	

Sediment		
Volume tested (ml)	Replicate	*E.coli* cfu/g
10	1	0.5
10	2	<0.5

NR – no result due to a laboratory error

Figure 6: *E. coli* concentrations in oyster samples



Statistical Analyses

Study 1

The greatest ratio of the geometric mean *E. coli* concentrations in unrinsed and rinsed samples seen in this work was 4.2. However, for all but three samples, the 95% confidence intervals of the ratios included the value of 1. The three samples for which this was not the case were all mussels, one from area 1 and two from area 2. Of these three samples, the greatest ratio found was 2.6.

Two-way Analysis of Variance (ANOVA) using $\log_{10}$ shellfish *E. coli* as the response variable and collection date and unrinsed/rinsed status as the explanatory variables gave the following p values:

		p-value from ANOVA for factor		
Area	**Species**	**Date collected**	**Unrinsed versus rinsed**	**Interaction**
1	Mussels	0.002	0.159	0.119
1	Cockles	0.001	0.178	0.306
2	Mussels	<0.001	0.989	0.014

The differences in the *E. coli* concentration of the shellfish sampled on different dates were therefore highly significant for all areas, as would be expected from practical experience. There was no significant effect of rinsing at time of sampling ($p<0.05$). The interaction between collection date and rinsing was significant for the mussels from area 2. That is, there was an effect on some sampling occasions and not others – this could not be explained from the information available.

Regression analysis showed no significant association between $\log_{10}$ shellfish *E. coli* for the "unrinsed" samples and $\log_{10}$ sediment *E. coli* concentrations. There were a number of sampling occasions on which sediment samples were not taken and therefore the data sets were rather small.

Study 2

The greatest ratio of the geometric mean *E. coli* concentrations in unrinsed and rinsed samples seen in this work was 4.04 and the smallest was 0.07. However, for all but three samples, the 95% confidence intervals of the ratios included the value of 1. The three sets of data for which this was not the case included the mussel subsamples which included drain water in the sample bags (ratio = 0.36), the oyster subsamples which included drain water in the sample bags (ratio = 4.04) and the oyster subsamples in the net bag (ratio = 0.53).

Two-way Analysis of Variance (ANOVA) using $\log_{10}$ shellfish *E. coli* as the response variable and drained status and unrinsed/rinsed status gave the following p values:

	P-value from ANOVA for factor		
Species	**Drained status**	**Unrinsed/rinsed**	**Interaction**
Mussels	0.611	0.316	0.132
Cockles	0.532	0.805	0.488
Oysters	0.170	0.161	0.004

There was no significant effect of rinsing the samples at the time of harvesting as all p values are greater than 0.1. There was also no significant effect with regards to the drainage status of the samples again all p values are greater than 0.1. There was however a highly significant interaction between the drained status and rinsed/unrinsed status of the oyster trial (p=0.004). It can be seen from Figure 6 that this is primarily due to the following:

- large differences for the subsamples without drain water, where the geometric mean *E. coli* concentration of the rinsed subsamples was markedly higher than in the unrinsed subsamples;
- slightly smaller differences for the subsamples with drain water, where the geometric mean *E. coli* concentration of the rinsed subsamples was somewhat lower than in the unrinsed subsamples.

It can be seen from Table 6 that the difference for the samples without drain water was solely due to the duplicate *E. coli* results from one of the two rinsed subsamples and that the other showed *E. coli* results much closer to those of the two unrinsed subsamples. The results for the samples that were transported in drain water were consistent and the results of the unrinsed subsamples were markedly and consistently higher than those of the rinsed subsamples.

Simple regression of $\log_{10}$ *E. coli* results in shellfish versus $\log_{10}$ sediment *E. coli* on the same sampling date showed a significant association with half of the variability in the shellfish *E. coli* results being explained by the sediment *E. coli* content (r^2(adj) = 50.0%).

Discussion

There was no evidence from Study 1 that the presence of sediment on transported samples contributed to increased *E. coli* concentrations in the shellfish. However, the shellfish were drained before being placed in sample bags and it was considered that an effect might be seen if residual seawater or other fluid were present to encourage uptake by the shellfish. Study 2 was designed to explore this possibility.

The results of Study 2 indicate that there was no consistent effect of either drain status or rinsed status on their own but that there was an interaction between the two for the oyster trial. The reason for the large difference in *E. coli* concentration between the duplicate rinsed subsamples transported without drain water is not apparent and the results of one subsample were markedly higher than all others in that trial. No conclusions can be drawn from those observations without further investigation. The difference in *E. coli* results between the unrinsed and rinsed oyster subsamples transported in drain water would support the possibility of uptake of contaminated material from the outside of the shell due to the presence of fluid. For the other species, there was no significant effect of either incorporating drain water or using net bags. It should be noted that the design of the laboratory experiments did not allow the determination of any contamination between separate samples held in net bags, or between the environment and samples in net bags.

It had been expected that there might be a general association between the concentrations of *E. coli* in bottom dwelling shellfish and those in the nearby sediment with ingested sediment contributing to the observed *E. coli* content. There was no evidence from Study 1 that this was the case and it must therefore be assumed that the *E. coli* in the shellfish largely derived from the water column. Most of the sediment samples showed very low *E. coli* concentrations and this may have reduced the likelihood of the sediment contributing significantly to the shellfish *E. coli* content. However, a significant association was seen in Study 2. It should be noted that this study only contained three sets of unrinsed shellfish/sediment sample data and the shellfish species differed for each collection date.

The highest *E. coli* concentration seen in sediment across the two studies was 20 per g. On this basis, at least 5 g of sediment would be required to contribute even 100 *E. coli* to the result from a routine shellfish analysis. As at least 50 g of shellfish flesh and intravalvular fluid are used for each analysis, twenty percent of the tested material would need to consist of sediment to give this outcome and this would be extremely unlikely to occur, unless both sampling and laboratory sample preparation techniques were markedly at fault.

On the basis of this study, there is no evidence to propose rejection of inadequately rinsed samples on receipt at the laboratory, as long as the sample bags/containers do not contain significant amounts of fluid. However, this does not detract from the continued recommendation of rinsing at time of sampling as good practice. The results from the work presented here show that the presence of fluid should not be a significant factor if the shellfish are well rinsed before being placed in sample bags. The use of net bags is not considered advisable due to the potential for cross-contamination, either from other samples present in the same coolbox, or from the inside of the coolbox itself.

References

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