Sources of variation of *Escherichia coli* concentrations in bivalve molluscs

R. J. Lee and R. Silk

ABSTRACT

Bivalve molluscs can concentrate contaminants, including pathogenic microorganisms, from the water column during their normal filter-feeding activity. In the European Union, the risk of human and animal faecal contamination in bivalves is estimated by determining the concentration of *Escherichia coli* in time-series samples from production areas. A structured field study was undertaken to determine the extent to which such concentrations varied between sites, sampling occasions and shellfish species and to determine the residual variability of the method. *E. coli* was enumerated in three species of bivalve mollusc (*Crassostrea gigas*, *Mytilus* spp. and *Pecten maximus*) co-located in each of three geographically separate commercial shellfisheries. The data were subjected to analysis of variance (ANOVA). This showed that the effects of site, sampling occasion, species and site/sampling occasion interaction were all significant. The proportion of variation due to site was markedly greater than that due to other factors. Post-ANOVA analysis showed that the concentration of *E. coli* in *P. maximus* was significantly higher than in the other two species. *Mytilus* spp. and *C. gigas* exhibited comparable levels of *E. coli.* The observed standard deviation of the most probable number method in the study was 0.33 $\log_{10}$.

Key words | bivalve, *E. coli*, factors, mussels, oysters, scallops

R. J. Lee (corresponding author)
R. Silk
Centre for Environment,
Fisheries and Aquaculture Science,
Weymouth Laboratory,
Barrack Road,
Weymouth, Dorset,
DT4 8UB,
UK
E-mail: *ron.lee@cefas.co.uk*

INTRODUCTION

Bivalve molluscs feed by filtering microalgae from the water column. During this activity, they can take up and concentrate a range of contaminants, including microorganisms. If these microorganisms include human pathogens, consumption of the bivalves may lead to illness, usually gastroenteritis (Rippey 1994; Lees 2000). Classification of bivalve mollusc harvesting areas is undertaken to provide an estimate of the risk of contamination from faecal sources: individual pathogens may or may not be present at a specific time depending on whether they are present in the contaminating sources. In the European Union, classification is based on the enumeration of *Escherichia coli* (a microbiological indicator of faecal contamination) in time-series samples from each production area (Anon 2004a, b, 2010). Under EU legislation, classification of bivalve mollusc harvesting areas is not necessarily required for *Pectinidae*. If *Pectinidae* are harvested from outside classified areas, then microbiological testing is undertaken after the landing of harvested bivalves. In the UK, aquaculture scallops are currently monitored and classified. The requirements of food hygiene legislation relating to bivalve molluscs and the background to the microbiological monitoring programmes have been described elsewhere (Lee & Murray 2010; Murray & Lee 2010; Oliveira *et al.* 2011). The classification requirements for production areas under the EU legislation, together with the subsequent post-harvest treatment requirements, are shown in Table 1.

It is known that many different environmental and biological factors can affect the extent to which bivalve molluscs are contaminated with *E. coli*. Lee & Morgan (2003) showed significant effects of season, rainfall, wind direction and the high/low tidal cycle in at least one of three areas studied: no significant effect of the spring/neap tidal cycle or wind direction was seen in any of the three areas.

Table 1 | Criteria for the classification of bivalve mollusc harvesting areas

Class	Microbiological standard[a]	Post-harvest treatment required
A	Live bivalve molluscs from these areas must not exceed 230 MPN *E. coli* per 100 g of flesh and intra-valvular liquid[b]	None
B	Live bivalve molluscs from these areas must not exceed, in 90% of the samples, 4,600 MPN *E. coli* per 100 g of flesh and intravalvular liquid. In the remaining 10% of samples, live bivalve molluscs must not exceed 46,000 MPN *E. coli* per 100 g of flesh and intravalvular liquid[c]	Purification, relaying or cooking by an approved method
C	Live bivalve molluscs from these areas must not exceed 46,000 *E . coli* MPN per 100 g of flesh and intravalvular liquid[d]	Relaying or cooking by an approved method
Prohibited	>46,000 *E. coli* MPN per 100 g of flesh and intravalvular fluid[e]	Harvesting not permitted

Notes:

[a]The reference method is given as ISO 16649-3.

[b]By cross-reference from Regulation (EC) No 854/2004, via Regulation (EC) No 853/2004, to Regulation (EC) No 2073/2005.

[c]From Regulation (EC) No 1021/2008 (Anon 2008).

[d]From Regulation (EC) No 854/2004.

[e]This level is not specifically given in the Regulation but does not comply with classes A, B or C. The competent authority has the power to prohibit any production and harvesting of bivalve molluscs in areas considered unsuitable for health reasons.

While production area monitoring reflects, in general, the microbiological water quality in an area, there is not a simple relationship between the concentration of faecal indicator bacteria in bivalve molluscs and in the surrounding seawater (Lees & Nicholson 1997). Analyses have also been undertaken to determine the relative levels of contamination between different species (Berry & Younger 2009; Amouroux & Soudant 2011). However, these studies were carried out on historical data from routine monitoring programmes and factors other than species effects may complicate the relationships that are seen. The present study was undertaken to determine whether aquaculture scallops concentrated faecal contamination to such an extent that they would require depuration prior to sale for consumption (Silk 2000). Depuration is undertaken to remove pathogenic bacteria of faecal origin but, as currently commercially practised, is not regarded as effective for the removal of viruses such as norovirus and hepatitis A virus (Lee *et al.* 2008; EFSA 2011).

The three species selected for the study were *Mytilus* spp. (mussels), *Crassostrea gigas* (Pacific oysters) and *Pecten maximus* (king scallops). The first two species are commonly raised in aquaculture in Europe, including the UK, and have been the subject of routine microbiological monitoring programmes and studies based on the data from such programmes. Scallops have not been included

in such studies and were the primary focus of the investigation and data analysis presented here.

MATERIALS AND METHODS

Fieldwork

Mytilus spp., *C. gigas* and *P. maximus* were placed in mesh bags at three geographically separate sites (designated K, L and M) in England and Wales. Duplicate samples of each species were taken from each site on a monthly basis for a period of eight months. The outside of the sample animals was rinsed in clean water, and the individuals were then drained, placed in a plastic bag and transported to the laboratory in a coolbox to maintain the temperature between 1 and 8 °C.

Bacteriological testing

Each sample was tested using the European reference method for *E. coli* in bivalve molluscs (Donovan *et al.* 1998; ISO 2004). Essentially, this consisted of a first stage of homogenisation of flesh and intravalvular fluid from at least ten individual animals in 0.1% peptone. Duplicate aliquots of each homogenate were then subjected to the

following: further 1 in 10 dilutions were prepared in the 0.1% peptone followed by a most probable number (MPN) technique with an initial stage in minerals modified glutamate broth (MMGB) incubated at 37 °C for 24 h and confirmation on tryptone bile glucuronide agar (TBGA) incubated at 44 °C for 22 h (as defined in the reference method). MPN values were determined according to the tables given in Donovan *et al.* (1998). The effectiveness of MMGB for the enumeration of *E. coli*, compared to some other traditional media, has been assumed to be due to the lack of an inhibitory shock to already stressed organisms (Blood & Curtis 1995). TBGA is used as the confirmatory medium in the reference method due to the specificity of the method for *E. coli*: this has been a general trend in food and water microbiology (Frampton & Restaino 1993).

Data analysis

The *E. coli* results were $\log_{10}$-transformed and an analysis of variance (ANOVA) was performed using the general linear modelling functionality within Minitab 15: site, sampling occasion and species were coded as factors.

RESULTS AND DISCUSSION

The output from the ANOVA is given in Table 2 and the main effects plot from the analysis is given in Figure 1. Diagnostic plots (not shown) were satisfactory. The adjusted R^2 value was 0.78: a large proportion of the variability in the $\log_{10}$ *E. coli* values was therefore explained by the identified factors and interactions. From Table 1 it can be seen that the geographical site, sampling date and bivalve species all had a highly significant effect on the levels of *E. coli* detected ($p < 0.001$). A highly significant interaction between site and sampling date was also detected ($p < 0.001$). Of the factors found to have a significant impact on *E. coli* levels, geographical site was responsible for the highest variability between samples (51%) followed by sample date (9%) then species (5%). The difference in *E. coli* results between shellfish species was shown to be independent of the geographical site and sample date.

To examine the relationship between the individual sites and species examined, Tukey post-ANOVA analyses were

Table 2 │ Results from an ANOVA for concentrations of *E. coli* in three species of bivalve molluscs from three sites in England and Wales

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	p
Site	35.50	2	17.75	166.51	0.000
Sample date	6.50	7	0.92	8.71	0.000
Species	3.62	2	1.81	16.96	0.000
Site × Sample date	8.94	14	0.64	5.99	0.000
Site × Species	0.41	4	0.10	0.96	0.436
Sample date × Species	2.47	14	0.18	1.66	0.085
Site × Sample date × Species	4.51	28	0.16	1.51	0.083
Error	7.68	72	0.11		
Total	69.62	143			

carried out (output not shown). The analyses showed that the mean *E. coli* values for all three sites differed significantly from each other. They also showed that the mean level of *E. coli* in *P. maximus* was significantly higher than those for both *C. gigas* and *Mytilus* spp. The latter two species were not found to significantly differ from each other.

The error mean square, an estimate of the variance of the method after removing the effects of the factors studied here, was 0.1066. The corresponding standard deviation is therefore approximately 0.33 $\log_{10}$. This includes the variability introduced by subsampling and sample preparation. The theoretical standard deviation of the 5-tube 3-dilution MPN test is stated to be approximately 0.24 $\log_{10}$, provided that the outcome is not an extreme tube combination (ISO 2010). The observed variability is obviously larger than the theoretical variability.

The factor explaining most of the variability in the *E. coli* data was site. The main effects plot (Figure 1) shows that each pair differed by more than 0.5 $\log_{10}$. This was not unexpected as the sites analysed were geographically distinct and were selected to reflect likely low (site L), medium (site K) and high (site M) levels of microbiological pollution. A previous geospatial study in France showed that faecal contamination varied significantly across a mussel farming area (Beliaeff & Cochard 1995). The types and loads of pollution sources, the distance between these and the monitoring points, and the hydrography of the
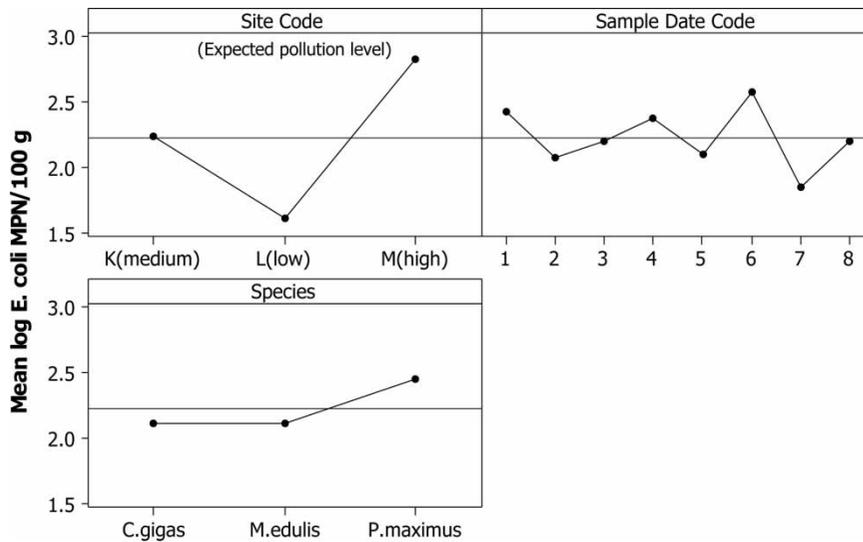
Figure 1 │ Main effects plot: fitted means of *E. coli* at each level of the three analysed factors.

areas will all differ greatly between sites and will thus influence the average levels of *E. coli* seen in the bivalves. These spatial effects reinforce the need to consider production areas, and even separate parts of harvesting areas, separately with regard to monitoring and classification under European Union regulations (Anon 2004a, b, 2010; Lee & Murray 2010).

Sampling date was also found to have a significant effect on within site variation. The interaction between site and sampling date can be influenced by numerous different environmental effects on both pollution sources and the relative uptake of contaminants by bivalves. Season, changing tidal currents and the effect of weather (rainfall and wind) have all been shown to affect the degree of contamination detected in microbiological monitoring of production areas (Lee & Morgan 2003; Campos *et al.* 2011). The results underline the need for frequent monitoring at individual sites to detect both seasonal effects and unpredicted contamination events.

Bivalve species were also found to be a significant factor affecting the observed levels of *E. coli*. The effect was found to be independent of the geographical site and sampling occasion. An extension of the initial ANOVA analysis showed that *Mytilus* spp. and *C. gigas* exhibited similar levels of contamination, while *P. maximus* showed significantly higher levels of contamination when grown under the same conditions.

Previous studies comparing the levels of *E. coli* contamination between bivalve shellfish species have shown inconsistent results. In some studies using monitoring data, *E. coli* concentrations in *Mytilus* spp. have been shown to be higher than in *C. gigas* grown in the same area (Younger *et al.* 2003; Berry & Younger 2009). In another, no significant difference was seen between the levels of contamination in these two species (Amouroux & Soudant 2011). The outcome obtained in the present study supports the latter observation.

The bivalves examined in this study were co-localised, grown by a standardised method and the samples tested were harvested, transported and analysed together, thereby minimising localised within site effects and technical variability. Samples of different species taken at a site had therefore been exposed to seawater of the same microbiological quality. The relationship between paired samples selected from routine monitoring data is less well defined and these samples are unlikely to comply with such rigid controls. Unaccounted factors, in addition to shellfish species, could contribute to the variability between sample sets and influence the final results. This may contribute to inconsistent results between published studies.

There is considerable interest in the relative uptake of faecal indicator bacteria by different bivalve species. By default, each commercially harvested bivalve species grown in each harvesting area is monitored separately.

However, it has been proposed that a single, commonly occurring indicator species, e.g. mussels, could be used to represent a range of other species. Public health protection would be provided by using a species which yields equivalent or higher results for the indicator bacteria (Anon 2010). The outcome of the present study shows that *Mytilus* spp. could be used to represent areas containing *C. gigas* but not *P. maximus*. An additional consequence for the latter species is that they are likely to show higher levels of contamination than some other bivalve species when grown in areas of equivalent microbiological quality. Thus, if an area is classified for this species, it may yield a worse classification than the other commonly farmed species. Even if *P. maximus* are harvested from outside classified areas, they will be likely to fail the standard given in the EU Microbiological Criteria Regulation (Anon 2005).

CONCLUSIONS

The study reported here was a structured experimental field study specifically designed to yield an assessment of the relative extent of contamination of three commercially important bivalve species. The most significant source of variability in the *E. coli* concentration in bivalve molluscs in this study was geographical location, followed by sample date and species. There was also a significant interaction detected between site and sampling date. Mussels and Pacific oysters were found to contain approximately equal concentrations of *E. coli* while king scallops contained significantly higher levels.

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