APPENDIX A8-1

Cefas

Relationship between the microbial quality of shellfish flesh and seawater in UK harvesting areas

Project WT1001

Factors affecting the microbial quality of shellfish

Authors: Carlos J. A. Campos, Allan Reese, Simon Kershaw and Ron J. Lee

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Executive summary

The relationship between levels of *E. coli* in shellfish flesh versus water was examined using a dataset of *E. coli* results quantified in 602 samples of shellfish (Pacific oysters, native oysters and mussels) and water collected from 40 sampling points within 6 production areas in England and Wales during the period 1991–1994.

Linear regression models of *E. coli* levels in shellfish versus water were fitted for each of the species tested and for the "pooled species" dataset. These models show that 18% of the variance in *E. coli* levels in native oysters and Pacific oysters and 44% of the variance in *E. coli* levels in mussels are explained by the variation in water values. These results highlight the importance of environmental and biological factors in determining inter-species differences in the accumulation of enteric bacteria by shellfish. These effects were also investigated as part of this project and are reported separately.

Logistic regression models were fitted for each of the three species and for the "pooled species" dataset with the aim of finding specific water quality threshold *E. coli* values that would ensure similar level of protection for shellfish beds as that currently given by the shellfish flesh guideline (G) standard (300 faecal coliforms per 100g fluid and intravalvular liquid) of the Shellfish Waters Directive (SWD). The models predicted that this would be achieved at a geometric mean of 10 and 90th percentile of 55 *E. coli* per 100ml water (at 75% compliance with the SWD G).

Significant differences in compliance rates between mussels and Pacific oysters emerged from the logistic models indicating that these relationships are indeed complex and require further investigation. Compliance with the class B threshold (≤4,600 *E. coli* 100g^{-1} FIL with 90% probability) in mussel, native oyster and Pacific oyster samples was predicted at 33, 177, and 4,200 *E. coli* levels in water, respectively.

Recommendations are given on the use of monitoring data from the Shellfish Hygiene monitoring programme for the purposes of informing water quality in shellfish waters (shellfish protected area status under WFD) in England and Wales.

Table of contents

1 Introduction

In the UK, there are two statutory mechanisms aimed at limiting the presence of microbial contaminants in shellfish and ensuring the quality of shellfish for human consumption. These are enforced through requirements in Directive 2006/113/EC (codified version) on the quality required of shellfish waters [which replaced 79/923/EC]) [hereafter referred Shellfish Waters Directive (SWD)] and European food hygiene legislation, namely Regulation (EC) No 854/2004 laying down rules for the organisation of official controls on products of animal origin intended for human consumption and associated legislation[1].

The SWD, adopted by the European Parliament and the Council of the European Union in December 2006, concerns the quality of shellfish waters and applies to coastal and brackish waters designated by European Union (EU) Member States as needing protection or improvement in order to support shellfish life and growth and thus contribute to the high quality of shellfish products directly edible by man (European Communities, 2006). Under the SWD, Member States are required to designate shellfish waters (SWs) and establish programmes in order to reduce pollution to ensure that designated shellfish waters conform, within six years following designation, with water quality parameters contained in the Annex to the Directive. From a microbiological point of view, the SWD specifies a Guideline (G) standard≤300 faecal coliforms per 100ml of shellfish flesh and intervalvular fluid (FIL) in 75% of samples collected on a quarterly basis (European Communities, 2006).

Chapter A of Annex II of Regulation (EC) No 854/2004 prescribes three classes of levels of *Escherichia coli* monitored in bivalve mollusc flesh and intravalvular liquid (FIL) and for which a level of post-harvest treatment is required before marketing of shellfish for human consumption (Table 1). A fourth class (Prohibited) is often used for those areas that do not comply with the requirements of the regulation.

In 1999, the UK Government set up a target in relation to the microbial quality of shellfisheries, which was expressed in the following statements for England:

[1] Regulation (EC) No 852 of the European Parliament and of the Council, Regulation (EC) No 853 of the European Parliament and of the Council, Regulation (EC) No 2073/2005, Regulation (EC) No 1666/2006.

"The Government's aim is that bacteriological standards should be achieved in designated waters which allow harvesting areas to achieve at least category B standard under the system applied by the Ministry of Agriculture, Fisheries and Food to classify shellfish harvesting areas for food safety purposes" (Michael Meacher).

...and Wales:

"Under this system, I intend that all designated waters should achieve at least category B standards under the quality criteria for shellfish taken from harvesting areas..." (Peter Law).

The Department for Environment, Food and Rural Affairs (Defra) is committed to improving water quality to a level where all designated SWs can support at least class B. This is considered an achievable interim target towards meeting the G faecal coliform standard for shellfish FIL under the SWD and thus providing significant benefits to the environment as well as to the shellfish industry (Defra, 2011).

Table 1. Microbiological standards for classification of bivalve mollusc production areas under Regulation (EC) No 854/2004.

Class	Microbiological standard[a]	Post-harvest treatment required
A	Live bivalve molluscs from these areas must not exceed 230 Most probable number (MPN) *E. coli* per 100g of FIL[b]	None
B	Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution MPN test of 4,600 *E. coli* per 100g of FIL in more than 10% of samples. In the remaining 10 % of samples, live bivalve molluscs must not exceed 46,000 MPN *E. coli* per 100g of FIL[c]	Purification, relaying or cooking by an approved method
C	Live bivalve molluscs from these areas must not exceed the limits of a five-tube, three dilution MPN test of 46,000 *E. coli* per 100g of FIL[d]	Relaying over a long period or cooking by an approved method
Prohibited	>46,000 *E. coli* per 100g of FIL[e]	Harvesting not permitted

[a] Reference method is given as ISO 16649-3 (2005).
[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 854/2004 as amended by Regulation (EC) 1021/2008.
[d] From Regulation (EC) No 854/2004.
[e] This class 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.

In 2000, the European Community adopted Directive 2000/60/EC of the European Parliament and of the Council of 23 October 2000 establishing a framework for Community action in the field of water policy [hereafter referred EU Water Framework Directive (WFD)]. The publication of the WFD followed a thorough review of the European Water Policy. This legislation is considered an important operational tool setting the objectives for future water protection within the European Union. The WFD will repeal the SWD in 2013. It seeks, among others, to streamline existing legislation, expand the scope of water protection to all waters and achieve "good status" for these waters. It incorporates new requirements for water management based on river basins.

According to recital 51 and Article 4.9 of the WFD, at least the same level of protection afforded by the old legislation should be achieved with the implementation of the WFD. However, currently the WFD does not incorporate a microbiological standard upon which this objective could be enforced with respect to SWs (which the WFD defines as protected areas). On this matter, the UK Technical Advisory Group on the WFD (UKTAG, 2007) has noted that the standards required to achieve the objective for the shellfish elements of protected areas under the WFD are currently specified in the SWD and that these standards may be reviewed after the repeal of the SWD in 2013.

Factors Affecting the Microbial Quality of Shellfish seeks to make recommendations regarding an *E. coli* standard (water column standard versus shellfish flesh standard) for shellfish protected areas (SPAs) under the WFD. An investigation into the relationship between levels of *E. coli* in shellfish flesh and overlying waters in UK shellfish production areas was undertaken to help inform Defra's policy on this matter. This report documents results from this investigation and discusses use of shellfish flesh data to inform water quality in SPAs.

2 Relationship between harvesting area seawater quality and shellfish flesh quality

2.1 Methods

2.1.1 Sampling and sample collection

The study was based on paired data where *E. coli* were enumerated in shellfish FIL and in nearby water samples by Public Health Laboratory Service (PHLS) laboratories during the period 1991–1994. The data were made available to Cefas[2] by six local authorities, to support an investigation on the relationship between bacterial levels in shellfish versus water and help inform bacteriological water quality standards equivalent to those set out in Directive 91/492/EEC[3].

The data covers native oysters (*Ostrea edulis*), Pacific oysters (*Crassostrea gigas*) and mussels; it includes but does not distinguish results for the common mussel (*Mytilus edulis*) and the Mediterranean mussel (*M. galloprovincialis*). Levels of *E. coli* in shellfish were quantified using the MPN technique as described by the standard protocol used in the official monitoring programme (MAFF *et al.*, 1992). Levels of *E. coli* in seawater were quantified using the standard method in use in each PHLS laboratory which was either membrane filtration (MF) or MPN.

The dataset used in this study consisted of 602 paired samples from 40 water and 40 flesh monitoring points within six different production areas (see Appendix 1). At 34 monitoring points (85% of the total number of points), only one species of shellfish was collected. The dataset includes *E. coli* results from two production areas (Conwy and Yealm) for which fluxes of faecal indicator organisms (FIOs) impacting on shellfish waters have been quantified as part of deliverable 3 of this project (see Interim Technical Report 3). This deliverable aims to inform to what extent previous water company investment programmes have contributed to improve the microbial quality of SWs.

An analysis of temporal trends in levels of *E. coli* in shellfish monitored under Regulation (EC) No 854/2004 undertaken for the purposes of this project revealed no upward or downward trends in geometric means of the microbiological indicator in beds within the wider Conwy production area.

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[2] Cefas manages the microbiological monitoring programme for bivalve mollusc harvesting areas in England and Wales on behalf of the competent authority (Food Standards Agency).

3 Superseded by Regulation (EC) No 854/2004.

Similarly, no consistent trends were found in geometric mean *E. coli* levels in Pacific oysters from the Yealm production area. However, *E. coli* levels in mussels from the Yealm were found to have increased significantly (Mann-Kendall test; Z=2.5 in mussels from Thorn; Z=1.97 in mussels from Fox Cove) in the production area[4].

The dataset does not contain information on how close, in space or time, water samples were collected in relation to shellfish samples. Therefore, on the basis of the data available it is not possible to relate the effect of environmental factors on the levels of *E. coli* contamination in the study sites.

A review of published literature was undertaken to enable a better understanding of potential causes and influences on FIO contamination of shellfish flesh and overlying waters. This review included re-analysis of other data resources held by Cefas and data supplied by the Environment Agency and forms a separate report within this research project.

2.1.2 Statistical analyses

Bacterial counts are conventionally converted to a log scale for analysis, so values described as *E. coli* may be assumed to refer to $\log_{10}$(MPN). Logarithmic transformation is commonly used to ensure a more symmetrical distribution of the data (see Velleman and Hoaglin, 1981; Helsel and Hirsch, 2002) and is justified by biological considerations (bacteria grow exponentially). Censored data at the limit of detection (LOD) for each method were taken at face value. After $\log_{10}$-transformation, both datasets remained significantly non-normal (Skewness/Kurtosis test; p=0.007 and 0.005, respectively). However, quantile plots indicate that the datasets have similar distributions as suggested by the good fit over most of the range of *E. coli* results (Appendix III).

Simple linear regression (also known as ordinary least squares) models were computed to investigate the co-variation between *E. coli* levels in shellfish flesh and *E. coli* levels in water. Linear regression is particularly useful when estimating or predicting values of one variable based on the knowledge of another variable, for which more data are available (Helsel and Hirsch, 2002). The assumptions of these linear regression models are described in the Appendix II.

[4] It was not possible to identify whether *E. coli* levels at this location changed significantly over the period 1992–1994 due to insufficient number of results during this period.

For the purposes of this analysis, the variable "*E. coli* levels in shellfish flesh" is considered the response because the mechanism of contamination is assumed to result from the filter-feeding mechanism of shellfish and accumulation of bacteria present in the seawater, i.e. the mechanism of contamination integrates contamination available during seawater flows over the preceding hours of the tidal cycle. It is assumed the *E. coli* do not multiply within the shellfish, but may be retained or washed out.

Logistic regression is used when the response variable is observed only as a binary characteristic: yes/no, present/absent, or in this case, comply/fail coded as 1/0. To assess the various degrees of association between the SWD faecal coliform G and levels of *E. coli* in seawater, weighted logistic regression models were computed for:

Compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the geometric mean of *E. coli* in seawater for all species tested;
Compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the geometric mean of *E. coli* in seawater for each species tested;
Compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the 90%-ile of *E. coli* in seawater for all species tested; and
Compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the 90%-ile of *E. coli* in seawater for each species tested.

The models test the relationship between the threshold levels used for the purposes of classifying harvesting areas under Regulation (EC) No 854/2004 and the levels of *E. coli* in seawater. Because no covariates were available for each flesh sample other than the monitoring point and time, the data are grouped at that level and the fitted value is the proportion of samples that come under the threshold for classification. This predicted response is the probability of a sample passing the test at each *E. coli* level in seawater.

All statistical analyses were undertaken using Stata data analysis and statistical software (Stata/IC version 11.1 for Windows, StataCorp LP, College Station, Tx 2010).

2.1.3 *Assumptions on data*

To enable statistical analyses on data collated from a variety of sources, a number of assumptions were made on the dataset, namely:

Measurements of *E. coli* and faecal coliforms were considered to be equivalent. As stated in the Environment Agency's Water Quality Consenting Guidance: Consenting Discharges to Achieve the Requirements of the Shellfish Waters Directive (Microbial Quality), the use of the ratio 1 *E. coli*: 1.3 faecal coliforms suggested in the repealed Shellfish Hygiene Directive (91/492/EEC) standard for class B 6,000 faecal coliforms or 4,600 *E. coli* per 100g of flesh in 90% of samples, is not supported by revisions on Bathing Water monitoring programme data, which suggest that faecal coliform numbers are broadly equivalent to *E. coli* numbers (Environment Agency, 2003). A recent review of these data added "with large datasets, a "rule of thumb" EC/FC ratio of ~0.9 (FC/EC = 1.1) is realistic, but it must be recognised that individual samples will show a wide variation from this mean." And "all the UK data examined (fresh and saline) has a *mean* ratio closer to 1 than the Canadian and USA recommendations" (Andrew Wither, 2009). In 2003, the UK National Reference Laboratory analysed a data set from England and Wales and determined that, for shellfish, the median faecal coliform: *E. coli* ratio was 1.00 (n = 13,058) while for seawater the median ratio was 3.05 (n=462).

MF and MPN methods used for *E. coli* enumeration in seawater provide equivalent results. Although other studies contradict this assumption (e.g. Green *et al.*, 1980; Jagals *et al.*, 2000), it has been considered that this variability is more due to the probabilistic basis for calculating the MPN than laboratory procedure variability (Gronewold and Wolpert, 2008).

Levels of *E. coli* in the common mussel *Mytilus edulis* and the Mediterranean mussel *M. galloprovincialis* provide equivalent results. We found no evidence in the literature indicating significant differences in FIO accumulation between these species. High levels of hybridisation and gene introgression occur between these species in the south west of England. In England and Wales, they have been classified at genus level under Regulation (EC) No 854/2004.

2.2 Results

Table 2 shows summary statistics for arithmetic (not logged) levels of *E. coli* in shellfish flesh and water from the six UK production areas. Minimum results correspond to the limit of detection of the enumeration methods. Overall, mussels were found to be more contaminated than oysters, as indicated by statistics representing the central tendency of the data. The higher levels of *E. coli* in mussels than those in oysters correspond to the general pattern detected in classified production areas in England and Wales (Younger and Reese, 2011).

Table 2. Summary statistics for* E. coli *levels each species tested in six UK production areas.

	Pacific oysters (*C. gigas*)		Mussels (*Mytilus* spp.)		Native oysters (*O. edulis*)	
	Shellfish flesh	Seawater	Shellfish flesh	Seawater	Shellfish flesh	Seawater
n	111	—	313	—	178	—
Minimum	20	<2	20	<2	20	<2
Median	280	160	2,400	600	500	66
Maximum	16,000	5,400	91,000	240,000	160,000	91,000
Geometric mean	797	512	4,578	3,102	2,792	940
Log_{10} standard deviation	0.641	0.908	0.780	1,112	0.723	0.799
Skewness	0.150	-0.364	-0.744	-0.622	0.229	0.491
Kurtosis	2.430	2.250	2.987	2.818	3.536	4.411

Linear regression of log_{10}-transformed *E. coli* levels in flesh versus log_{10}-transformed *E. coli* levels in seawater shows that a very significant proportion of *E. coli* results lie above the line of equality (Figure 1). This is expected as the mechanism of *E. coli* in shellfish often determines higher levels of *E. coli* in the shellfish flesh than those found in seawater. Therefore, the correlation coefficient detected (r=0.59) is indicative of the level of agreement between variables. A test for non-linearity (Ramsey RESET test) shows no significant curvature in the relationship. Overall, the tendency for *E. coli* levels in shellfish to increase with *E. coli* levels in the seawater and the wide spread of values around the regression line are evident.

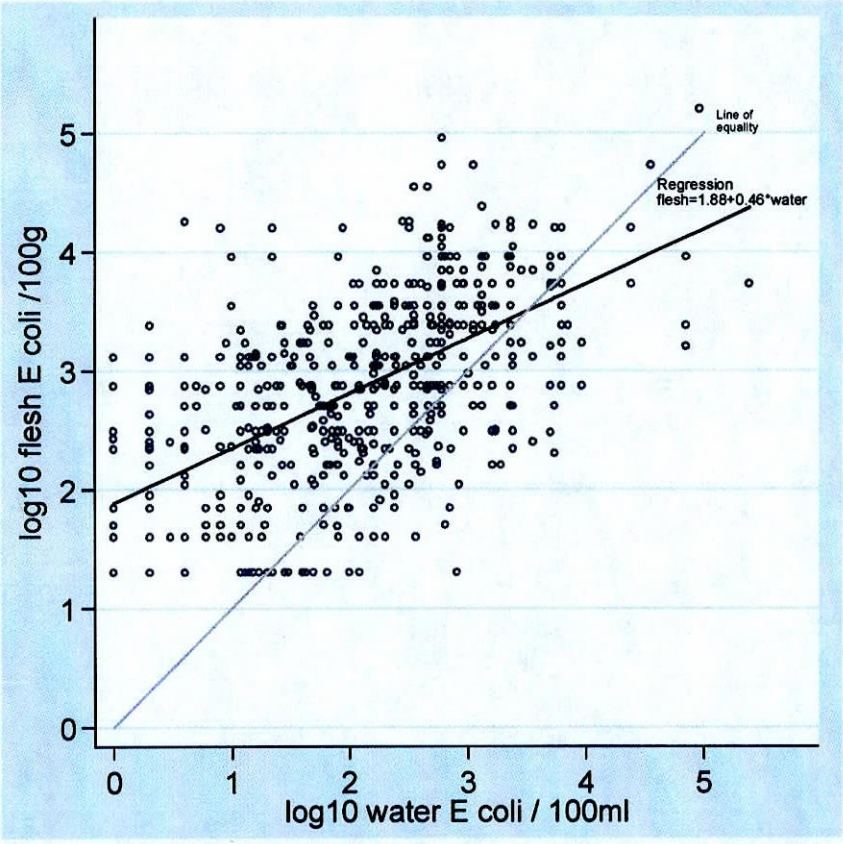

Figure 1. Scatterplot of* E. coli *levels in shellfish flesh and seawater for 602 paired samples from six production areas in the UK (all species).

The coefficient of determination (R^2) shows that the regression accounts for 35% of the variation observed in water values, suggesting that other factors would explain the variance between variables. This coefficient is higher than most coefficients obtained for environmental studies published in the literature (Table 3). A "moderate" R^2 is typical of data obtained under natural environmental conditions, i.e. the relationship between FIOs in shellfish and growing waters is influenced by various factors, including physiological mechanisms influencing bacterial accumulation in shellfish and environmental factors determining FIO survival and transport in the marine environment. However, we argue that the model is sufficiently robust to infer further conclusions.

Figure 2 highlights the variation of *E. coli* results from the Yealm and Conwy, two of the study areas for which fluxes of FIOs have been quantified as part of the project.

Table 3. Linear regression models for levels of faecal indicator organisms in shellfish flesh versus water published in the literature.

Shellfish species	Faecal indicator*	Regression equation	Variance (explained) R^2	Reference
Clams, Oysters, Mussels	FC/EC	n/a	0.3	Burkhardt III *et al.* (2009)
Mussels	FC/EC	n/a	0.2	Plusquellec *et al.* (1983)
Mussels	FC	log FC (*M. galloprovincialis*) $100g^{-1}$=0.79log FC (seawater) + 0.79	0.6	Šolić *et al.* (1999)
Oysters	FC/EC	$\log_{10}$ FC (seawater) $100ml^{-1}$=0.7495$\log_{10}$ EC(shellfish) $100g^{-1}$ + 0.5215	0.2	Ogburn and White (2009)
†Mussels	EC	$\log_{10}$ EC (*Mytilus* spp.) $100g^{-1}$=0.8087$\log_{10}$ EC (water)+1.7312	0.8	Kay *et al.* (2007)
†Oysters	EC	$\log_{10}$ EC (*C. gigas*) $100g^{-1}$=0.4651$\log_{10}$ *E. coli* (water)+2.8717	0.9	Kay *et al.* (2007)

*FC (faecal coliforms); EC (*E. coli*). †Microcosm studies. n/a - not stated.

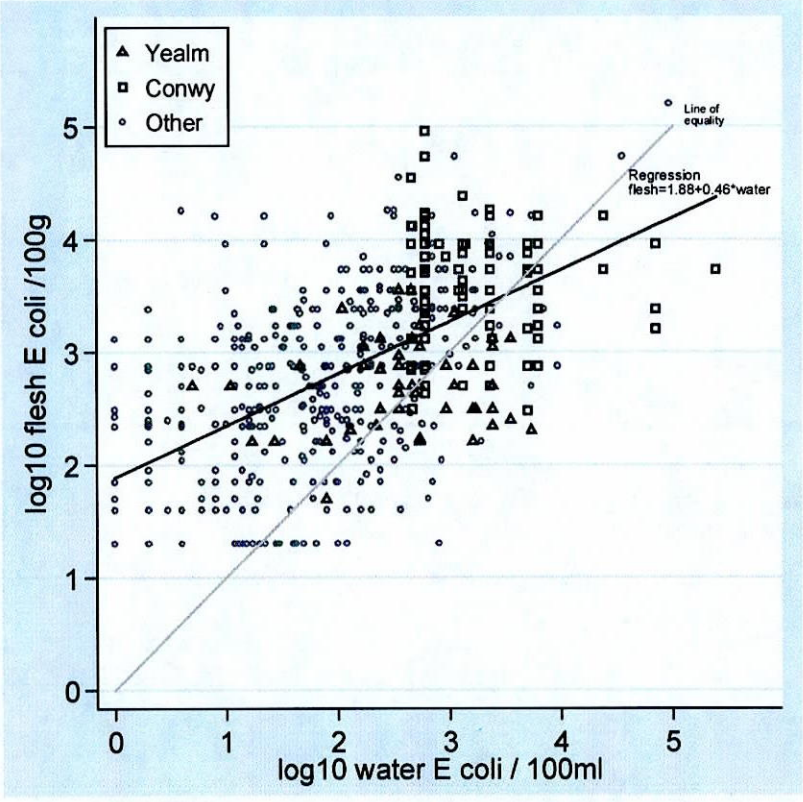

Figure 2. Scatterplot of E. coli *levels in shellfish flesh and seawater for 602 paired samples from six production areas in the UK.*

The spread of *E. coli* results between these production areas appears to be distinct with water samples from Conwy more contaminated than those from the Yealm. This is consistent with higher fluxes of FIOs impacting on the Conwy SW than those impacting on the Yealm SW.

Although the data points from each production area form clusters within the overall cloud, the results fall into the general band of points. This is highlighted in the "convex hull" graph (Figure 3), in which each production area has a more limited range of *E. coli* results in seawater and forms a more spherical clump than the overall distribution pattern of *E. coli* results. This suggest that fitting regression lines separately for each production area would be spurious and would reflect local environmental conditions and/or the small range of *E. coli* levels in seawater in that area.

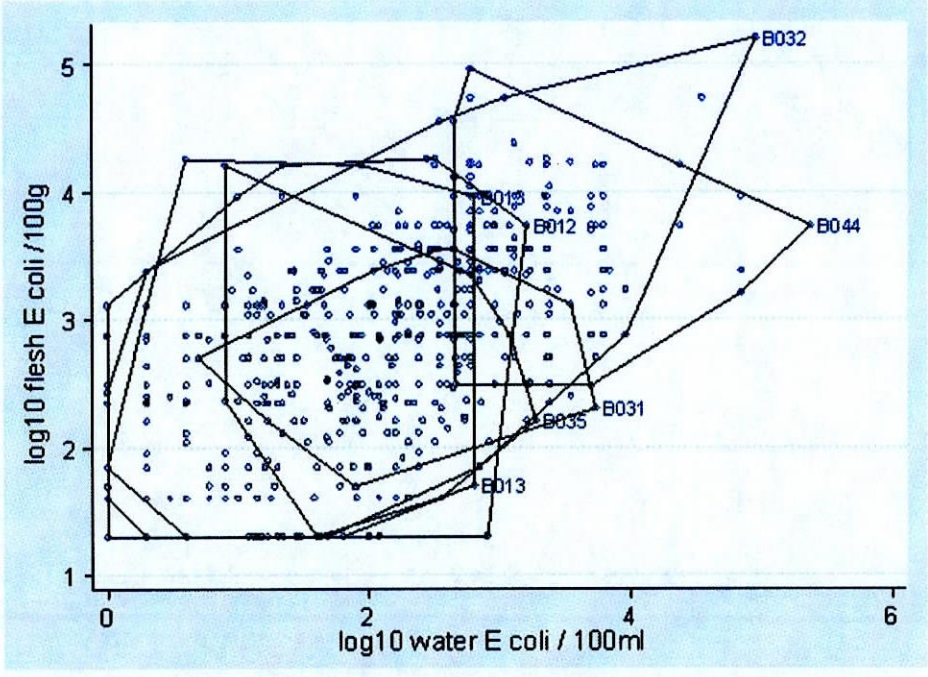

Figure 3. Convex hull graph for levels of* E. coli *in shellfish flesh and seawater from six production areas in the UK. B012 - Colne; B013 - West Mersea; B031 - Yealm; B032 - Plymouth; B035 - Camel; B044 - Conwy. The "convex hull" is a polygon enclosing the outermost points for each area.

Figure 4 shows the linear regression fit thematically represented by species. This model shows the good spread of *E. coli* results around the regression line for all three species. The majority of results above the class B threshold (4,600 *E. coli* $100g^{-1}$ FIL; $\log_{10} = 3.663$) correspond to mussel samples.

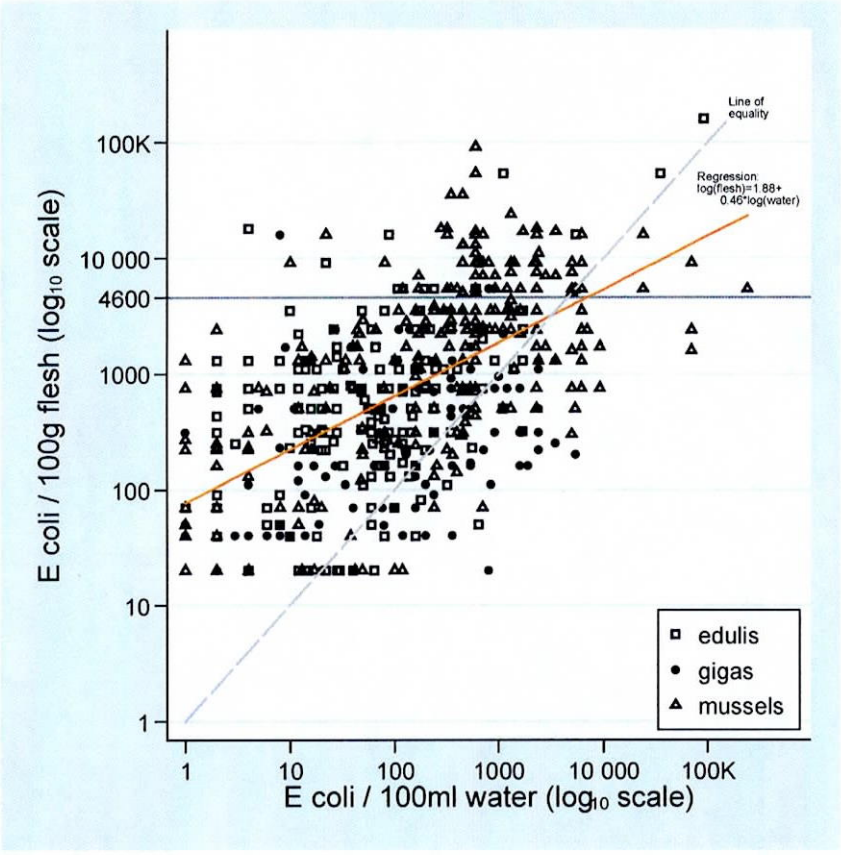

Figure 4. Scatterplot of* E. coli *levels in shellfish flesh and seawater for 602 paired samples from six production areas in the UK for each species tested.

Table 3 summarises linear regression models for *E. coli* levels in shellfish flesh versus water.

Table 3. Regression models for levels of* E. coli *in shellfish flesh versus seawater for each species tested.

Species	Log_{10} *E. coli* in seawater: Geometric mean	Standard deviation	n	Regression equation	Variance (explained) R^2
Pacific oysters (*C. gigas*)	4.75	2.09	111	$\log_{10}$ shellfish flesh=1.821+0.299$\log_{10}$ seawater	0.18
Mussels (*Mytilus* spp.)	5.78	2.56	313	$\log_{10}$ shellfish flesh=2.027+0.464$\log_{10}$ seawater	0.44
Native oysters (*O. edulis*)	4.12	1.84	178	$\log_{10}$ shellfish flesh=1.999+0.389$\log_{10}$ seawater	0.18
"Pooled species" model	5.10	2.40	602	$\log_{10}$ shellfish flesh=0.299+1.653$\log_{10}$ seawater	0.35

The regression model for mussels shows that *E. coli* levels in seawater explain a higher proportion of the variation of *E. coli* levels in that species than that in the model for the three species combined. This is probably due to the fact that mussel samples represent approximately 52% of the total number of samples. In contrast, levels of *E. coli* in Pacific oysters and native oysters explain relatively less proportion of the variation of *E. coli* in seawater. Furthermore, the difference between *E. coli* levels in mussels and in Pacific oysters is highly significant (one-way ANOVA; Scheffé's test) whereas the difference in *E. coli* levels between Pacific oysters and native oysters is marginal (p=0.07).

2.3 Results of more complex relationships

Figures 5–6 show logistic regression models of compliance with SWD G for all species tested (hereafter referred to as "pooled species" model) and for each species tested, respectively versus geometric mean of *E. coli* in seawater. The "pooled species" model is supported by mussel and native oyster *E. coli* results across the range of *E. coli* values in seawater.

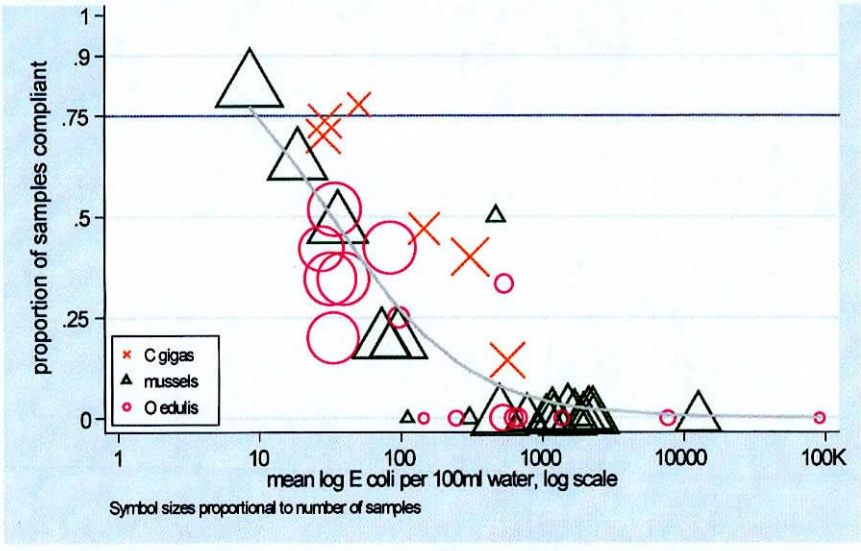

Figure 5. Logistic regression model of compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the geometric mean of* E. coli *in seawater for pooled species. *Each symbol represents a site average.*

When fitted for each species separately, Pacific oysters achieve higher compliance rates at each water quality than mussels and native oysters (Figure 6). Models individually fitted for each species show a better fit than the "pooled species" model as indicated by the lower values of information coefficients [Akaike's Information Coefficients) and Bayesian Information Coefficients] (Appendix

IV).The "pooled species" model of compliance with SWD G versus 90th percentile of *E. coli* in seawater are supported by a smaller range of *E. coli* results in water (Figure 7).

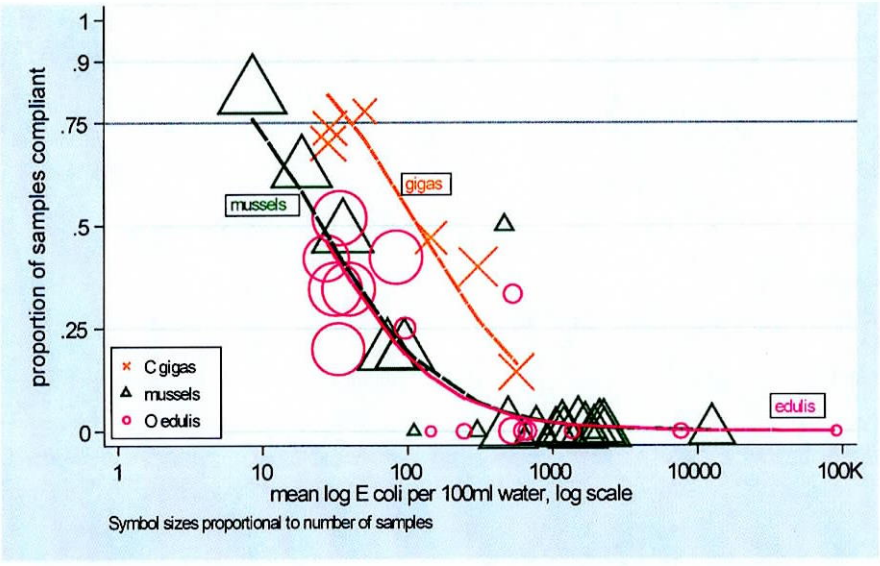

Figure 6. Logistic regression model of compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the geometric mean of* E. coli *in seawater for each species. *Each symbol represents a site average.*

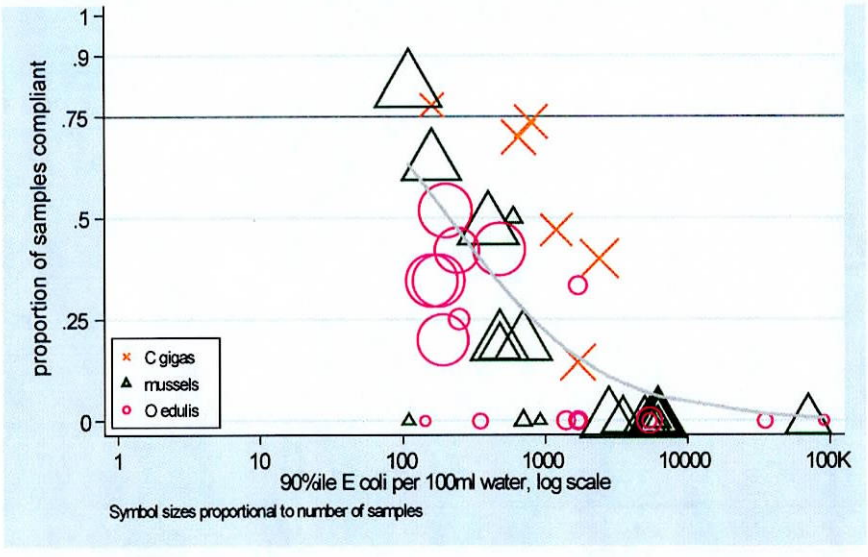

Figure 7. Logistic regression model of compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the 90%-ile of* E. coli *in seawater for all species. *Each symbol represents a site average.*

Figure 8 shows differences in compliance curves for individual models fitted for each species tested. Pacific oysters achieve higher compliance rates (>90%) than mussels and native oysters when the 90th percentile of *E. coli* in seawater is considered.

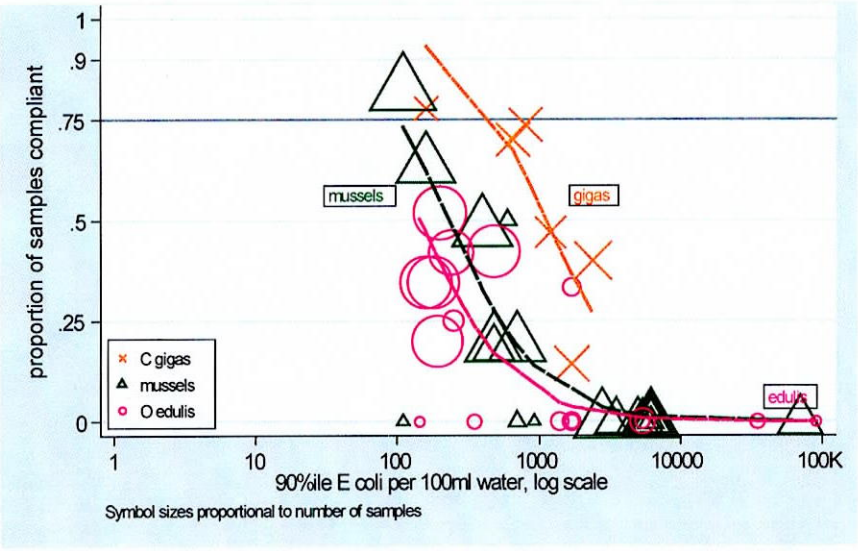

Figure 8. Logistic regression model of compliance with 300 faecal coliforms per 100ml of shellfish flesh in 75% of samples versus the 90%-ile of* E. coli *in seawater for each species. *Each symbol represents a site average.*

The R^2 obtained in the "pooled species" model demonstrates a moderate level of agreement between variables. Table 4 indicates that a geometric mean and 90th percentile of *E. coli* of 10 and 55 respectively would be equivalent to the SWD G standard.

Table 4. Estimated geometric means and 90th percentile of* E. coli *in seawater at 75% compliance with 300 faecal coliforms in shellfish fluid and intravalvular liquid.

Species	Geometric mean of *E. coli* in seawater	90th percentile of *E. coli* in seawater	Sample Pairs (n)
Mussels (*Mytilus* spp.)	8.9	102	313
Native oysters (*O. edulis*)	8.3	64	178
Pacific oysters (*C. gigas*)	41	492	111
"Pooled species" model	9.6	55	602

The logistic regression models computed to assess whether *E. coli* levels in the seawater would come under the thresholds for class B and class A are shown in Figures 9–10. The *E. coli* levels in seawater for which 90% of compliance with threshold 4,600 *E. coli* 100g^{-1} FIL (class B compliance) is predicted to be achieved as follows: 33 *E. coli* 100ml^{-1} (mussels), 177 *E. coli* 100ml^{-1} (native oysters) and 4,200 *E. coli* 100ml^{-1} (Pacific oysters). The high *E. coli* level tolerated in the water for class B compliance in Pacific oyster samples is due to the fact that only three samples failed the pass/fail test (Figure 9).

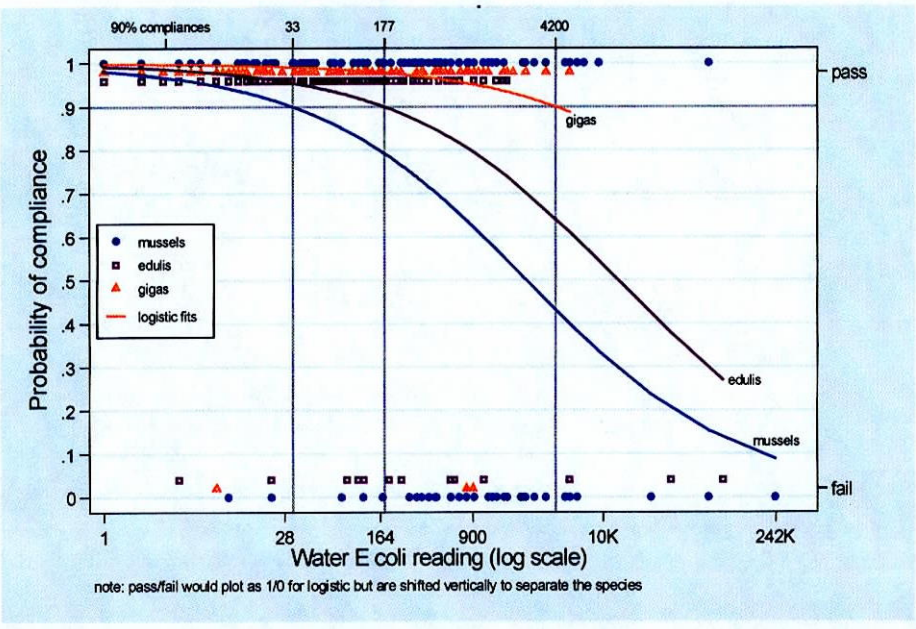

Figure 9. Logistic regression models of compliance (pass/fail) of levels of* E. coli *in seawater with the class B threshold (≤4,600* E. coli *$100g^{-1}$ FIL) under Regulation (EC) No 854/2004. *Each point represents an individual sample.*

In terms of compliance with the class A threshold, none of the samples met the 95% probability criteria demanded (Figure 10) and extrapolation of the model is not firmly grounded. The logistic models for mussels and native oysters are not significantly different from each other.

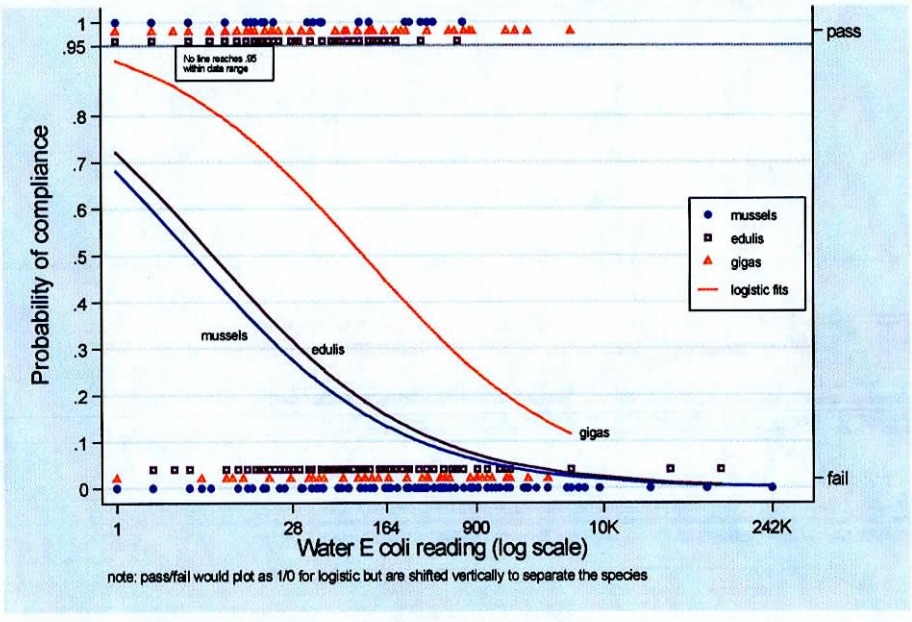

Figure 10. Logistic regression models of compliance (pass/fail) of levels of* E. coli *in seawater with the class A threshold (≤230* E. coli *$100g^{-1}$ FIL) under Regulation (EC) No 854/2004. *Each point represents an individual sample.*

3 Recommendations on a microbial standard for shellfish protected areas under the Water Framework Directive

3.1 Water column standard versus shellfish flesh standard

The main aim of the SWD G standard (300 faecal coliforms per 100ml of shellfish flesh and intervalvular fluid in 75% of samples) is to contribute to the high quality of shellfish products directly edible by man. The UK Technical Advisory Group on the WFD has noted that a revised microbiological standard for the purposes of the WFD may be more stringent (e.g. nearer existing G) or less stringent than the existing standard (UKTAG, 2007). The level of stringency is determined by the strength of the association between the levels of FIOs in shellfish and those in the overlaying waters. Different species of shellfish growing in the same area may differ in the degree to which they accumulate FIOs. These differences are often attributed to biological and/or physiological characteristics of shellfish species (e.g. age, size, maturity, nutritional condition, physiological mechanisms regulating feeding and digestion) and the combined effect of prevailing environmental conditions. Furthermore, the growing methods used for the commercial production of shellfish determine the time during which shellfish are immersed in the water column and hence their temporal and spatial exposure to microbial contaminants.

The differences between water *E. coli* geometric means in mussel/native oysters and those in the "pooled species" model are deemed of no practical effect in terms of microbial quality of shellfish. The difference between the 90th percentile in mussels and that in the "pooled species" model could be associated with the high standard deviation of *E. coli* results in mussels, reflecting the fact that some of the areas where mussels grow are/have been affected by high levels of microbiological contamination.

The ability of Pacific oysters to be compliant at higher water contamination levels could be explained by differences in physiological mechanisms determining the uptake or retention of microbial contaminants; different growing methods (e.g. oysters more commonly grown on trestles and mussels commonly grown on sea/riverbed); and differences in impacting pollution sources. Other considerations on the practical application of a microbial standard are discussed in section 4.

3.2 Discussion on the possibility of using shellfish flesh data from the Shellfish Hygiene monitoring programme to inform water quality in shellfish protected areas

In the UK, there are substantial differences in the way that the Shellfish Hygiene (SH) and Shellfish Waters (SWs) monitoring programmes are undertaken to ensure compliance with the requirements of Directive 2006/113/EC and Regulation No 854/2004, respectively.

Under the SH monitoring programme in England and Wales, individual classifications are attributed to each species commercially harvested from each production area. In most cases, these classifications are attributed on the basis of *E. coli* monitoring undertaken separately for each of these species. There are however some circumstances where a sentinel species (often mussels) is used to represent levels of contamination of other species within the same production area. In such cases, the sentinel species must be at least as protective as the species to be classified. Consequently, the number/location of sampling points and the rationale for their selection varies according to the location and extent of beds/production areas for each of these species. Consideration when selecting sampling points is also given to proximity of significant pollution sources and hydrodynamic effects determining the circulation of pollutants. Designated Shellfish Waters tend to encompass several shellfish beds.

Under the SWs monitoring programme, surface water monitoring points are chosen to represent the shellfish water as a whole (Environment Agency, 2003). SW flesh monitoring points are selected on the basis of identifying the nearest hygiene monitoring to the designated water column monitoring point which may not be predictive of the water as a whole.

The second important element requiring consideration is the tolerance around monitoring point position. In England and Wales, the recommended maximum acceptable tolerance around monitoring points may be from 10m up to 50m for hand-picked/hand-raked shellfish and up to 250m for dredged shellfish, although this varies according to local circumstances.

The use of shellfish flesh data to inform water quality of a shellfish SPA would be dependent on the availability of monitoring data from a "species likely to be most protective" of all species sampled from a point within the tolerance established for that species. From the results shown in Table 4, mussels would be a "protective" species if a microbial water column standard based on geometric

mean *E. coli* is to be used for SPA under the WFD. It would therefore be reasonable to assess the overall water quality using *E. coli* monitoring data in mussels on the basis of models developed in this study.

A third aspect is the comparability of monitoring results, which has been constrained by the different monitoring frequencies used in the SH (weekly basis for provisional classification of beds; monthly basis for ongoing monitoring) and SWs (quarterly) monitoring programmes. These differences imply that the probabilities of detecting episodes of deteriorated water quality are necessarily different. This problem could be overcome by harmonising with the monthly sampling regime required by the WFD for monitoring physico-chemical priority substances in rivers, lakes, transitional and coastal waters (European Communities, 2000).

4 Considerations on the practical application of a microbial standard for shellfish protected areas under the Water Framework Directive

In England and Wales, compliance with the SWD G has been assessed on the basis of monitoring one species from a single point deemed to be representative in the SW. The WFD provides more specification on the approach for sampling point selection as follows:

> *For bodies at risk from significant point sources, sufficient monitoring points within the body of water in order to assess the magnitude and impact of the point source and where a body is subject to a number of point sources, monitoring points may be selected to assess the magnitude and impact of these sources as a whole.*
>
> *For bodies at risk from significant diffuse sources, sufficient monitoring points within a selection of the bodies in order to assess the magnitude and impact of the diffuse sources. The selection bodies shall be made such that they are representative of the relative risks of the occurrence of the diffuse source pressures, and the relative risks of the failure to achieve good surface water status.*
>
> *For bodies at risk from significant hydromorphological pressure, sufficient monitoring points within a selection of the bodies in order to assess the magnitude and impact of the hydromorphological pressures. The selection of bodies shall be indicative of the overall impact of the hydromorphological pressure to which all the bodies are subject.*

Further considerations on the selection of monitoring points in SPAs are given below:

a) Identify a single species and single monitoring point that protects the SPA as a whole.
   Advantage: relatively simple to adopt, easier to administer and enforce.
   Disadvantages: May not be representative of larger SPAs such as wild shellfisheries, in which the location/extent of shellfish beds change considerably over short timescales.

b) Identify more than one species to protect all beds of that species and any additional species for which it can act as a sentinel of sub-divisions of the SPA.

Advantage: More likely to ensure adequate protection for sub-divided SPAs than that expected from (a).

Disadvantage: technically more difficult to administer than (a).

c) Identify multiple monitoring points and one/more species that protect sub-divisions of the SPA.

Advantage: More likely to ensure protection for sub-divided SPAs than that expected from (a).

Disadvantage: technically more difficult to administer than (a).

d) Identify a water column monitoring point that protects the SPA as a whole.

Advantage: relatively simple to adopt as it requires fewer resources, easier to administer and enforce.

Disadvantage: may not adequately represent the spatial/temporal variations of microbial contamination in shellfish across the SPA.

e) Identify more than one water monitoring point representative of sub-divisions of the SW.

Advantage: more likely to ensure adequate protection for sub-divided SPAs than that expected from hypothesis (c).

Disadvantage: technically more difficult to administer than (c).

f) Identify water column monitoring points that reflect the point in the water column in which shellfish are grown.

Advantage: more likely to adequately represent the distribution of microbial contaminants in the water column than (c).

Disadvantage: interpretation of monitoring programme data for the purposes of compliance assessment could be confounded by results from multiple points taken at different depths.

g) Harmonise the timing of water column sampling under WFD with flesh sampling under SH monitoring programme.

Advantage: ensures data comparability between monitoring programmes.

Disadvantage: technically difficult to administer as it requires high level of collaboration between authorities.

5 Conclusions

1. Simple linear regression modelling was used to study the relationship between levels of *E. coli* in shellfish flesh versus levels of the indicator in overlying waters from six UK production areas. The "pooled species" model explained approximately 35% of the variance observed in the variables. This is higher than that reported in the literature for some environmental studies. There was no evidence for a non-linear relationship within the relevant ranges.

2. No appreciable differences between harvesting areas were found in the levels of the microbial indicator in shellfish and overlying waters suggesting that the sample data represents the range of contaminating levels found in UK harvesting areas. However, it was not possible to investigate spatial and temporal sampling bias, as data on time and precise location of sampling were not available.

3. Logistic regression models were used to determine targets for geometric mean and 90th percentile of *E. coli* in seawater compliant with the SWD G standard. A geometric mean of 10 and 90 percentile of 55 *E. coli* per 100ml water are predicted to be equivalent to the SWD G standard (300 faecal coliforms per 100g FIL). We recommend that these thresholds should be validated on a more up-to-date dataset and compared to results from microcosm experiments undertaken under project *Impact of Chronic Microbial Pollution on Shellfish* (WT0923) by Cefas and CREH for Defra.

4. Differences in compliance rates between mussels and Pacific oysters emerged from the logistic regression models indicating that these relationships are indeed complex and require further investigation, namely on the role of environmental factors and physiological mechanisms determining the uptake of FIOs by shellfish.

5. Compliance with the class B threshold (≤4,600 *E. coli* 100g^{-1} FIL with 90% probability) in mussel, native oyster and Pacific oyster samples was predicted at 33, 177, and 4,200 *E. coli* levels in seawater, respectively. The high *E. coli* level in Pacific oyster samples reflects the fact that the vast majority of samples passed the test of class B threshold compliance. This suggests that

Pacific oysters are less appropriate than mussels as sentinel species for the purposes of monitoring shellfish water quality.

6. In terms of compliance with the class A threshold (230 *E. coli* $100g^{-1}$ FIL), none of the logistic models achieved 95% compliance rates within the range of *E. coli* levels detected in the water.

7. We discuss the use of shellfish flesh data from the Shellfish Hygiene monitoring programme for the purposes of informing water quality in SPAs under the WFD. Important differences are identified in the way these monitoring programmes have been undertaken in the UK namely in terms of: the number/location of sampling points and the rationale for their selection, tolerance around the sampling points, and frequency of sampling. It is argued that harmonisation of monitoring practices would facilitate data comparability between monitoring programmes.

8. The evidence presented in this report will be complemented by the literature review on environmental factors influencing the microbial quality of shellfish undertaken as part of this project and the results from microcosm and field studies under project WT0923.

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7 Appendices

Appendix I. Number of samples from each monitoring point and for each species in the dataset.

Production area	Monitoring point	Shellfish species			
		C. gigas	*Mytilus* spp.	*O. edulis*	Total
Colne	B012A	0	23	0	23
	B012B	0	11	0	11
	B012C	9	0	0	9
	B012D	0	21	0	21
	B012E	19	0	0	19
	B012F	20	0	1	21
	B012G	0	22	0	22
West Mersea	B013A	0	0	26	26
	B013B	0	0	25	25
	B013D	0	0	26	26
	B013E	0	0	19	19
	B013F	0	0	27	27
	B013G	0	0	26	26
	B013H	0	0	4	4
Yealm	B031A	21	0	0	21
	B031B	25	0	2	27
Plymouth	B032E	0	0	2	2
	B032G	0	21	6	27
	B032K	0	1	0	1
	B032L	0	10	3	13
	B032M	0	11	3	14
	B032N	0	2	0	2
	B032O	0	21	0	21
	B032P	0	28	0	28
	B032Q	0	1	3	4
	B032R	0	0	2	2
	B032S	0	0	2	2
	B032U	0	0	1	1
Camel	B035A	17	0	0	17
	B035B	0	2	0	2

Conwy	B044A	0	18	0	18
	B044B	0	10	0	10
	B044C	0	15	0	15
	B044D	0	18	0	18
	B044E	0	12	0	12
	B044F	0	11	0	11
	B044G	0	4	0	4
	B044H	0	17	0	17
	B044I	0	21	0	21
	B044M	0	13	0	13
	Total	111	313	178	602

Appendix II. Assumptions of linear regression models.

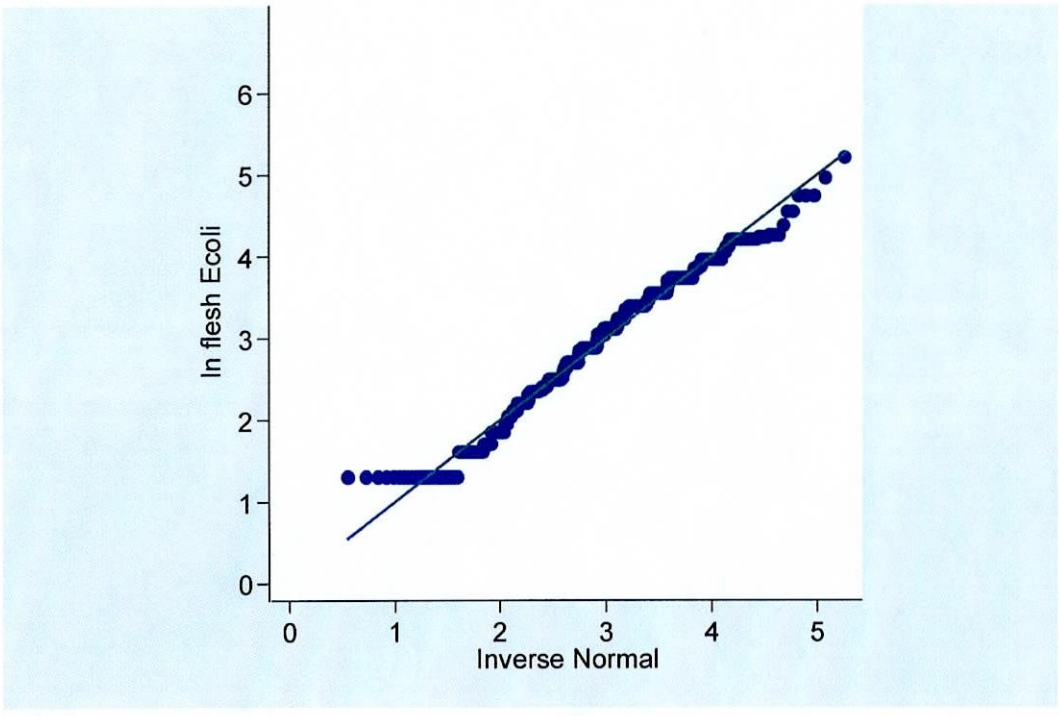
	Purpose			
Assumption	Predict *E. coli* levels in shellfish flesh given *E. coli* levels in water	Predict *E. coli* levels in shellfish flesh and a variance for the prediction	Obtain best linear unbiased estimator of *E. coli* levels in shellfish flesh	Test hypotheses, estimate confidence or prediction intervals
E. coli levels in shellfish flesh are linearly related to *E. coli* levels in water	+	+	+	+
Data used to fit the model are representative of data of interest	+	+	+	+
Variance of the residuals is constant. It does not vary with *E. coli* levels in water or on anything else (e.g. time)		+	+	+
The residuals are independent between observations			+	+
The residuals are normally distributed				+

+ Assumptions necessary for the purposes to which the linear model is determined.

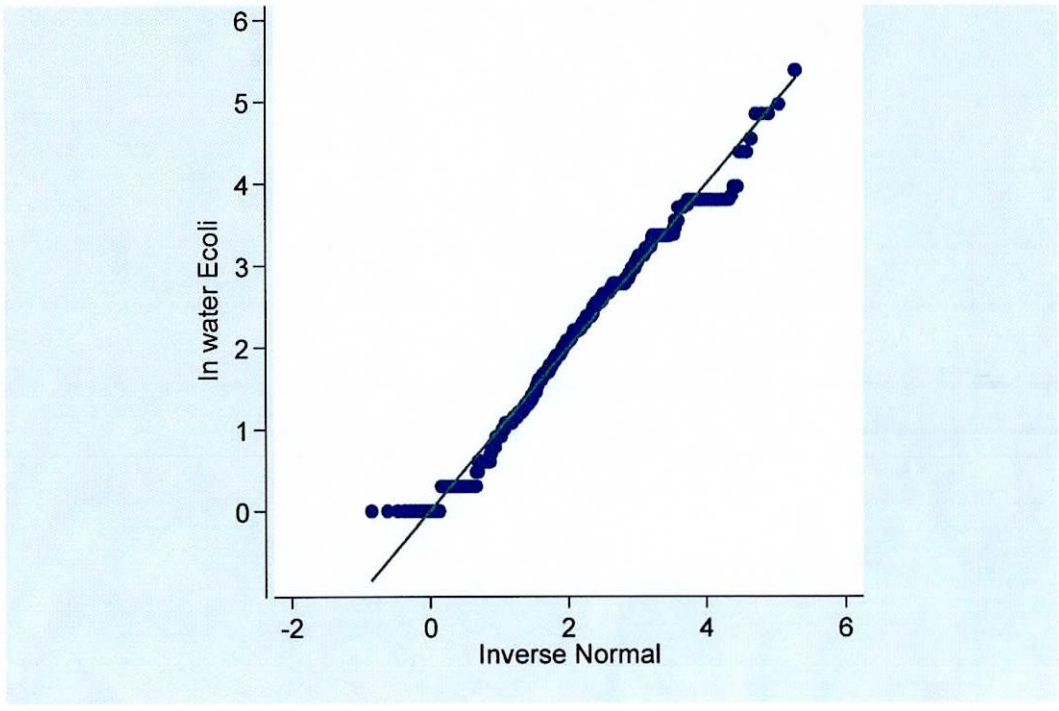
Adapted from Helsel and Hirsch (2002).

Appendix III. Quantile plots of Log_{10}-transformed *E. coli* results in shellfish flesh (A) and seawater (B) for UK dataset.

A



B



Appendix IV. Outputs from the Logistic regression model of compliance with 300 faecal coliforms in 75% of samples versus geometric mean of *E. coli* in seawater for pooled species.

```
Deviance: 72.7682454
Pearson: 74.74581186
Variance function: V(u) = u*(1-u/sample)
Link function: g(u) = Ln (u/(sample-u))
Log likelihood = -68.22829948
No. observations: 46
Residual df = 44
Scale parameter: 1
(1/df) Deviance: 1.653824
(1/df) Pearson = 1.698768

(Binomial)
(Logit)
AIC = 3.053404
BIC = -95.69198
```

Compliance 300 faecal coliforms	OIM Coefficient	Standard error	Z	P>IzI	95% confidence interval	
E. coli in seawater	-0.9013193	0.0901207	-10.0	0.000	-1.077953	-0.7246859
Constant	3.140463	0.3766187	8.34	0.000	2.402304	3.878622

Appendix V. Outputs from the logistic regression model of compliance with 300 faecal coliforms in 75% of samples versus geometric mean of *E. coli* in seawater for each species tested.

```
Deviance: 34.49347494
Pearson: 41.6055635
Variance function: V(u) = u*(1-u/sample)
Link function: g(u) = Ln (u/(sample-u))
Log likelihood = -49.09091425
No. observations: 46
Residual df = 42
Scale parameter: 1
(1/df) Deviance: 0.8212732
(1/df) Pearson = 0.9906087

(Binomial)
(Logit)
AIC = 2.308301
BIC = -126.3095
```

Compliance 300 faecal coliforms	OIM Coefficient	Standard error	z	P>|z|	95% confidence interval	
E. coli in seawater	-1.044341	0.1068213	-9.78	0.000	-1.253707	-0.8349751
C. gigas	1.600619	0.3090013	5.18	0.000	0.9949871	2.20625
O. edulis	-0.0791923	0.2515881	-0.31	0.753	-0.5722959	0.4139112
Constant	3.385913	0.4511417	7.51	0.000	2.501692	4.270135

Appendix VI. Outputs from the logistic regression model of compliance with 300 faecal coliforms in 75% of samples versus 90%-ile of *E. coli* in seawater for pooled species.

```
Deviance: 123.9274286
Pearson: 120.0467182
Variance function: V(u) = u*(1-u/sample)
Link function: g(u) = Ln (u/(sample-u))
Log likelihood = -93.8078911
No. observations: 46
Residual df = 44
Scale parameter: 1
(1/df) Deviance: 2.816532
(1/df) Pearson = 2.728335

(Binomial)
(Logit)
AIC = 4.16556
BIC = -44.53279
```

Compliance 300 faecal coliforms	OIM Coefficient	Standard error	Z	P>IzI	95% confidence interval	
90th percentile of *E. coli* in seawater	-0.8140287	0.0857238	-9.50	0.000	-0.9820444	-0.6460131
Constant	4.363091	0.5349935	8.16	0.000	3.314523	5.411659

Appendix VII. Outputs from the logistic regression model of compliance with 300 faecal coliforms in 75% of samples versus 90%-ile of *E. coli* in seawater for each species.

```
Deviance: 50.92550812
Pearson: 53.72191013
Variance function: V(u) = u*(1-u/sample)
Link function: g(u) = Ln (u/(sample-u))
Log likelihood = -57.30693084
No. observations: 46
Residual df = 42
Scale parameter: 1
(1/df) Deviance: 1.212512
(1/df) Pearson = 1.279093

(Binomial)
(Logit)
AIC = 2.665519
BIC = -109.8774
```

Compliance 300 faecal coliforms	OIM Coefficient	Standard error	Z	P>IzI	95% confidence interval	
90th percentile of E. coli in seawater	-1.328646	0.1476527	-9.00	0.000	-1.61804	-1.039252
C. gigas	2.092913	0.3243272	6.45	0.000	1.457243	2.728583
O. edulis	-0.6230634	0.2628621	-2.37	0.018	-1.138264	-0.1078631
Constant	7.241959	0.8943404	8.10	0.000	5.489084	8.994834

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