APPENDIX A8-2

Cefas

Impact of chronic microbial pollution on shellfish

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Executive Summary

The dynamics of *Escherichia coli* (*E. coli*) accumulation, retention and clearance were examined in three shellfish species (mussels, Pacific oysters and cockles) in a series of microcosm experiments using seawater microcosms (target temperature 10.5°C and target salinity 30 practical salinity units) in the Cefas Weymouth Laboratory experimental facility. The principal aim of the experiment was to identify water concentrations of this faecal indicator organism that resulted in shellfish flesh values around the 300 colony forming units (cfu) per 100 ml Shellfish Waters Directive (SWD) faecal coliform "guideline" standard and to inform policy on an appropriate national standard under the Water Framework Directive (WFD).

To simulate shellfish exposure to prolonged ('chronic') microbiological pollution, six flow-through tank seawater microcosms were established in which shellfish were exposed to six different target concentrations of *E. coli* in seawater ranging from 1 to 330 cfu/100ml. Levels of *E. coli* in sewage, tank water and shellfish flesh were measured prior to, during and following exposure.

Linear regression models of *E. coli* levels in shellfish versus water were fitted for the six target water concentrations. These models show that 52% of the variance observed in *E. coli* levels in mussels and Pacific oysters and 60% of the variance in *E. coli* levels in cockles are explained by the variation of *E. coli* levels in the water.

On exposure to sewage, shellfish exhibited relatively rapid accumulation of *E. coli* to a maximum 'equilibrium' state in each tank and, following end of dosing, a relatively rapid clearance phase. Over the range of concentrations studied, maximum levels accumulated in shellfish during the exposure phase are shown to be proportional to the level of water contamination. Overall, cockles accumulated the bacteria to a higher level than mussels and Pacific oysters. Mean accumulation factors calculated as the geometric mean indicator concentration of the organism in shellfish divided by the corresponding geometric mean concentration in the overlying water are 330 (cockles), 15 (mussels) and 12 (Pacific oysters). At the end of eight days exposure to sewage contamination, mussels and Pacific oysters were more efficient in clearing the bacteria accumulated in their tissues. Cockles were less efficient, particularly when exposed to more contaminated water.

Environmental investigations were undertaken to verify whether the results implied by the microcosm experiments can be confirmed in shellfish growing waters. Levels of *E. coli* were monitored in samples of the same three species used in the microcosms collected from 20 netlon bags laid in the intertidal zone beneath Mumbles Pier (Swansea Bay) and in water samples collected along a transect adjacent to the western side of the pier during the period 5-15 September 2011. These investigations were complemented by hydrodynamic modelling (DIVAST) designed to provide near-real-time prediction of *E. coli* concentrations for the site where shellfish were laid. The sewage input used for model predictions was a sewage pumping station at Knab Rock, Mumbles Head.

The relative ordering of inter-species *E. coli* accumulation was consistent with those obtained in the microcosm studies and the literature. However, both modelled and measured *E. coli* levels in water sampled during the preceding tidal incursion impacting upon the shellfish bags were not significantly correlated with the measured *E. coli* levels in shellfish flesh.

The empirical results also indicated ubiquitous and high (over 2 $\log_{10}$ orders) 'natural' temporal variability in *E. coli* concentrations over a diurnal cycle, even under dry-weather conditions. Exploratory data analysis of *E. coli* levels monitored during the 2011 summer bathing season in Swansea Bay indicate that high-flow events elevate daily mean concentrations of the faecal indicator but not their range at a monitoring site in Swansea Bay.

On the assumption that most inshore shellfish waters around England and Wales will show variability in *E. coli* of 100 fold or more (over 2 $\log_{10}$ orders) in 'normal' conditions, it is concluded that low levels of microbiological pollution such as those below the SWD faecal coliform standard cannot be characterised as constant faecal indicator concentrations. It is therefore recommended that any sampling regime designed for regulatory purposes should be able to accommodate and characterise this variability. One possible approach would be to consider the 'chronic' water quality condition as a probability density function (pdf) and use the observed *E. coli* accumulation factors to derive an associated pdf shellfish flesh concentrations from any given water concentration.

Glossary

Accumulation:	Uptake and storage of faecal indicator organisms (FIOs) within the cells of living bivalve shellfish (see below).
Accumulation factor:	Measure of the intensity of the accumulation of FIOs in bivalve shellfish. This measure is given by the ratio between the concentration of FIOs in shellfish relative to the concentration of FIOs in the overlying water.
Bivalve filter pump:	Groups or bands of lateral cilia on filaments arranged in parallel within the mantle cavity of the bivalve.
Bivalve mollusc	Any marine or freshwater mollusc of the class Pelecypoda (formerly Bivalvia or Lamellibranchia), having a laterally compressed body, a shell consisting of two hinged valves and gills for respiration. The group includes clams, cockles, oysters and mussels.
Chronic exposure:	Contact of bivalve shellfish with FIOs in the overlying waters that occurs over a long time (more than 5 days).
Clearance:	In the context of this report, the process by which shellfish eliminate FIOs during filter-feeding when exposed to normal conditions of salinity and temperature for the species.
Concentration:	Amount of FIOs present in a certain amount of shellfish flesh and intravalvular liquid or water.
Confidence interval (CI)	Two numbers that surround a statististical estimate (eg the mean value) such that, on average, a given proportion of estimates from similar samples will lie between the two values. CIs are often based on maximum likelihood and the *t* distribution, so are quoted as estimate +/- constant, but CIs do not have to be symmetrical. In particular, the confidence interval for a proportion cannot lie outside [0 1].
Escherichia coli (*E. coli*)	A species of bacterium that is a member of the faecal coliform group (see below). It is more specifically associated with the intestines of warm-blooded animals and birds than other members of the faecal coliform group.
Faecal coliforms	A group of bacteria found in faeces and used as a parameter in the Hygiene Regulations, Shellfish and Bathing Water Directives, *E. coli* is the most common example of faecal coliform. Coliforms (see above) which can produce their characteristic reactions (e.g. production of acid from lactose) at 44°C as well as 37°C. Usually, but not exclusively, associated with the intestines of warm-blooded animals and birds.

Faecal indicator organism (FIO)	Bacteria or groups of bacteria (usually faecal coliforms, *Escherichia coli*, enterococcus) normally residing in the intestinal tract of warm-blooded animals and used to demonstrate the potential presence or absence of microbial pathogens.
Geometric mean	The geometric mean of a series of N numbers is the Nth root of the product of those numbers. It is more usually calculated by obtaining the mean of the logarithms of the numbers and then taking the anti-log of that mean. It is often used to describe the typical values of a skewed data such as one following a log-normal distribution.
Sewage	Sewage can be defined as liquid, of whatever quality that is or has been in a sewer. It consists of waterborne waste from domestic, trade and industrial sources together with rainfall from subsoil and surface water.
Wastewater Treatment Works (WwTW)	Facility for treating the wastewater from predominantly domestic and trade premises.

List of Abbreviations

BST	British summer time (UMT+1 hour)
Cefas	Centre for Environment, Fisheries & Aquaculture Science
cfu	Colony Forming Unit
CREH	Centre for Research into Environmental Health
CSO	Combined sewer overflow
Defra	Department for Environment, Food and Rural Affairs
DO	Dissolved oxygen
DSP	Designated sampling point
EC	European Commission
E. coli	*Escherichia coli*
FIO	Faecal indicator organism
FIL	Flesh and intravalvular liquid
FC	Faecal coliforms
LoD	Limit of detection
LoQ	Limit of quantification
MMGB	minerals modified glutamate broth
MPN	Most probable number
MLSB	membrane lauryl sulphate broth
pdf	probability density function
psu	practical salinity units
spp.	Species
SPS	sewage pumping station
SWD	Shellfish Waters Directive
TBGA	tryptone bile glucuronide agar
UKTAG	UK Technical Advisory Group on the Water Framework Directive
WFD	Water Framework Directive
WwTW	Wastewater Treatment Works

Figures

Tables

Table of contents

1 Introduction

1.1 Context

As part of this Defra funded project WT0923 "*Impact of chronic microbial pollution on shellfish*", a series of 21 flow-through tank "controlled microcosm" experiments were undertaken to provide data on the dynamics of accumulation[1] and clearance[2] of *Escherichia coli*[3] (*E. coli*) in three species of bivalves commercially harvested in England and Wales: mussels (*Mytilus* spp.), Pacific oyster (*Crassostrea gigas*) and the common cockle (*Cerastoderma edule*). These studies were undertaken using seawater tank microcosms in the Cefas Weymouth Laboratory experimental facility. Laboratory microcosms were considered to offer the best means of simulating a 'chronic' (i.e. constant) exposure of shellfish to sewage derived faecal indicator organisms, thus, to determine the concentration factors between the water and flesh concentrations under controlled conditions.

An environmental investigation was then undertaken by CREH to investigate whether the results implied by the microcosm experiments can be confirmed in environmental waters. In reality, environmental concentrations of faecal indicator organisms (FIOs) are notoriously variable and respond rapidly to pollution source impacts and environmental drivers such as tidal state, rainfall, solar irradiance and turbidity (Kay *et al.*, 2005; 2007; 2008a; 2008b). This normal 'environmental' variability and the lag between water and flesh concentration cycles, has made the search for operationally useful relationships between the two matrices and, thus, an environmental water concentration value to guide regulatory practice, scientifically challenging for the reasons explored in Kershaw *et al.* (2012b: Section 4, Figure 2 and Table 2).

1.2 Objectives

The principal aim of the experiment was to identify water concentrations of this faecal indicator organism which would be compliant with the SWD faecal coliform "guideline" standard of 300 cfu 100/ml and to inform policy on an appropriate national standard for Shellfish Protected Areas under the WFD. Specifically, the project aims to:

A. Determine which levels of water contamination cause build-up of contamination in shellfish flesh.

B. Explain how the twin factors of length of exposure and level of contamination are linked in determining the microbial quality of harvested shellfish.

C. Describe the dynamics of self-cleansing during periods of good water quality.

D. Identify if there is a balance between level of exposure and time of exposure.

E. Inform the sampling strategy required in a given shellfish water to allow us to predict shellfish quality.

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[1] Uptake and retention of faecal indicator organisms within the tissues of live bivalve shellfish.

[2] The process by which shellfish eliminate faecal indicator organisms during filter-feeding when exposed to normal conditions of salinity and temperature for the species.

[3] Indicator of contamination of faecal origin recommended by Defra to be used for the purposes of monitoring Shellfish Protected Areas.

2 Methods

2.1 Microcosm experiments

2.1.1 Experimental design

Key variables considered in the experimental design were: source of FIOs; target seawater FIO concentrations; target seawater temperature and salinity; selection of bivalve mollusc species; stocking density/acclimatisation and sampling strategy.

A pilot study was undertaken to a) ascertain the concentration of FIOs in locally sourced sewage, b) establish the change in concentration of FIOs in a stock sample of sewage held over a number of days and c) to establish and test the experimental microcosm set-up used in the subsequent experimental work. This work included: assaying indicative levels of *E. coli* and faecal coliforms in sewage; testing microcosm tank water flow-through control and salinity/temperature/aeration control; testing and calibration of sewage dosing equipment and investigating water FIO contamination and flesh uptake in three species of shellfish in order to target water FIO concentrations in the subsequent experimental work.

Following the pilot study, a series of three experiments, each using a different species of shellfish, were undertaken in which bivalve molluscs were exposed to sewage contaminated seawater over a range of target water *E. coli* concentrations for a period of eight days. For each experiment animals were placed into six identical sea-water tanks set up in parallel in the Cefas Experimental Facility. Following a 48h period of shellfish acclimatisation, each tank was dosed with dilute sewage derived from a common sewage stock. Each of the six tanks was exposed to a different target concentration of sewage derived *E. coli* ranging from 1 to 330 bacteria per 100 ml. Levels of *E. coli* concentrations in shellfish flesh and the overlying tank water were analysed prior to and during the 8-day chronic exposure phase and a 2-day post-exposure coliform clearance phase. The clearance phase provided information regarding the efficacy of *E. coli* removal from shellfish flesh following exposure to chronic pollution.

2.1.2 Health and safety

Cefas risk management procedures were followed in relation to bio-hazard management, and health and safety requirements met for safe working, handling and disposal of contaminated materials.

2.1.3 Shellfish sourcing and handling

Farmed rope-grown mussels (total wet weight 93.00 kg) harvested from Portland Harbour and farmed Pacific oysters (total wet weight 237.44 kg) harvested from Portland Harbour and the Fleet Lagoon were supplied by Lyme Bay Shellfish Ltd. Wild cockles (total wet weight 124.33 kg) from Poole Harbour were supplied by Lakeside Shellfish Ltd.

The shellfish were divided equally by weight into six tanks. The number of animals used took into account the numbers required for sampling (55 cockles, 18 oysters and 35 mussels for each sample) while allowing for 50% mortality throughout the course of the experiments. The stocking density was designed such that water volume in each tank was enough that, at any time, the weight of water in the tank was greater than ten times higher than the weight of shellfish. The time between shellfish harvesting and the commencement of shellfish acclimatisation in the seawater tank microcosms did not exceed 6 hours.

2.1.4 *Sewage collection and dosing*

It was necessary to use sewage stock containing a sufficiently high titre of FIOs in order to achieve the desired range of target water FIO concentrations in the microcosms. Untreated 'crude' sewage was therefore used as a sewage stock of FIOs. Samples of crude sewage were collected from the works inlet at Dorchester (Louds Mill) Wastewater Treatment Works (WwTW) in Dorset (population equivalent c. 47,000). This process involved collecting sewage in 20-litre plastic containers under the supervision of Wessex Water staff between 08:00 and 10:00 on the day prior to each experiment (Figure 1).

Figure 1 - Sewage collection point at Dorchester (Louds Mill) WwTW.

The sewage was transported to the laboratory immediately after collection and stored refrigerated at temperature 4°C. A sample of the sewage stock was assayed in duplicate on arrival at the laboratory (within two hours of collection) and again on any subsequent days prior to its use for the experiments.

Two reservoirs of sewage with different dilutions of the sewage stock were used to facilitate delivery of *E. coli* target concentrations into the experimental tanks (Figure 2). Sewage stock assay results were used to determine the target level of dilution required in each reservoir. Precise volumetric dilutions were made on the basis of FIO results[4] obtained from assays of a refrigerated sewage stock sample taken on the preceding day: i.e. the most recent result available to inform volumetric calculations. The calculations are given in the Appendix I.

[4] Refrigerated sewage stock was assayed for presumptive faecal coliforms and *E. coli* on a daily basis. A conversion factor of 0.85 was applied to the results of presumptive faecal coliforms to derive an assumed *E. coli* concentration. This concentration was then used to calculate the dilutions required for each sewage reservoir.

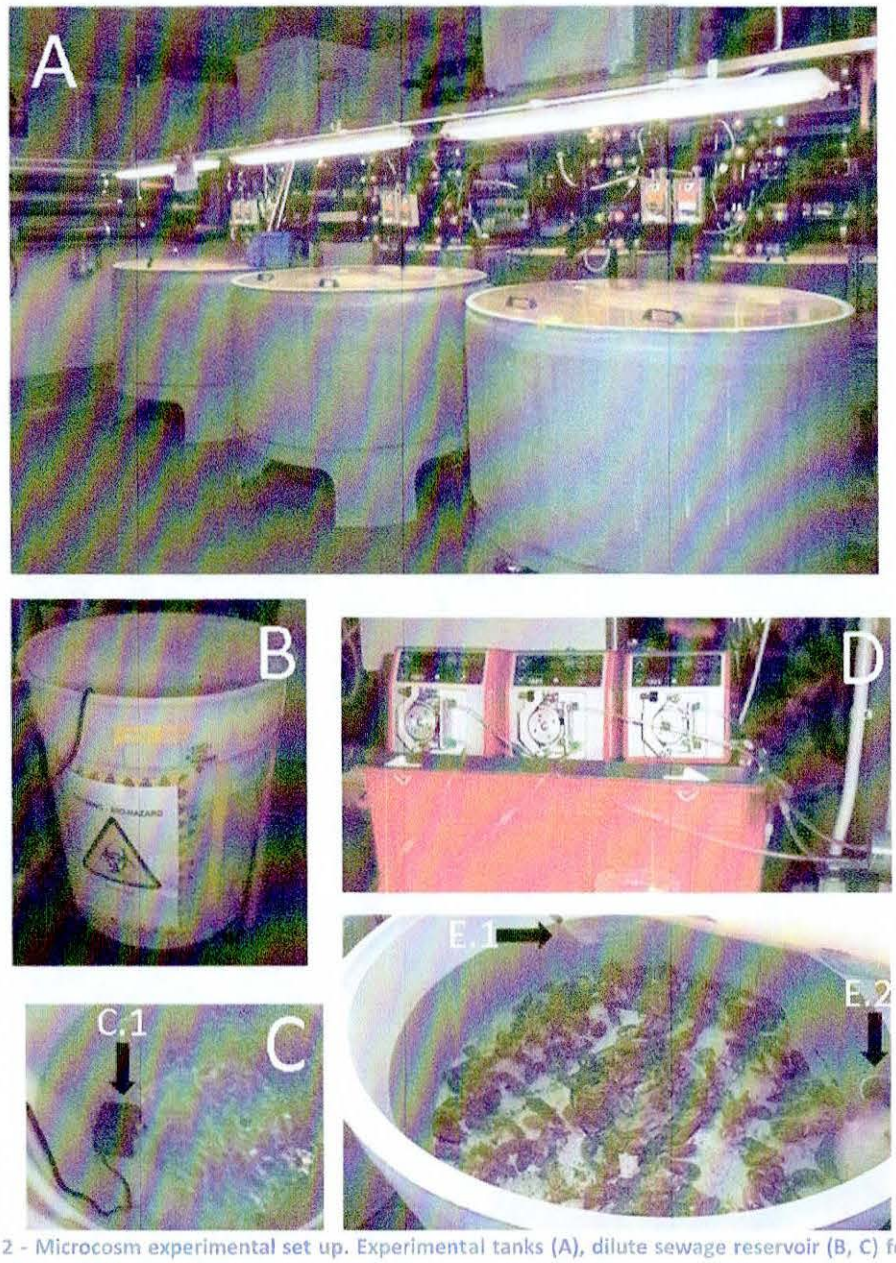


Figure 2 - Microcosm experimental set up. Experimental tanks (A), dilute sewage reservoir (B, C) for tanks with 1, 3.3 and 10 cfu/100ml target *E. coli* levels, reservoir circulation pump (C.1), peristaltic pumps (D), tank seawater inlet spray bar (E.1), tank aeration streamer (E.2).

The procedure for dilution of sewage into reservoirs was achieved as follows. Containers of the sewage stock were rotated 180 degrees about the vertical axis and back five times followed by inversion and righting about the horizontal axis five times to ensure homogeneity of the sewage stock effluent prior to use. A quantity of the sewage stock was then diluted with de-chlorinated potable water in each of the two reservoirs to achieve the desired target sewage concentrations. The reservoirs each contained a submersible pump (model Rio 1100) to provide continual mixing. Sewage was dosed from the reservoirs to the microcosm tanks using peristaltic pumps to lift sewage from the reservoirs and transfer it through PVC grade manifold and transfer tubing.

Dilute sewage in the reservoirs was replenished every 48 hours or less and fresh sewage stock was collected and assayed for use every other day. This combination of storing sewage stock under

refrigerated conditions, utilising it within and replenishing it at least every other day, was undertaken to minimise the effects of change in bacterial numbers in the stock over time.

Each reservoir served three dosing peristaltic pumps (Watson-Marlow 120 S/DM2) (6 pumps in total). Each pump served one microcosm tank and was calibrated and set to dose at a specific rate to achieve the target microcosm *E. coli* water concentration.

2.1.5 Experimental tank set-up

All experimental work was conducted using cylindrical flow-through experimental tanks of 900 litre capacity each containing approximately 820 litres of water (Figure 2A, 3).

Seawater used for this study was mechanically filtered to ≈50 µm, with an inline single pass UV dosing system then passed into the header tanks which fed the sewage dosing points. De-chlorinated potable water, again treated through a single pass UV system prior to header tanks was added to control salinity to ± 2 psu thereby achieving brackish conditions. Flow of seawater out of the tank was via a gravity draw-off pipe located in the middle of each tank. This was covered by a perforated filter plate to prevent loss of shellfish or blockage.

Individual tank volumes were measured to within 100ml of their operational capacity and flow control set points adjusted accordingly. The tank flow regime was visually tested using food grade dye prior to commencement of the pilot experiment (see Section 2.3 below) and again prior to undertaking the subsequent experiments. Rapid mixing of the dye was observed following inoculation with visual homogeneity achieved within 5 minutes in all tests. In each tank, seawater was introduced via a 20cm spray bar located horizontally perpendicular to the tank wall and 20cm above the tank water surface. The spray bar was perforated with ('sparge') holes that were angled to direct the influent seawater at approximately 45° to the water surface imparting a circular flow to the water on entry. This mechanism provided some oxygenation of the influent seawater. An air line and terminal stone diffuser, housed in a plastic tubular 'streamer' was attached to the opposite side of the tank to the spray bar in a vertical orientation. A cut away section in the top of the streamer tube provided a directional stream of aerated water. This arrangement introduced oxygenation to water at the base of the tank which was then lifted vertically through the streamer and circulated. For all experiments, oxygen levels were maintained at 8 ± 2 mg/l and 90 ± 10% saturation.

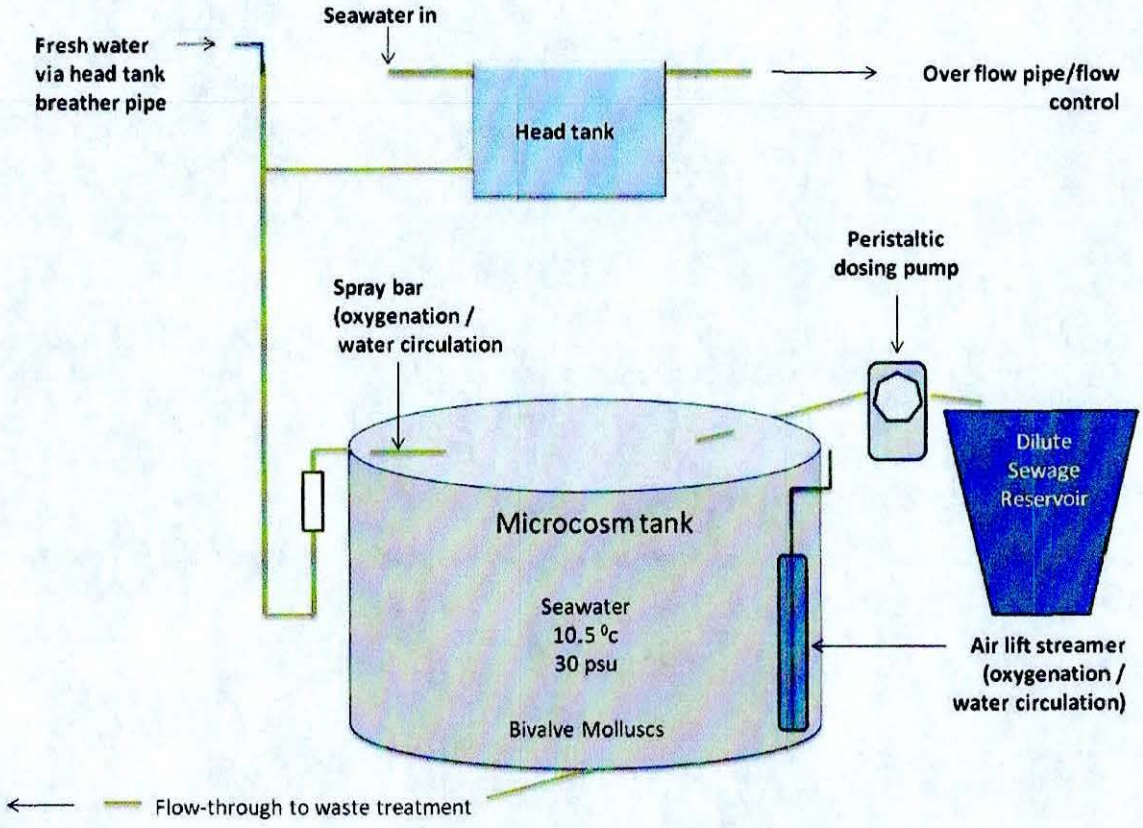


Figure 3 - Microcosm experimental set up - experimental tanks.

Salinity and temperature (linked to a telemetry alarm) were monitored throughout the experiments. Temperature was controlled by blending seawater at ambient temperature with either a chilled or heated supply as required. A fixed target temperature (10.5°C) and salinity (30 psu) were used which were within the known tolerance limits for each of the three species used in the experiments (Cefas, 2010; Kershaw *et al.*, 2012). These target parameters were commensurate with those achieved during previous short-term microbiological contamination experiments conducted by Cefas (Kay *et. al.*, 2007) and were employed throughout the experimental work.

2.1.6 Target water E. coli *concentrations*

A range of *E. coli* concentrations in seawater were targeted to produce shellfish flesh microbial concentrations equivalent to those above and below the current SWD "guideline" standard (300 cfu/100ml). Half-$\log_{10}$ concentration increments of *E. coli* in tank seawater ranging from 1 to 330 cfu per 100 ml were targeted to achieve this.

2.1.7 Water and shellfish sampling, observations and measurements

Sampling of microcosms always proceeded from the least contaminated tank, ending in the most contaminated tank as part of the strategy to avoid cross-contamination between tanks. Water samples were collected before flesh samples to avoid the possibility of water sample contamination with shellfish 'pseudo faeces'.

Water samples were collected using sterile polystyrene sampling containers (Sterilin©) clamped to a sampling pole. On each sampling occasion two (duplicate) samples were collected from mid-tank depth in the centre of each of the six tanks. This sampling point was considered to be equidistant

between the inlet and outlet of tank and representative of the tank as a whole. In order to ensure an appropriate sample volume was obtained for analysis, sample of 500ml volume were collected initial 'background' samples of tank water pre-sewage dosing and tanks dosed with sewage to target concentrations of 1, 3.3 and 10 cfu/100ml. Samples of 300 ml volume were collected for tanks dosed with sewage to target concentration of 33, 100 and 330 cfu/100ml.

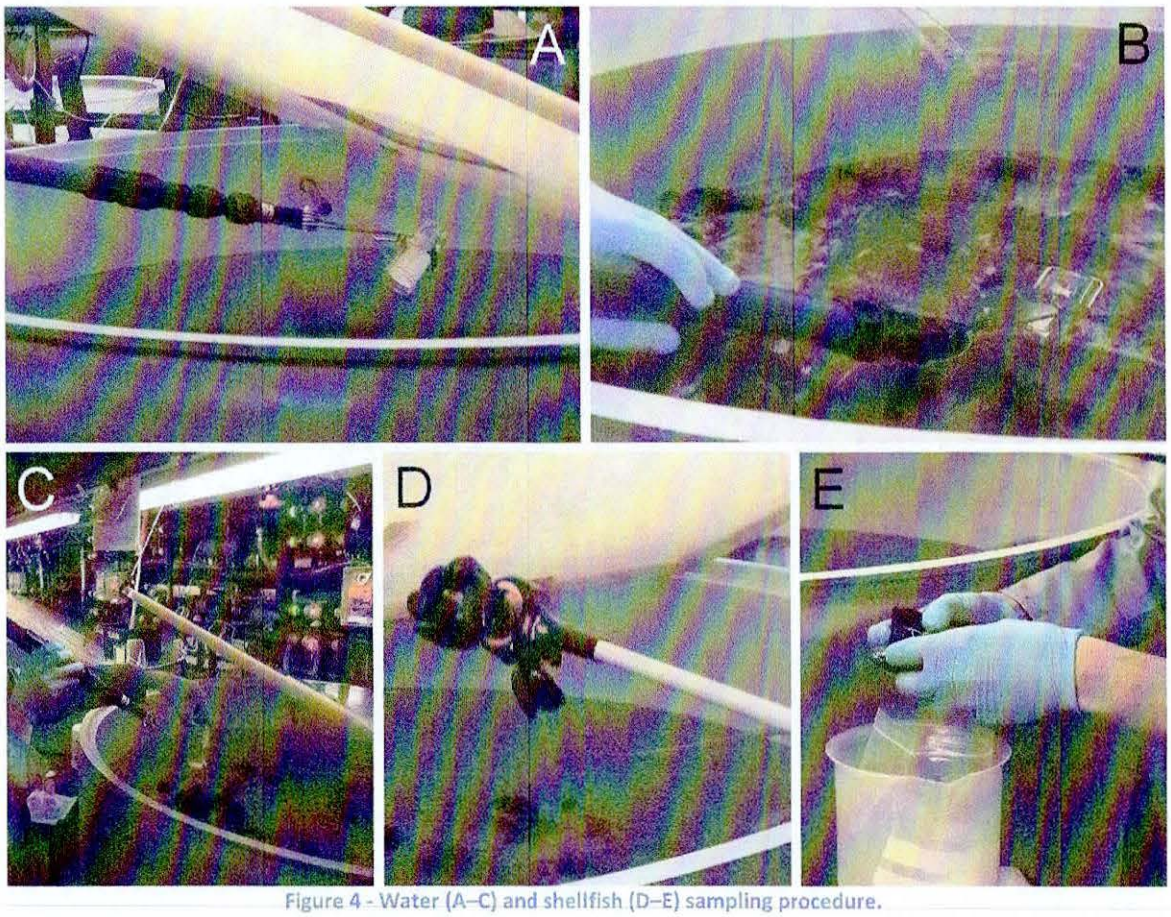


Figure 4 - Water (A–C) and shellfish (D–E) sampling procedure.

Single samples of shellfish were collected from each tank using a long handled 'Streetmaster' litter picker (Figure 4D) and immediately placed into double plastic bags. Use of the 'picker' sampling device enabled individual shellfish to be carefully removed with minimum disturbance to adjacent animals. Where present, any excess liquid was drained prior to securing the bags using cable ties before conveyance to the laboratory for microbiological analyses. Dead specimens were then removed from tanks, counted and recorded before being placed into separate plastic bags for disposal.

Sampling poles and pickers were thoroughly rinsed with tap water and then with de-chlorinated water prior to sampling individual tanks and in between each sampling event. Care was taken to sample bivalves from across all areas of each tank.

Prior to each sampling event, observations were made with respect to sampling time, tank number, shellfish species, shellfish mortalities (Appendix II), water circulation, visual evidence of sewage dosing delivery to tanks, level in sewage reservoir and operation of circulation pumps. Spot salinity and temperature measurements were made in each tank using a portable conductivity meter (WTW Profiline 197i). Dissolved oxygen was also measured in each tank using a hand-held DO meter (OxyGuard® Handy Polaris). Two conductivity/temperature loggers were also placed in each

experimental tank for monitoring the variation in temperature, salinity and dissolved oxygen during the course of the experiments.

2.1.8 Microbiological analyses

Levels of *E. coli* in sewage stock, sewage reservoir and tank water samples were quantified for presumptive faecal coliforms and *E. coli* using a modified membrane filtration technique (Environment Agency, 2000). This technique used membrane lauryl sulphate broth (MLSB) as a recovery medium.

The total filtered volumes were 1 ml in the case of sewage and up to 500 ml of water depending on the anticipated *E. coli* in water concentration. Serial dilutions of the filtered samples were then prepared in 0.1% peptone (0.1% P).

Petri dishes were prepared for each dilution under test with a pad absorbed in MLSB and 1 Petri dish for the control. The membrane filtration equipment was set up using a 0.45µm pore-size filter. For each dilution, 1ml volume of sample was filtered and placed onto Petri dishes. For the control, 10 ml of 0.1% P was inoculated using a sterile 1 µl inoculating loop with *E.coli* NCTC 12241 and filtered as previously described.

Samples were incubated for 2 hours at 37°C followed by 18 hours at 44°C. After incubation, membranes were examined for the presence of yellow colonies which indicated the presumptive presence of faecal coliforms. Sub-cultures of 10 or fewer of these colonies were cultivated onto tryptone bile glucuronide agar (TBGA) and incubated for 24 hours at 44°C. TBGA plates were examined for the presence of blue-green colonies which indicated the presence of *E. coli*.

Levels of this indicator in shellfish flesh were quantified in duplicate using the most probable number technique ISO/TS 16649-3 (International Organization for Standardization, 2005). This method recovered *E. coli* in minerals modified glutamate broth (MMGB) and incubated for 24 hours at 37°C with confirmation by detection of ß-glucuronidase on TBGA at 44°C for 22 hours.

Sewage and tank water results were expressed as colony forming units (cfu) per 100 ml and in shellfish flesh as most probable number (MPN) per 100 g.

2.1.9 Statistical analyses

The microbiological dataset contained 152 out of 396 (38%) water results and 144 out of 395 (36%) shellfish flesh results for *E. coli* measurements at the limits of detection and therefore censored. The effects of censored data on the relationship between *E. coli* levels in shellfish flesh and water were adjusted for using a Tobit regression model (Appendix III). For the purposes of this analysis, the variable "*E. coli* levels in shellfish flesh" was considered the response because the direction of contamination is assumed to run from the levels of bacteria in the overlying waters to the shellfish because of their filter-feeding activity.

Bacterial numbers in measured samples are usually assumed to follow a log-normal distribution[5] because they reflect exponential growth. The assumption is consistent with the generally observed highly right-skewed sampling distributions. MPN/cfu values were therefore $\log_{10}$ transformed prior to statistical analyses to ensure a more symmetrical distribution of the data (see Velleman and Hoaglin, 1981; Helsel and Hirsch, 2002).

[5] The logarithm of its probability distribution is normally distributed (bell shaped).

Simple linear regression models were computed to investigate the correlation between *E. coli* levels in shellfish flesh and *E. coli* levels in water.

Line plots of *E. coli* in water and shellfish flesh against time were overlaid as an exploratory tool, and immediately revealed the parallel development of the two series.

Descriptive statistics (minimum, maximum, geometric mean and standard deviation of Log_{10} transformed results) were calculated and bar charts showing the mortalities that occurred as a percentage of total number of individuals per tank were produced using Microsoft Excel. The remaining graphical data analyses were undertaken using Stata (Stata/IC version 11.1 for Windows, StataCorp LP, College Station, Tx 2010) and Minitab (version 15) statistical software.

2.2 Environmental investigations

First, the requirement for a licence to deploy the shellfish in Swansea Bay was explored with the relevant authority, the Marine Consents Unit of the Welsh Government. It was agreed that the project requirement to lay commercial shellfish species in Netlon® bags fell under Article 4, Exemption 16 of the Marine Licensing (Exempted Activities)(Wales) Order 2011. Thus, the appropriate regulatory authority judged that the activity did not require a formal licence application.

Second, the source of shellfish had to be chosen to ensure that no movement restrictions were contravened and, here, expert advice was sought from appropriate sections within Cefas. The chosen commercial shellfish grower, able to supply mussels (*M. edulis*), cockles (*C. edule*) and Pacific oysters (*C. gigas*) in a single consignment was based in Brancaster Bay, Norfolk. The shellfish were harvested and immediately transferred from Brancaster on 5th September 2011 in cold boxes with ice packs separated from the shellfish to prevent frost damage to the live shellfish. The three species were laid in the intertidal zone within netlon bags attached beneath Mumbles Pier during low water on 6th September from 06:00 to 07:30 (Figure 5). The cockles were partially covered in sand and the area protected by large stones to prevent the sand being removed by normal wave action.

The number of animals laid in each bag was based on the requirements of the analytical laboratory (Cefas, Weymouth) and the expected attrition rate of the animals during the laying period. Table 1 shows the distribution of each species and numbers involved.

Table 1 - Calculation of the number of shellfish needed for the experiment.

Species	No animals required per sample	Samples required	Animals required for analysis	Contingency i.e. loss factor expected	Total	Animals in each bag	Number in 20 bags
Mussels	18	14	252	1.5	378	27	540
Pacific oysters	12	14	168	1.5	252	18	360
Cockles	35	14	490	2	980	70	1400

The numbers per bag were determined to facilitate harvesting of complete bags on each low tide collection event during the ten day experimental period (Table 2).

Table 2 - Sampling protocol and timing of 14 batches of shellfish, each containing 3 species, laid on 6th September 2011, thence collected between 7th and 15th September 2011 for analyses in Cefas, Weymouth laboratory.

Analytical results for two replicate samples of each batch are presented as MPN/100g. Please note, higher than expected cockle mortality precluded analysis following collections 13 and 14: see text below for explanation.

Batch No.	Collection No.	Day	Date	Low water (BST)	No. Mussels	*E. coli* sample 1	*E. coli* sample 2	No. Oysters	*E. coli* sample 1	*E. coli* sample 2	No. Cockles	*E. coli* sample 1	*E. coli* sample 2
1	1	Tuesday	06/09/2011	20:01	27 (1bag)	16000	3500	18 (1bag)	2200	790	70 (1bag)	16000	9200
	2	Wednesday	07/09/2011	08:53	27 (1bag)	5400	5400	18 (1bag)	2400	790	70 (1bag)	9200	3500
2	3	Friday	09/09/2011	23:49	27 (1bag)	5400	24000	18 (1bag)	35000	35000	70 (1bag)	16000	54000
3	4	Saturday	10/09/2011	12:07	27 (1bag)	9200	9200	18 (1bag)	1300	3500	70 (1bag)	54000	54000
	5	Sunday	11/09/2011	00:26	27 (1bag)	16000	16000	18 (1bag)	9200	16000	70 (1bag)	54000	16000
4	6	Sunday	11/09/2011	12:40	27 (1bag)	92000	16000	18 (1bag)	16000	9200	70 (1bag)	3500	16000
	7	Monday	12/09/2011	00:57	27 (1bag)	24000	9200	18 (1bag)	5400	9200	70 (1bag)	9200	5400
5	8	Monday	12/09/2011	13:09	27 (1bag)	16000	16000	18 (1bag)	3500	3500	70 (1bag)	16000	35000
	9	Tuesday	13/09/2011	01:26	27 (1bag)	16000	24000	18 (1bag)	9200	5400	70 (1bag)	9200	9200
6	10	Tuesday	13/09/2011	13:40	27 (1bag)	9200	16000	18 (1bag)	5400	16000	70 (1bag)	9200	24000
	11	Wednesday	14/09/2011	01:57	27 (1bag)	3500	1700	18 (1bag)	2400	9200	70 (1bag)	1700	3500
7	12	Wednesday	14/09/2011	14:10	27 (1bag)	9200	3500	18 (1bag)	5400	9200	70 (1bag)	9200	9200
	13	Thursday	15/09/2011	02:25	27 (1bag)	3500	3500	18 (1bag)	5400	5400	70 (1bag)	-	-
8	14	Thursday	15/09/2011	14:39	27 (1bag)	2400	3500	18 (1bag)	3500	16000	70 (1bag)	-	-

After harvesting, the shellfish were stored and transported in insulated cool boxes, without freezing material in contact with the shellfish, and driven directly to the analytical laboratory in compliance with National Reference Laboratory recommendations on shellfish sample handling and storage[6].

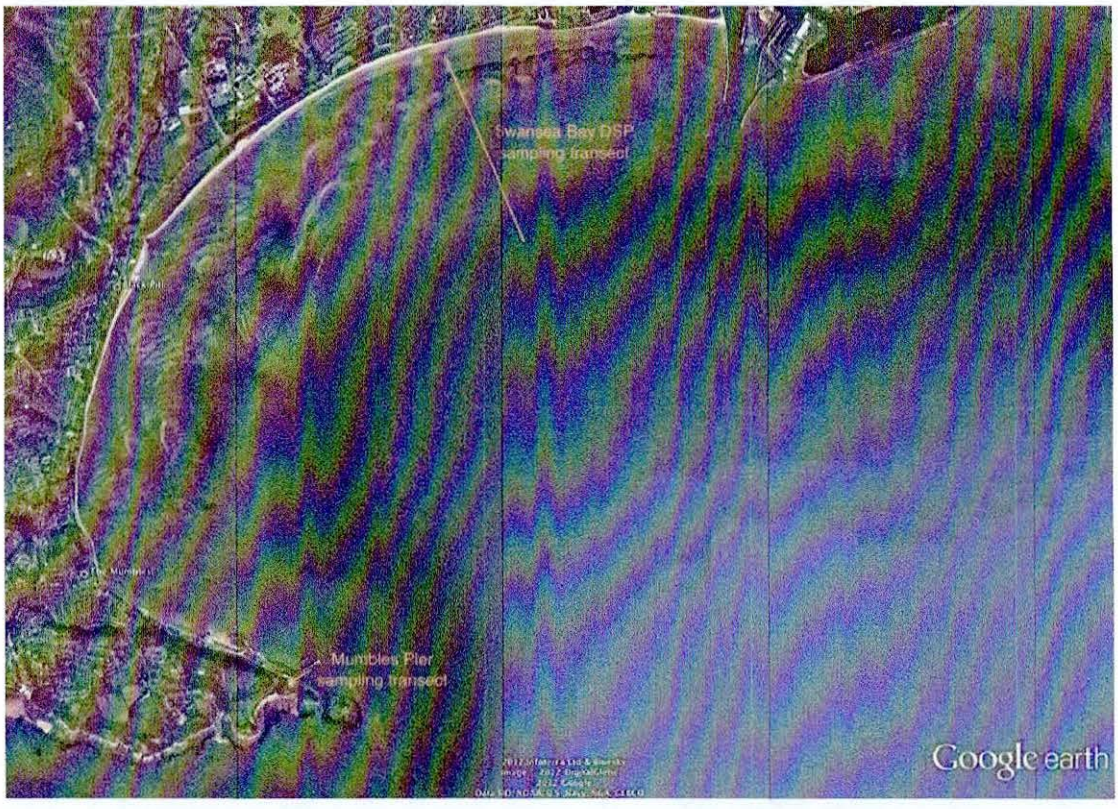
Water samples for *E. coli* analyses were collected along a tidal transect adjacent to the western side of Mumbles pier, between 08:00 and 18:10 on each day from 5th to 15th September 2011 (Figure 6A).

[6] National Reference Laboratory Recommendations for the collection and transport of Bivalve Mollusc Harvesting Areas under (EC) Regulation No 854/2004
http://www.cefas.defra.gov.uk/media/455947/recommendationssampling.pdf

Figure 5 - Mumbles pier where shellfish were laid between 6th and 15th September 2011.

The relationship between the experimental site and the bathing water sampling transect in Swansea Bay is shown in Figure 6B.

A



B

Figure 6 - The shellfish laying location in relation to the designated bathing water sampling point (DSP) (A) and the water sampling transect and shellfish laying location at Mumbles Pier near Knab Rock (B), in Swansea Bay.

Shellfish samples were transported in dark cool boxes, then stored in a laboratory refrigerator and analysed within 24 hours of collection in all cases.

3 Results

3.1 Microcosm experiments

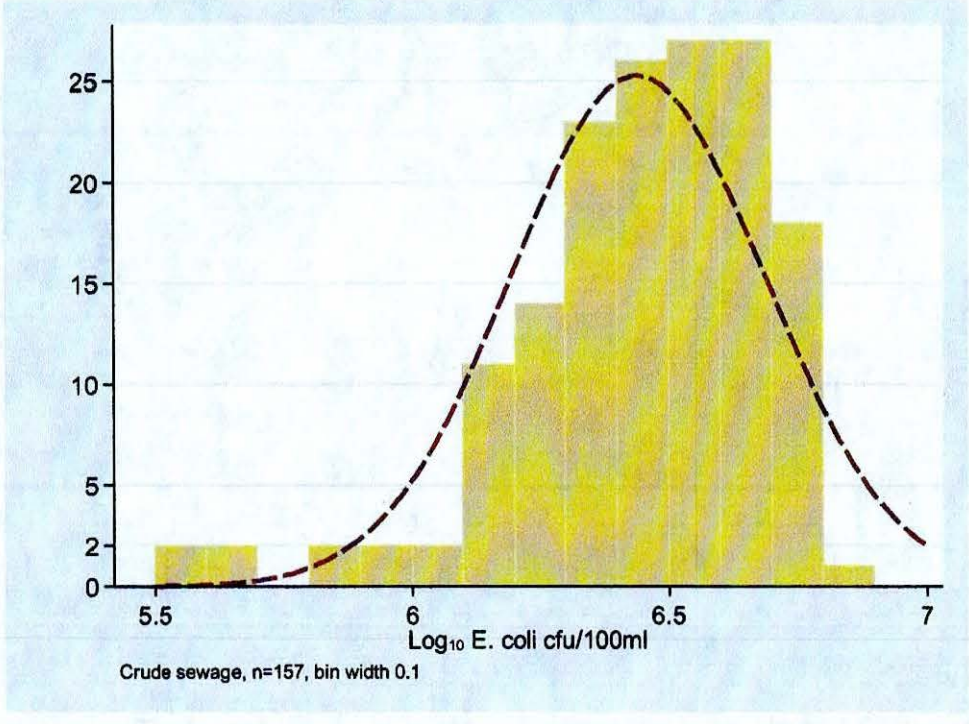
3.1.1 E. coli *levels in sewage and decay*

The enumerations of FIOs in the crude sewage stock, when log transformed, did not strictly follow a normal (bell shaped) distribution, having both tails heavier than expected when compared to the normal curve with sample mean and standard deviation (Figure 7A). This can be explained by bacteria in sewage usually occurring in clumps. The upper tail is also affected by very high values being above the upper limit of quantification (LoQ) of the assay.

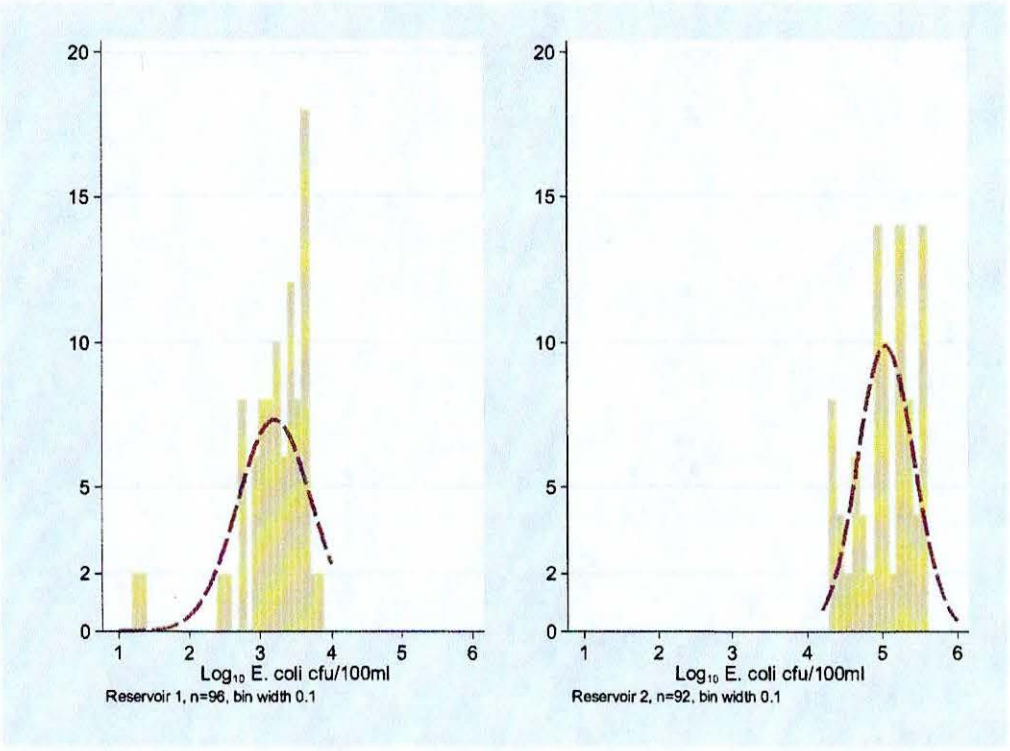
Levels of *E. coli* in the reservoirs were also not strictly (log) normally distributed, with outlier *E. coli* levels on the high side of the distribution in one reservoir (Figure 7B).

The skewed distributions of *E. coli* levels shown in Figure 7C correspond to concentrations of the indicator below the limit of detection in tank water in the absence of sewage dosing. As expected, the distribution flattened when sewage was dosed as laboratorial control of the sewage dilution process necessarily increased the variability of *E. coli* levels.

7A



7B



7C

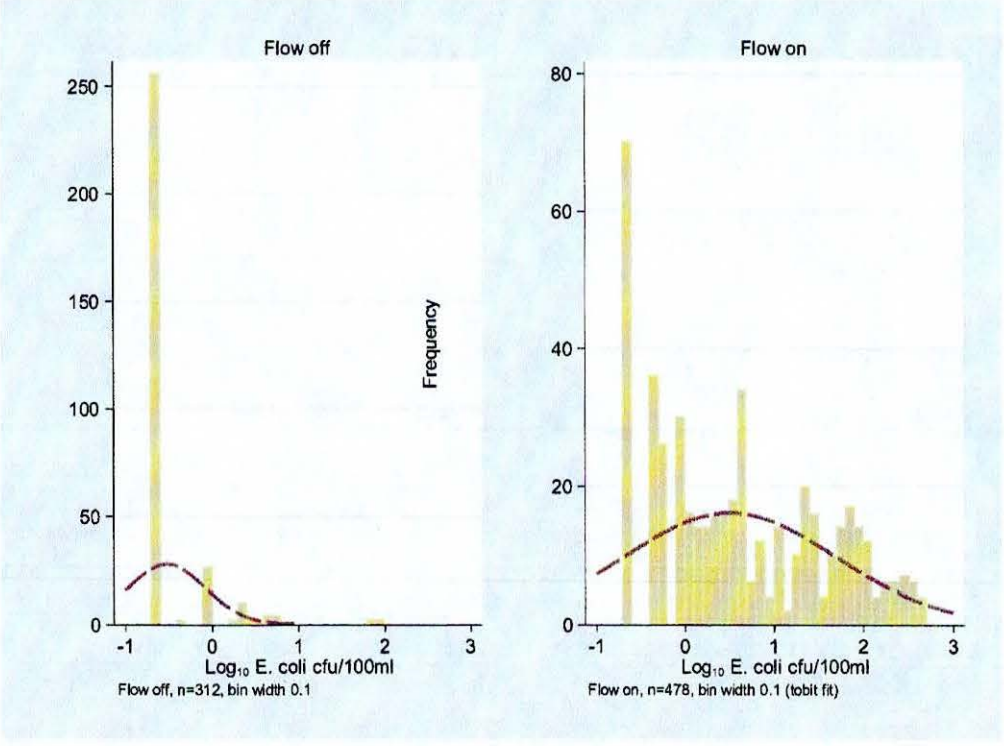


Figure 7 - Frequency distributions of *E. coli* levels in sewage stock (A), reservoirs (B) and tank water (C) with superimposed normal distribution (dashed lines).

3.1.2 *Relationship between faecal coliforms and E. coli*

Linear regression models indicate a similarity between faecal coliform and *E. coli* results obtained in crude sewage, reservoir and tank water samples during the experiments as might be expected (Figure 8).

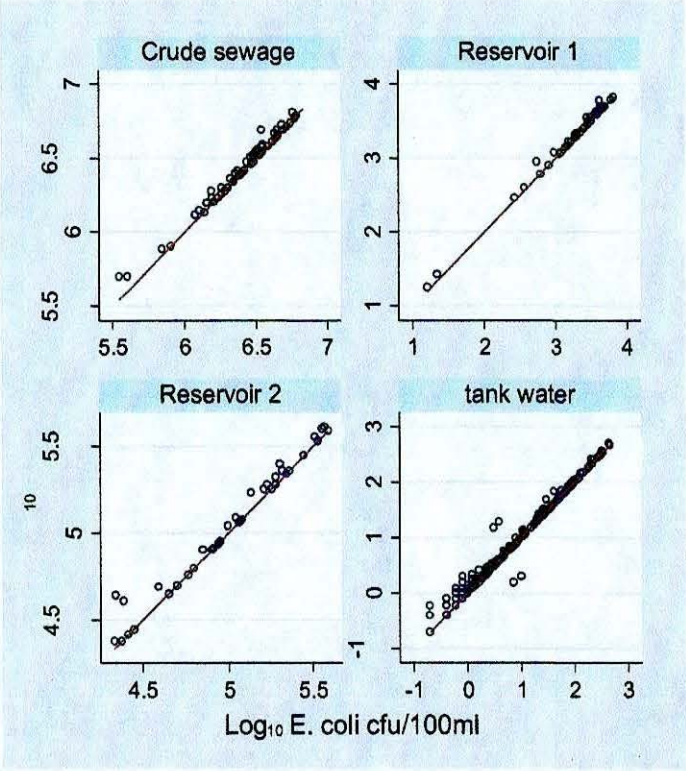


Figure 8 - Relationship between levels of faecal coliforms and *E. coli* in sewage stock, sewage reservoirs and tank water. Line of equality shown in red.

The Bland-Altman method (Bland and Altman, 1986) was used to evaluate the agreement between the ratios of faecal coliforms: *E. coli* (Appendix IV). No significant differences were found over the range of water values indicating that either group of bacteria could be used in the analyses. However, *E. coli* is specifically associated with the intestines of warm-blooded animals and, for this reason, is considered a more reliable indicator of contamination of faecal origin. This bacterium was used in this study because it is the currently-used indicator of the risk of microbiological contamination in shellfish intended for human consumption (European Communities, 2004). It is also the indicator recommended by UKTAG to replace the faecal coliform standard currently used to monitor shellfish waters under the SWD, which will be revoked in the UK by the WFD in 2013 (Warn *et al.*, 2010).

3.1.3 *Relationships between* E. coli *shellfish flesh versus water*

Levels of *E. coli* in water and flesh were considered as coincident in time and plotted as paired observations as water and flesh samples were usually taken from each tank within minutes of each other and the interval between sampling points was several hours. Linear regression models fitted with separate coefficients for the six target water concentrations indicate a single regression slope at least within each species (Figure 9).

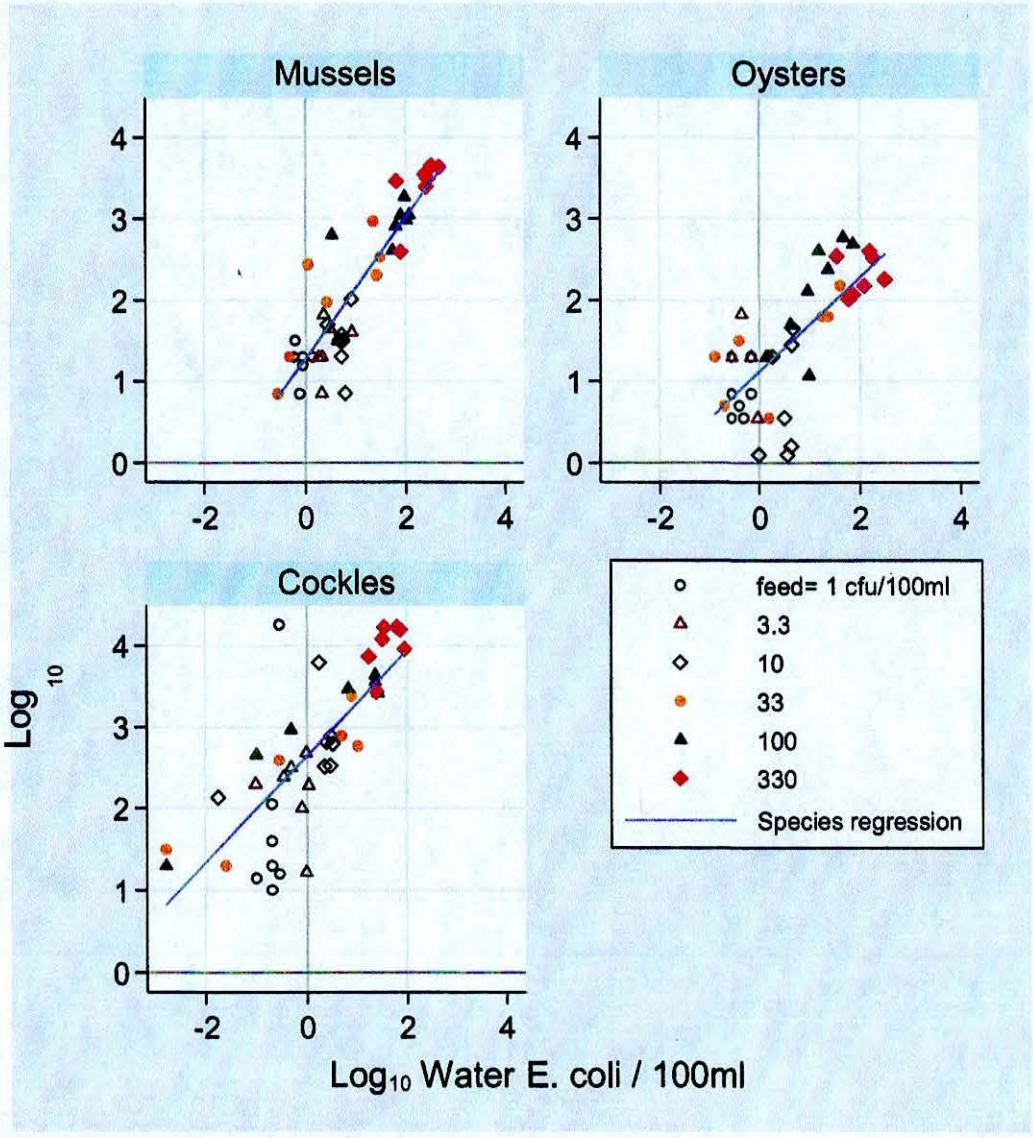


Figure 9 - Linear regression models for levels of *E. coli* in shellfish flesh and water for the three species tested.

Values recorded as below the limit of detection (LoD) of the MPN method were adjusted before plotting to avoid an artefact at the LoD. Please refer to Appendix V for explanation on the adjustment method.

Fitting a joint regression model suggests the parameters for cockles (slope and intercept) are significantly different from oysters and mussels, and that oysters are borderline significantly different from mussels. From examination of Figure 9 (ie post-hoc analysis and therefore not strictly testable by p values), it is apparent that the water values for cockles cover a wider range than the others; in particular there are water values of <-1 log, which are LoD values and unreliable. Restricting the regression to the range of water values >-1 suggests the slope for oysters is les steep than the others, while the intercept for cockles is higher than the others. Both these result would be consistent with other studies (e.g. Younger and Reese, 2011)

The linear regression models for *E. coli* results in shellfish flesh versus water for the three species tested are shown in Table 3. The R^2 values (i.e. variance explained are mid-range, suggesting uncontrolled factors are still operating. The pooled-species model over the whole water range is

plainly biased and not representative of any species, but the model for water of log values above -1 is realistic.

Table 3 - Linear regression models of *E. coli* levels in shellfish flesh and water for the three species tested. All models and parameters significant at p<.001

Species	Regression model	Variance explained (R^2)
Mussels (*Mytilus* spp.)	Log_{10} MPN *E. coli* flesh=0.86*Log_{10} cfu *E. coli* water+1.28	0.79
Pacific oysters (*C. gigas*)	Log_{10} MPN *E. coli* flesh=0.58*Log_{10} cfu *E. coli* water+1.12	0.52
Common cockle (*C. edule*)	Log_{10} MPN *E. coli* flesh=0.65*Log_{10} cfu *E. coli* water+12.65	0.60
Pooled species	Log_{10} MPN *E. coli* flesh=0.54*Log_{10} cfu *E. coli* water+1.77	0.62
Pooled species if water>-1	Log_{10} MPN *E. coli* flesh=0.64*Log_{10} cfu *E. coli* water+1.64	0.43

3.1.4 Time taken to reach equilibrium of E. coli in shellfish

The levels of *E. coli* accumulated by the three shellfish species during the course of the experiments are broadly determined by the concentration of the indicator in the tank water (Figures 10-12 below).

The figures show that shellfish had reached the peak level of accumulation by the time when the first samples were collected and in general the lines for flesh closely mirror those for water, regardless of the gap between samples. This implies that they are efficient in accumulating *E. coli*. Hence they reach equilibrium with bacterial concentrations in the waters at an undetermined time but in less than 18 hours.

Further attempts to derive rates of change and rates of reaction from the data were unsuccessful. Shellfish are therefore likely to respond to quite short term stimulus such as high rainfall incident that could be overlooked by occasional water sampling.

Graphs of dosed sewage, microcosm tank water and shellfish flesh concentrations for experiments on each shellfish species are given in Figures 10 to 12 below, in each followed by general and specific observations relating to each microcosm. For each experiment 'Reservoir 1' supplied sewage to the less contaminated tanks (water targets 1, 3.3 and 10 cfu/100ml); 'Reservoir 2' supplied sewage to the most contaminated tanks (water targets 33, 100 and 330 cfu/100ml). Each point in the graphs represents a sample mean, with the lines joining the average of *E. coli* results from duplicate samples (water) or duplicate assays (flesh). 0.1 on the scale has been used arbitrarily to plot points at the LoD, which may therefore in fact be anywhere from here to 0.

EXPERIMENT 1 - MUSSELS

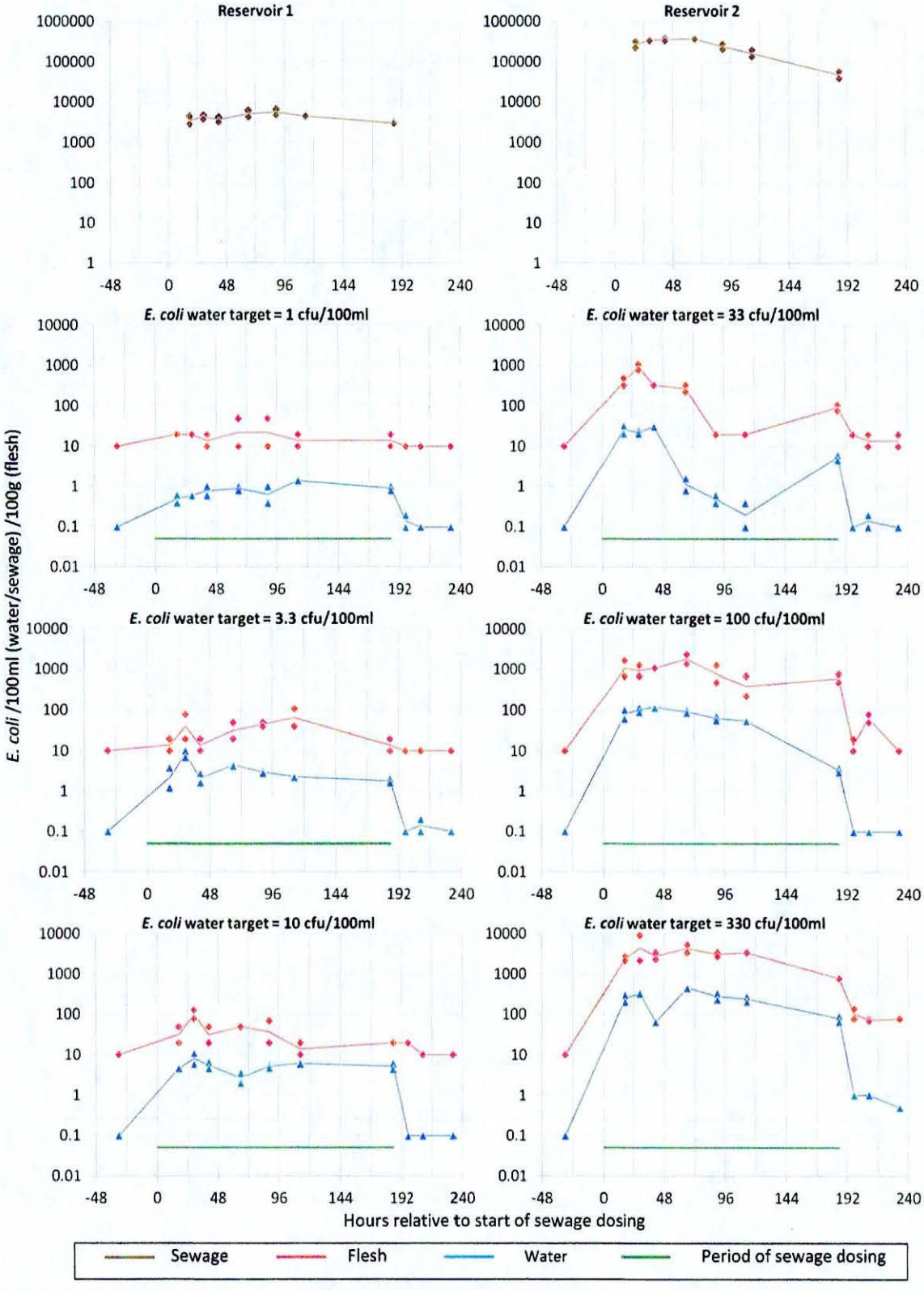


Figure 10 - Time series of levels of *E. coli* in the two sewage dosing reservoirs (top graphs), tank water and mussels for six target tank water concentrations (bottom six graphs)

GENERAL OBSERVATIONS

During the equilibrium phase, levels of *E. coli* in mussels in the less contaminated tanks (water targets 1, 3.3 and 10 cfu/100ml) generally followed the pattern of water contamination delivered from sewage reservoir 1. In reservoir 1, the lowest concentration of the indicator was detected after 17.4 hours of sewage dosing. In reservoir 2, the lowest concentration was detected at the end of the experiment (185.3 hours).

The *E. coli* content in shellfish tissues detected at the end of the exposure period reduced to levels <20 cfu per 100g in samples collected at approximately 11.5 hours (water targets 1, 3.3 and 100 cfu/100ml) and after 23.5 hours (water targets 10 and 33 cfu/100ml) of sewage dosing.

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 1 CFU/100ML

Levels of *E. coli* in tank water ranged between 0.2 and 1.4 cfu/100ml. In this tank, the maximum *E. coli* level in mussels (50 MPN/100g) was recorded after 66.3 hours of sewage dosing. The concentration of the indicator in mussel flesh decreased to below the limit of detection (LoD) in the sample taken after 89.5 hours of sewage dosing and remained at this level thereafter.

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 3.3 CFU/100ML

Levels of *E. coli* in tank water ranged between <0.2 and 4.6 cfu/100ml. The maximum level in water was observed after 65.5 hours of sewage dosing. Levels of the indicator in tank water decreased slightly during the exposure period to a minimum level of <0.2 cfu/100ml at 196.8 hours. The peak level in shellfish flesh (110 MPN/100g) was detected after 112.7 hours of sewage dosing.

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 10 CFU/100ML

Levels of *E. coli* in tank water increased to a peak level of 11 cfu/100ml detected at 28.7 hours corresponding to the peak level of accumulation in shellfish flesh (130 MPN/100g after 29.1 hours of sewage dosing).

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 33 CFU/100ML

Levels of *E. coli* in tank water accumulated to exceed the target concentration after 17.0 hours of sewage dosing. At this time, the corresponding concentration in shellfish flesh was 490 MPN/100g. Mussels accumulated to a maximum 1,100 *E. coli* MPN/100g after 29.0 hours of sewage dosing. A second lower peak level of 110 MPN/100g was detected at 185.0 hours.

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 100 CFU/100ML

Levels of *E. coli* in the tank water exceeded the target concentration during the exposure phase (17 to 66 hours) and were below this concentration outside this period. Again, the pattern of *E. coli* accumulation in mussel flesh closely followed the increase in water levels, with a maximum of 2,400 MPN/100g detected after 66.8 hours of sewage dosing. Mussels cleared this contamination thereafter until the end of the post-exposure phase, when levels of the indicator were below the limit of detection.

MUSSELS MICROCOSM - TARGET WATER CONCENTRATION 330 CFU/100ML

Levels of *E. coli* in tank water exceeded the target concentration in samples collected after 28.5, 66 and 89.3 hours of sewage dosing. Levels of *E. coli* in mussel flesh increased to peak (9,204 MPN/100g) in samples collected after 29.3 hours of sewage dosing and decreased slightly during the exposure period and more sharply during the clearance phase. No results below the lower limit of detection were obtained in shellfish flesh in this tank.

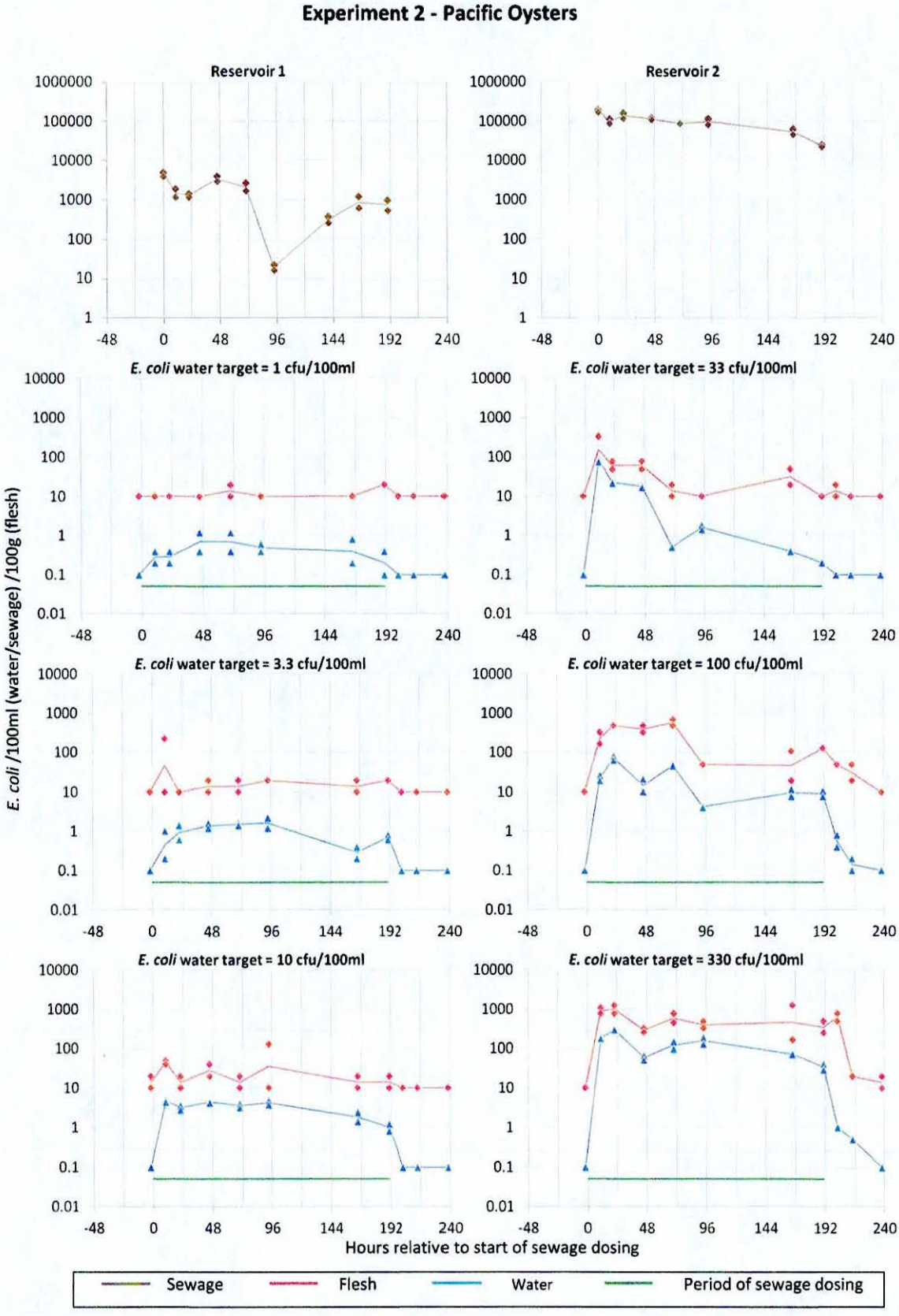


Figure 11 - Time series of levels of *E. coli* in the two sewage dosing reservoirs (top graphs), tank water and Pacific oysters for six target tank water concentrations (bottom six graphs)

GENERAL OBSERVATIONS

During the equilibrium phase ("plateau"), levels of *E. coli* in oysters kept in the most contaminated tanks (water targets 100 and 330 cfu/100ml) generally followed the pattern of contamination detected in the corresponding sewage reservoir (No. 2). In the least contaminated tanks (water targets 1 and 3.3 and 10 cfu/100ml) a dip in the reservoir *E. coli* concentration is observed at sample time 94.8 hours. This was attributed to a temporary blockage in the dosing delivery system.

At the end of the exposure period after dosing sewage stopped, the *E. coli* content in shellfish tissues detected was cleared to levels <20 MPN/100g in samples collected after approximately 11.0 hours in the least contaminated tanks (water targets 1, 3.3 and 10 cfu/100ml) and in samples collected at 47.0 hours in the two most contaminated tanks (water targets 100 and 330 cfu/100ml).

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 1 CFU/100ML

Levels of *E. coli* in tank water exceeded the target concentration at 45.3 and 69.4 hours. Levels of the indicator in oyster flesh did not exceed 20 MPN/100g during both the exposure or post-exposure periods.

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 3.3 CFU/100ML

Levels of *E. coli* in tank water did not exceed the target concentration during the experiment. The peak oyster flesh sample (230 MPN/100g) was collected at 10.4 hours. All other shellfish samples were at or below the limit of detection (20 MPN/100g).

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 10 CFU/100ML

Levels of *E. coli* in tank water exceeded the target concentration. Four flesh samples exceeded 20 MPN/100g: 40 MPN/100g, 50 MPN/100g (10.5 hours), 40 MPN/100g (45.7 hours) and 130 MPN/100g (93.7 hours).

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 33 CFU/100ML

In this tank only one water sample taken at 10.0 hours exceeded the target *E. coli* concentration. Oysters in this tank quickly accumulated *E. coli* to a maximum level of 330 MPN/100g (sample taken at 10.6 hours). From this time, the levels of the indicator in oysters generally decreased during the exposure period reaching <20 MPN/100g at 69.9 hours and reached a second peak level of 50 MPN/100g at 165.5 hours.

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 100 CFU/100ML

Levels of *E. coli* in this tank peaked at 82 cfu/100ml in the sample collected after 21.6 hours of sewage dosing and generally decreased during the exposure period. In this tank, oysters accumulated *E. coli* to a peak level of 700 MPN/100g in the sample collected after 70 hours of sewage dosing; contamination levels decreased to 130 MPN/100g at the end of the experiment (191 hours). The sample collected at 214.1 hours (approximately 12.3 hrs following the end of dosing) showed this contamination had reduced to 50 MPN/100g, and clearance continued to levels below the limit of detection at the end of the clearance period.

PACIFIC OYSTERS MICROCOSM - TARGET WATER CONCENTRATION 330 CFU/100ML

A very similar pattern of contamination/clearance was detected in oysters kept in this tank. In this tank, the period of time required to achieve the peak contamination level was shorter (22.1 hours) and the decrease observed during the exposure period took longer (214.2 hours). The peak *E. coli* level accumulated in oyster flesh was 1,300 MPN/100g (at 22.1 and 165.7 hours, although the level decreased between these times).

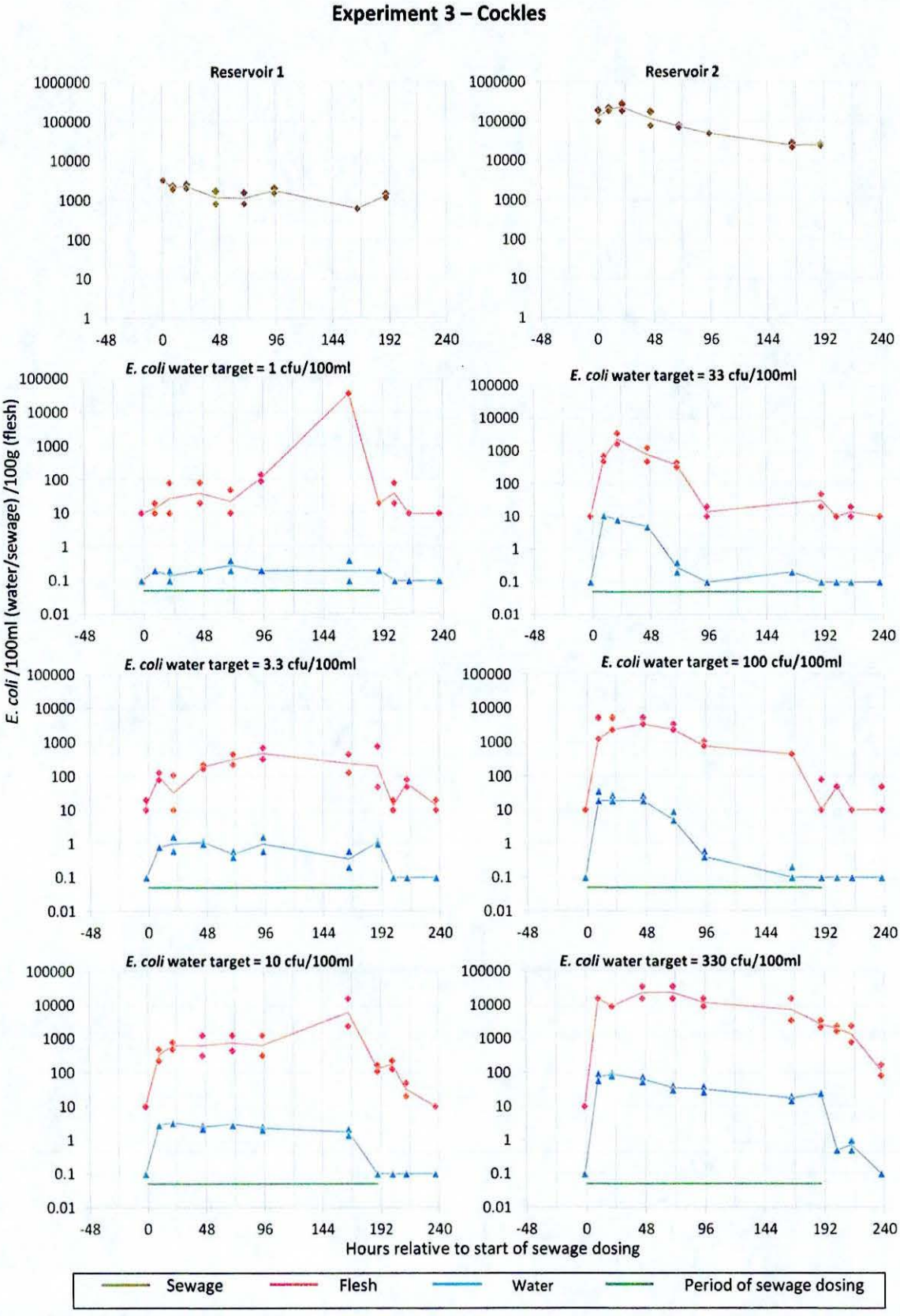


Figure 12- Time series of levels of *E. coli* in the two sewage dosing reservoirs (top graphs), tank water and cockles for six target tank water concentrations (bottom six graphs)

GENERAL OBSERVATIONS

During the equilibrium phase ("plateau"), levels of *E. coli* in cockles kept in the most contaminated tanks (water targets 33, 100 and 330 cfu/100ml) generally followed the pattern of contamination in reservoir 2. In both reservoir 1 and reservoir 2, the lowest concentration of the indicator was detected near the end of the experiment after 164.8 and 164.9 hours (respectively) of sewage dosing.

The times observed in which cockles had cleared their *E. coli* content accumulated at the end of the exposure period to levels <20 MPN/100g ranged between 12.5 (water target 3.3 cfu/100ml) and 48.32 hours (water target 10 cfu/100ml). *E. coli* contamination in cockles exposed to the most contaminated waters (330 cfu per 100g) did not achieve levels below the LoD.

In the experiments with cockles, none of the tanks exceeded the target water concentration at any time during the experiments. Levels of *E. coli* in tank water targeted to achieve 33 cfu/100ml decreased from 10.8 cfu/100ml to <0.2 cfu/100ml during the period 9-94 hours. Similarly, in the tank targeted to achieve 100 cfu/100ml, levels of the indicator decreased from 37 to <0.2 cfu/100ml during the period 9-165 hours.

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 1 CFU/100ML

Cockles in this tank accumulated *E. coli* to a level of 140 MPN/100g detected after 94.2 hours. The peak level of *E. coli* >18,000 MPN/100g (above LoD) was in the sample collected after 165.1 hours of sewage dosing; the causes of these high results are not known. Samples collected after 189.0 hours of sewage dosing returned *E. coli* levels in the lower limit of detection (20 MPN/100g).

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 3.3 CFU/100ML

The maximum *E. coli* in cockles was 791 MPN/100g after 189.1 hours of accumulation.

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 10 CFU/100ML

In this tank cockles accumulated *E. coli* to a maximum of 16,000 MPN/100g after 165.4 hours of sewage dosing. Levels of the ind
icator in cockles decreased in the later stages of the exposure period reaching levels below the limit of detection at the end of the post-exposure period 45 hours after sewage dosing was stopped (T = 237 h).

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 33 CFU/100ML

In this tank cockles accumulated *E. coli* to a concentration of 3,500 MPN/100g at 21 hours of sewage dosing thereafter levels decreased to the LoD (20 MPN/100g) at 94.8 hours peaking again at >18,000 MPN/100g at 165.2 hours. Bacterial levels decreased thereafter until the end of the experiment remaining below 50 MPN/100g after 189.3 hours of sewage dosing.

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 100 CFU/100ML

Cockles rapidly accumulated *E. coli* to a level of c. 5,400 MPN/100g between 9.5 and 46.0 hours of sewage dosing. Thereafter levels in cockles decreased.

COCKLES MICROCOSM - TARGET WATER CONCENTRATION 330 CFU/100ML

Cockles in this tank accumulated very high levels of the indicator during most of the exposure period. Cockles sampled after 9.8 hours had accumulated 16,000 MPN/100g. Samples collected after 46 and 71 hours of sewage dosing returned levels exceeding the upper limit of detection (>18,000 MPN/100g). No results below the limit of detection were detected in cockles maintained in this tank.

3.1.5 *Relative E. coli accumulation factors in different shellfish species*

The shellfish flesh data was analysed against the sampling time interval for evidence of a maximum rate of change in accumulation, however no specific rate of change was evident. The flesh values were also plotted against the water values for successive samples which confirmed the parallel nature of the changes, with the one finding that increases were better correlated than decreases. (Figure 13). Changes in accumulation were proportional to water concentration and changes in clearance were less proportionate to changes in water concentration. Changes observed between successive water readings reflected fluctuations in delivery of the sewage dosing regime, part from the explicit start and stop of dosing.

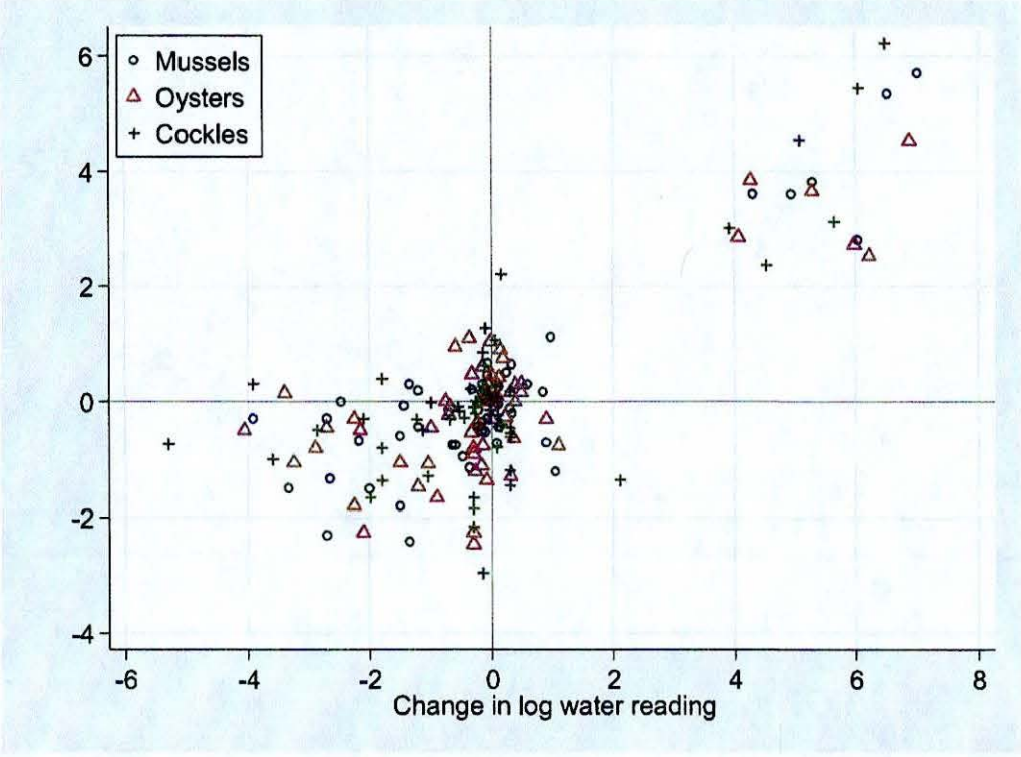


Figure 13 - Positive changes in water reading were generally reflected in proportionate increases in flesh readings. Decreases in water readings generally led to a decrease in the flesh reading but less obviously in direct proportion

Table 4 shows that cockles accumulated *E. coli* to a much greater level than mussels and Pacific oysters, but there were no patterns apparent in accumulation rates with the applied stimulus.

Table 4 - *E. coli* accumulation factors in the three species of shellfish tested.

Species	Target water concentration (cfu/100ml)	Water geometric mean (cfu/100ml)	Flesh geometric mean (MPN/100ml)	Accumulation factor - by individual microcosm*	Accumulation factor - all results for species*
Mussels (*Mytilus* spp.)	1	0.8	18	25	15.2
	3.3	2.5	30	12	
	10	5.1	37	7	
	33	3.6	155	43	
	100	83.7	936	11	
	330	236.6	3,399	14	
Pacific oysters (*C. gigas*)	1	0.4	12	29	11.7
	3.3	0.8	18	21	
	10	2.9	21	7	
	33	3.0	31	10	
	100	17.2	188	11	
	330	107.0	525	5	
Common cockle (*C. edule*)	1	0.2	84	419	330.0
	3.3	0.8	174	227	
	10	1.6	667	413	
	33	0.8	494	605	
	100	2.5	1055	418	
	330	41.4	11,143	269	

Sample times (hours elapsed from start of sewage dosing) = mussels ~16-192, oysters ~10-192, cockles ~10-192.

** Calculated as the geometric mean indicator concentration of the organism in shellfish flesh divided by the corresponding geometric mean concentration in the overlying water.*

The range of *E. coli* accumulation factors for individual water targets was similarly greater in cockles than that in mussels and Pacific oysters. Whilst the geometric mean *E. coli* level in shellfish flesh increases linearly with that in the water, no direct association is apparent between the latter variable and the accumulation factor.

3.2 Derivation of a water standard

In environmental monitoring, several samples are taken over a period of time. The information from the samples can be combined semi-quantitatively or fully-quantitatively. The former record each sample as a pass or fail and bases the compliance rule on the proportion of samples that pass within the period; this does not distinguish between marginal pass/fail and readings that are well away from the target. This approach has to date been used for Shellfish Waters. The fully-quantitative approach looks at the overall distribution of readings and derives parameters for a theoretical underlying distribution. This approach has been adopted for Bathing Waters.

The sampling regime and compliance standard are both relevant to a compliance regulation. In Table 5, it is assumed that samples are taken equally spaced through the year and are independent events. Risk-based sampling or any assumed bias would alter the conclusions. Demonstrating compliance rates by the semi-quantitative method requires that of n samples at least r should pass, where r/n equals or exceeds the target compliance rate – that is a simple decision ruler applicable to single sites. With four samples, for example, a 75% compliance rate is met with 0 or 1 failures, but any higher compliance rate allows no failures. With 12 samples, 1 failure is now allowed under a 90% compliance rule. The pass rate for individual samples, assuming this to be constant, can be calculated from a binomial distribution: specifically, the inverse of the cumulative binomial which is the probability of success on one trial such that the probability of observing k or fewer successes in n trials is p.

Table 5 - Indicative water standards required to achieve shellfish flesh standard of 300 *E. coli* MPN/100g)

Species	No samples annual	Target annual compliance rate (%)	Compliance required in individual samples (%)	Geomean required in flesh (MPN/100g)	Estimated geomean *E. coli* in seawater (cfu/100ml)	Estimated 90%ile *E. coli* in seawater (cfu/100ml)
Mussels	4	95	99	28	2.2	8
	4	90	97	45	3.4	13
	4	80	95	57	4.3	16
	4	75	76	149	10	38
	12	90	95	57	4.3	16
	12	80	87	97	7	26
	12	75	76	149	10	38
Pacific oysters	4	95	99	14	2.1	16
	4	90	97	26	3.6	27
	4	80	95	36	4.8	36
	4	75	76	122	14	108
	12	90	95	36	4.8	36
	12	80	87	71	9	66
	12	75	78	112	13	100
Cockles	4	95	99	8	0.03	0.3
	4	90	97	16	0.05	0.5
	4	80	95	23	0.07	0.7
	4	75	76	102	0.28	2.8
	12	90	95	23	0.07	0.7
	12	80	87	53	0.16	1.5
	12	75	78	93	0.26	2.5
All species	4	95	99	2.8	0.39	5.6
	4	90	97	7.1	0.66	9.5
	4	80	95	11	0.88	13
	4	75	76	74	2.7	38
	12	95	99	2.8	0.39	5.6
	12	90	95	11	0.88	13
	12	80	87	32	1.6	23
	12	75	78	74	2.7	38

The compliance rate for individual samples can be treated as the upper percentile of the confidence interval (CI) around the regression linking flesh and water levels in the microcosms. For a given compliance rate in flesh, the intersection with the upper CI defines the equivalent water standard and the estimated geomean for flesh samples can be calculated (Figure 14 and 15).

Figure 15 use the same data as Figure 9. P% compliance implies (1-P) values both above and below the confidence interval, which is therefore set as (2P-1). The flesh values are regressed on the water values to locate the flesh mean; then the water values are regressed on the flesh values to obtain the CI in water at the flesh mean; note that again the 90% CI gives the 95%ile. The graphs are shown only for the calculation based on 99% compliance of samples for one species.

In Table 6 indicative water standards required to achieve a shellfish flesh standard of 230 *E. coli* MPN/100g are shown i.e. the threshold level for shellfish hygiene 'class A' compliance.

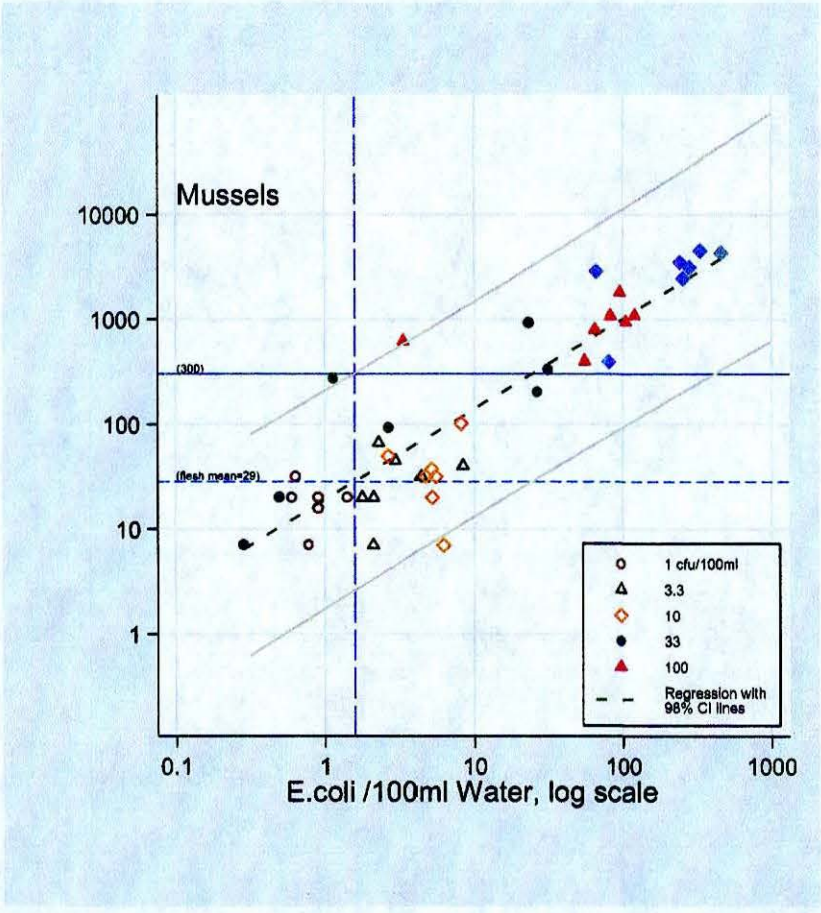


Figure 14 - Regress flesh level on water and derive mean flesh when 99%ile=300

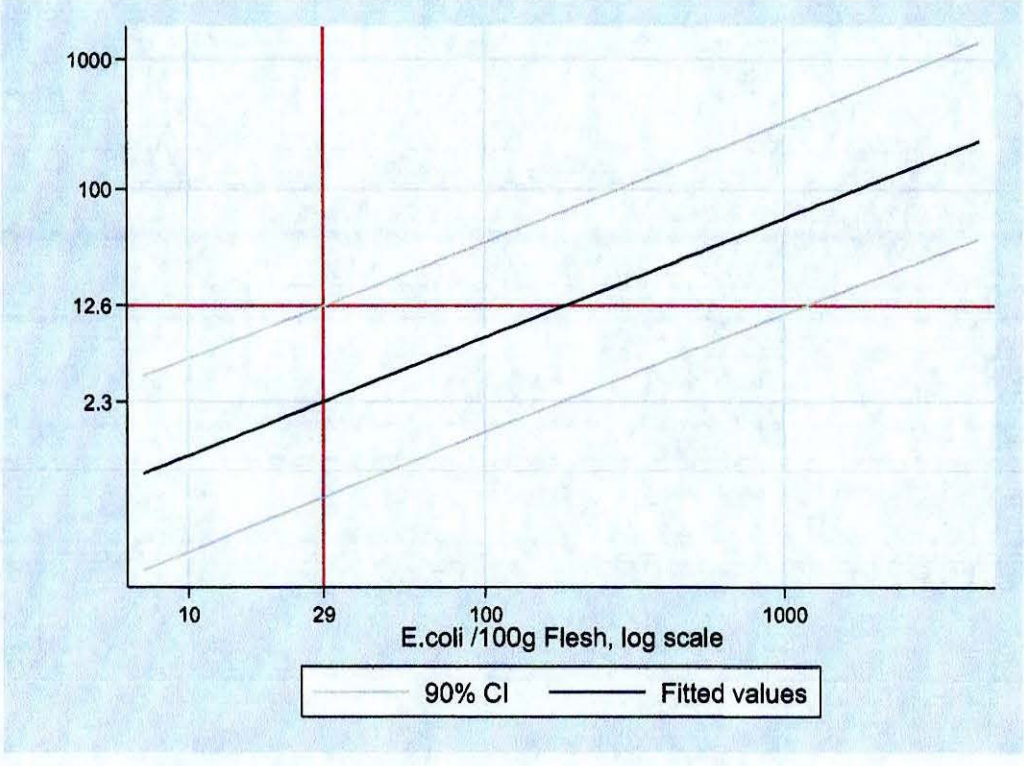


Figure 15 - Inverse regression to estimate water parameters

Table 6 - Indicative water standards required to achieve shellfish flesh standard of 230 *E. coli* MPN/100g

Species	No. samples /annum	Target annual compliance rate (%)	Compliance required in individual samples (%)	Geomean required in flesh (MPN/100g)	Estimated geomean *E. coli* in seawater (cfu/100ml)	Estimated 90%ile *E. coli* in seawater (cfu/100ml)
Mussels	4	95	99	21	1.7	6
	4	90	97	34	2.7	10
	4	80	95	44	3.4	12
	4	75	76	114	8	30
	12	90	95	44	3.4	12
	12	80	87	75	5.5	20
	12	75	76	114	8	30
Pacific oysters	4	95	99	11	1.7	12
	4	90	97	20	2.9	21
	4	80	95	28	3.8	28
	4	75	76	94	11	85
	12	90	95	28	3.8	28
	12	80	87	55	7	52
	12	75	78	86	11	79
Cockles	4	95	99	5.8	0.02	0.2
	4	90	97	12	0.04	0.4
	4	80	95	18	0.06	0.6
	4	75	76	79	0.22	2.2
	12	90	95	18	0.06	0.6
	12	80	87	41	0.12	1.2
	12	75	78	71	0.2	2.0
All species	4	95	99	2.2	0.33	4.8
	4	90	97	5.4	0.57	8
	4	80	95	8.7	0.75	11
	4	75	76	57	2.3	33
	12	95	99	2.2	0.33	4.8
	12	90	95	8.7	0.75	11
	12	80	87	25	1.4	20
	12	75	78	50	2.1	30

3.3 Environmental investigations

Shellfish flesh *E. coli* concentrations for each batch are presented in Table 7 and the geometric mean *E. coli* concentrations are presented in Table 8.

Table 7 - Daily geometric mean concentrations of *E. coli* in water from Mumbles Pier sampling transect in Figure 1. Six samples were collected each day between 08:00 and 19:00.

Geometric mean of six samples/100ml	Date of sample collection
66	05/09/2011
227	06/09/2011
96	07/09/2011
201	08/09/2011
155	09/09/2011
317	10/09/2011
381	11/09/2011
184	12/09/2011
183	13/09/2011
264	14/09/2011
134	15/09/2011

One significant problem encountered was the high mortality rate of the cockles during the experiment. Even though 20 bags were laid (i.e. not 14 as required in Table 2), there were insufficient live shellfish remaining to complete analyses of the last two batch collections on 15th September, hence the blank cells in Table 2 (these were treated as 'missing values' subject to 'pairwise deletion' in the statistical analyses below).

The initial exploratory analysis to determine the gross concentration factors for each of the three species over the full period of the experiment is presented in Table 8.

Table 8 - Accumulation factors between geometric mean *E. coli* concentration in the water and shellfish flesh using all data through the study period.

Matrix	Geomean	Accumulation Factor Expressed as a Geomean/100ml	Accumulation Factor Expressed as a Log_{10} Value
E. coli Water	204	-	-
E. coli Pacific oysters	5750	29	1.45
E. coli Mussels	8933	45	1.64
E. coli Cockles	12,326	62	1.78

This table uses water and shellfish flesh data sampled from 6th to 15th September 2011.

It is interesting to note that the relative ordering of inter-species *E. coli* accumulation is as would be predicted from Beucher (1993) and Kershaw *et al.* (2012: Figure 3) and the observed accumulation factors in this experiment are consistent with the literature sources summarised in Kershaw *et al.* (2012: Table 2).

The water and flesh concentration data approximate to a log_{10} normal probability density function with all Shapiro Wilk normality test significance values greater than the critical value of 0.05 (Table 9).

Table 9 - Shapiro Wilk normality test on water and flesh microbial distributions for all data collected in the experimental period.

	Shapiro-Wilk test of Normality		
	Statistic	df	Sig.
Log $_{10}$ *E. coli* Water	.939	12	.482
Log $_{10}$ *E. coli* Pacific oysters	.953	12	.677
Log $_{10}$ *E. coli* Mussels	.974	12	.951
Log $_{10}$ *E. coli* Cockles	.976	12	.959

These data are displayed graphically in Figure 16 which presents mean $\log_{10}$ *E. coli* concentration and 95% confidence intervals around each mean value for each species and for the water during the full period of the experiment.

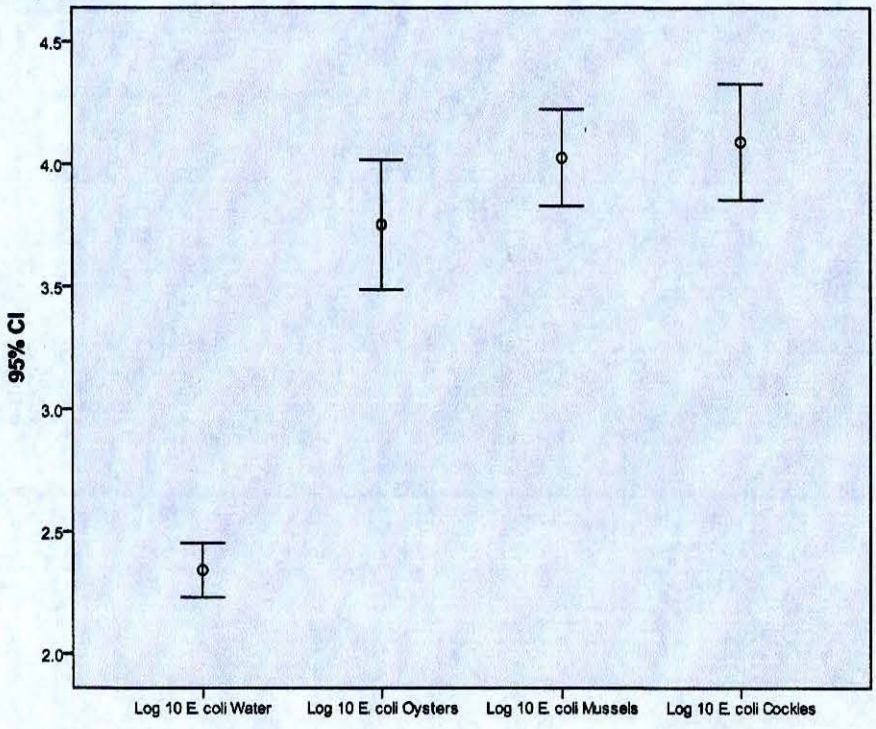


Figure 16 - Mean $\log_{10}$ *E. coli* concentrations and 95% confidence intervals around each mean value for each species and for the water during the full period of the experiment.

This representation of the gross accumulation rate data, suggests no statistically significant difference in the accumulation rates between species but significant differences between all species and the water concentration (this observation is confirmed by the analysis of variance outlined in the Appendix VII). It should be noted, however, that these data reflect a 10-day exposure period and the apparent variation in both water and flesh concentrations will reflect the normal environmental variability in *E. coli* concentrations in environmental waters. Thus, the observed accumulation factors in Table 8 will be affected by this variability and may not adequately reflect a 'constant' *E. coli* concentration in overlying water which might be expected to produce tighter confidence intervals about the mean values resulting in a higher probability of statistically significant differences in accumulation between species. This 'natural' variability in water concentrations of the faecal indicators is further explored below.

Two approaches were investigated to examine how the water variability influenced the accumulation of *E. coli* in shellfish flesh.

The first approach was to investigate correlations between *E. coli* in the shellfish flesh and the measured water quality collected along the transect adjacent to Mumbles Pier.

Table 10 shows water correlation between shellfish flesh quality and water quality collected on the day of sampling and Table 11 shows parallel correlations for water quality during the previous day.

Table 10 - Pearson correlation analysis between measured water quality on the day of collection and shellfish flesh Log_{10} *E. coli* concentration.

		Log_{10} *E. coli* Mussels	Log_{10} *E. coli* Pacific oysters	Log_{10} *E. coli* Cockles
Measured Log_{10} *E. coli* day of shellfish sample collection	Pearson Correlation	.420	.168	.195
	Sig. (2-tailed)	.135	.567	.544
	N	14	14	12

Table 11 - Pearson correlation analysis between measured water quality on the day prior to the day of shellfish collection (arithmetic mean of the log_{10} concentrations) and shellfish flesh *E. coli* concentration (arithmetic mean of the log_{10} concentrations).

		Log_{10} *E. coli* Mussels	Log_{10} *E. coli* Pacific oysters	Log_{10} *E. coli* Cockles
Measured Log_{10} *E. coli* day prior to shellfish sample collection	Pearson Correlation	.297	.220	-.276
	Sig. (2-tailed)	.303	.449	.385
	N	14	14	12

Neither analysis produced statistically significant correlations and $p>0.05$ in all cases.

The second approach employed modelled *E. coli* data predicted using a hydrodynamic two dimensional water quality model (DIVAST) for Swansea Bay which was under construction as part of the Smart Coasts project. Model runs were conducted for the immediate area adjacent to Mumbles Head and Mumbles Pier designed to provide near-real-time prediction of *E. coli* concentrations at Mumbles Pier where the shellfish were laid for the experiment. The principal local input for this location is the sewage pumping station (SPS) at Knab Rock, Mumbles Head. There is no direct measurement and recording of flux to sea from this intermittent discharge. However, periods of pumping to a storage tank, which then discharges on a falling tide, are recorded by Dŵr Cymru Welsh Water.

The full Severn Estuary model domain and the area of interest are shown in Figure 17 with a more detailed representation of the sites modelled in Figure 18. Site P28 in Figure 18 represents Mumbles Pier.

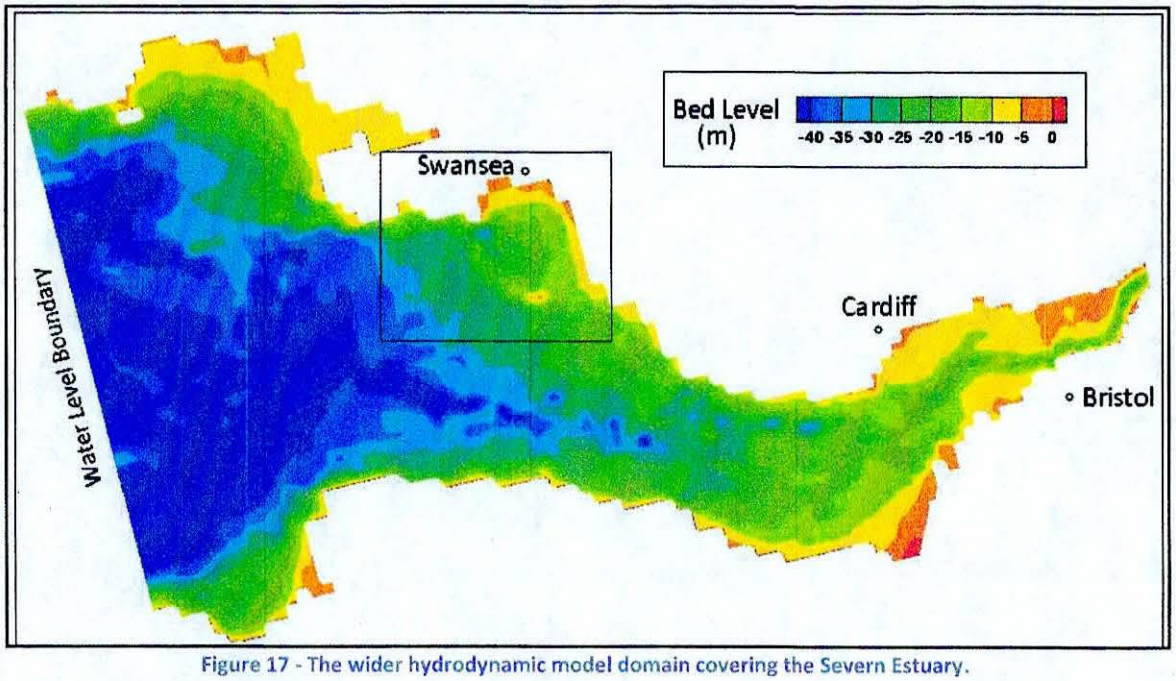


Figure 17 - The wider hydrodynamic model domain covering the Severn Estuary.

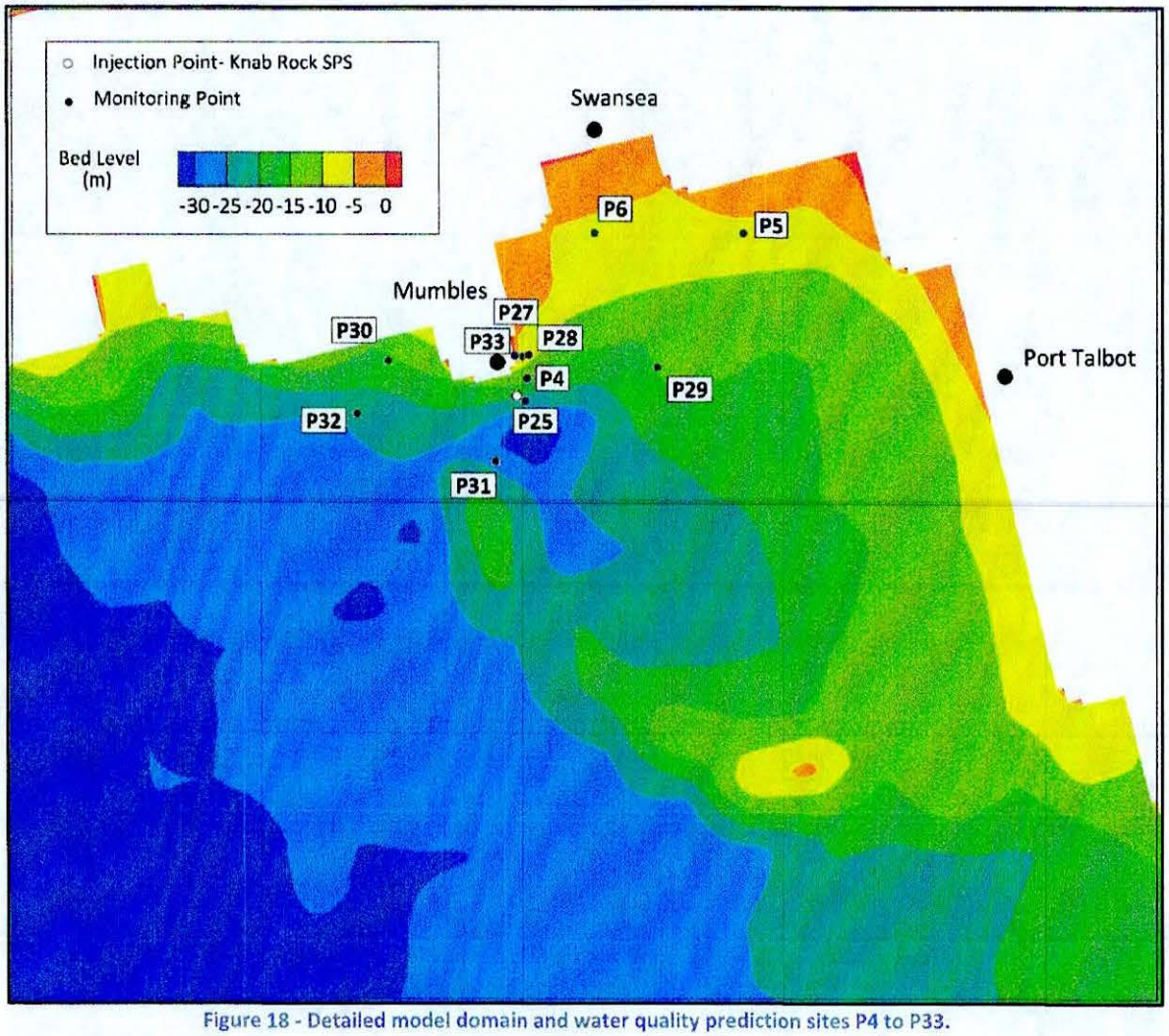


Figure 18 - Detailed model domain and water quality prediction sites P4 to P33.

For illustrative purposes, pump operation from 05:23 on 5th September through to 13:23 on 13th September are shown in Figure 19 together with measured *E. coli* concentrations in the sampling transect.

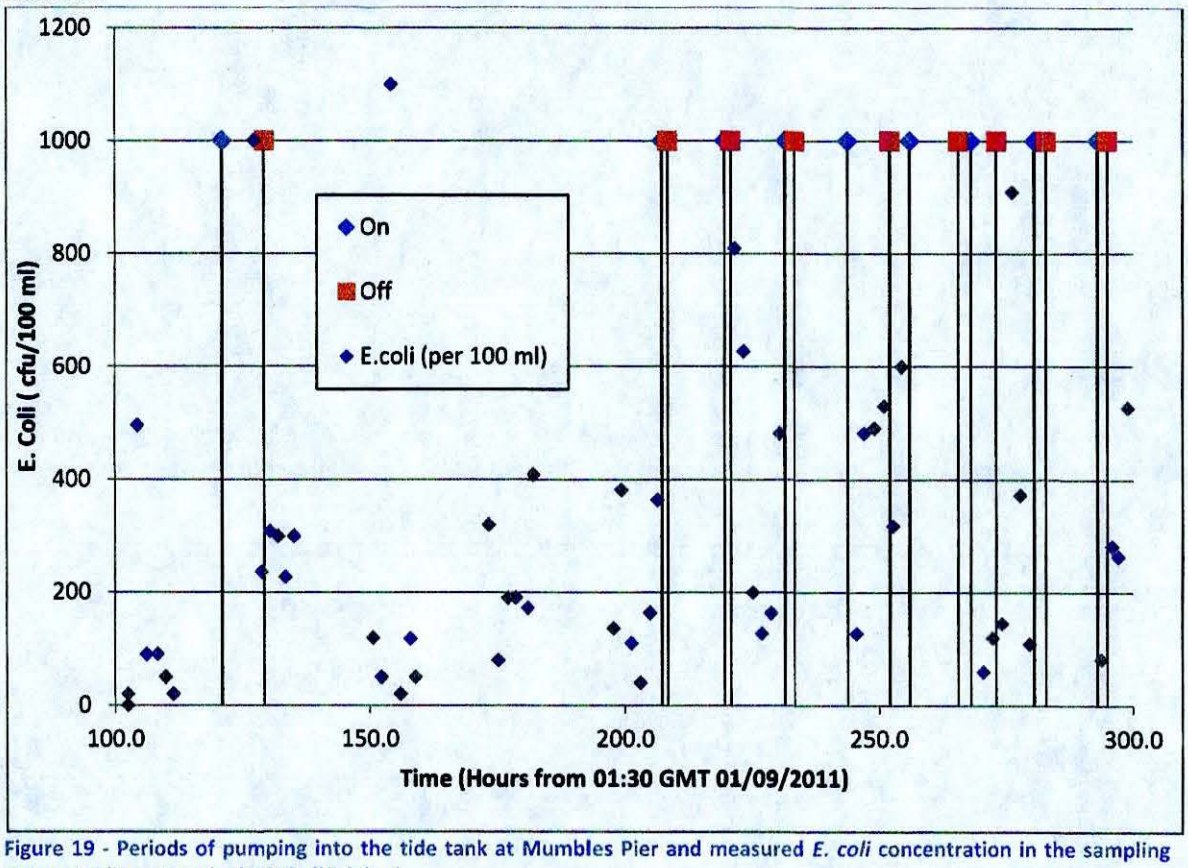


Figure 19 - Periods of pumping into the tide tank at Mumbles Pier and measured *E. coli* concentration in the sampling transect adjacent to the laid shellfish bed.

It was assumed that the tide tanks discharged on the falling tide following the start of any pumping period and this is illustrated in Figure 20 which covers the period from 13:23 on 13th September 2011 to 15:23 on 15th September 2011.

The predicted *E. coli* at Mumbles Pier (solid red line) and measured *E. coli* (solid blue circles) are shown on this figure together with pumping periods and tide tank discharges. For these model runs, fixed T_{90} values were used for *E. coli* of 40 hours (night time) and 20 hours (daylight). These values being chosen to match model outputs with measured values.

The modelled data were generated for the full study period at 0.1 hour resolution. The individual *E. coli* predictions were then used to generate a geometric mean value for the period of inundation of the shellfish site (i.e. tide height >4.90m ACD) during the previous high tide.

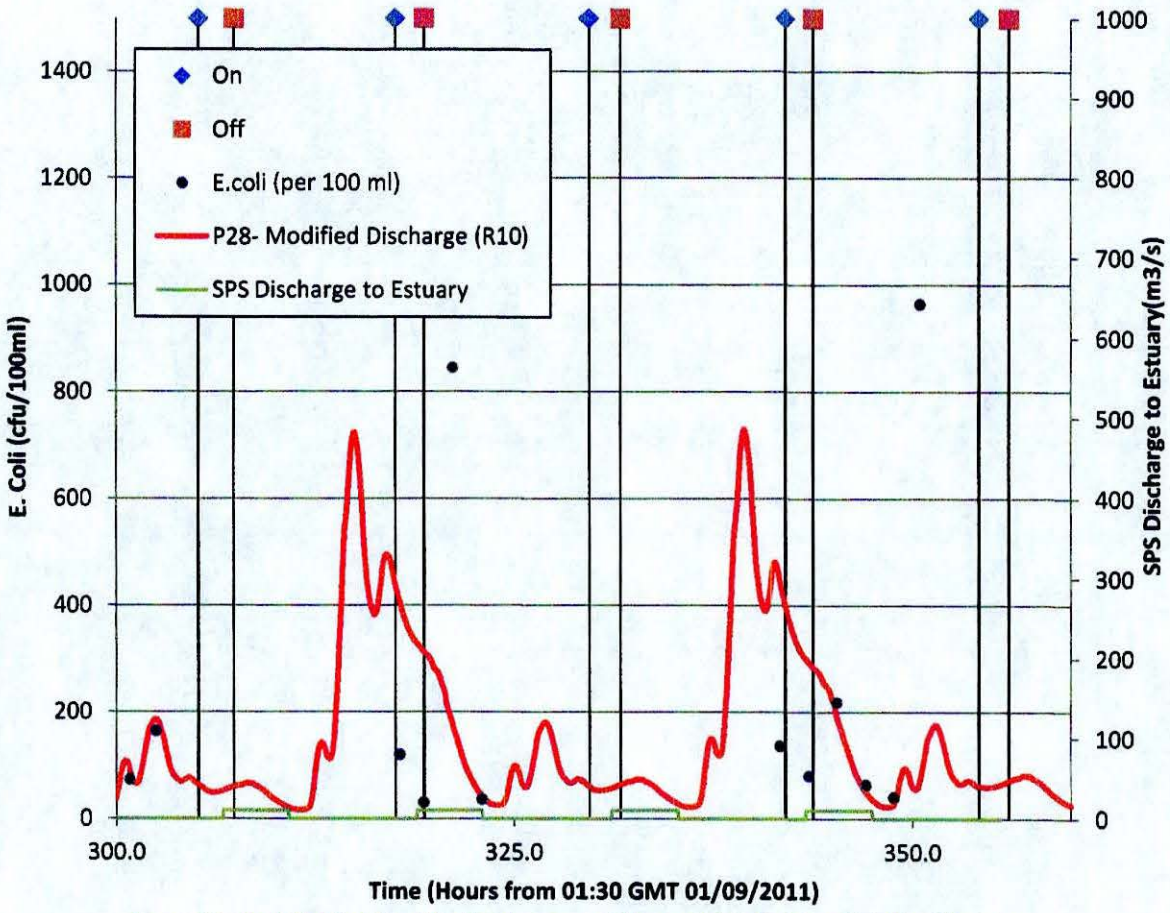


Figure 20 - Predicted (solid red line) and measured (blue solid circles) *E. coli* at Mumbles Pier.

Table 12 shows a parametric correlation analysis of the predicted mean log_{10} *E. coli* concentration during the preceding inundation period prior to each shellfish collection shown in Table 2. None of the correlation coefficients exhibit a statistically significant relationship and, indeed, that of Pacific oysters is negative. This analysis does not suggest a strong correlation with modelled water quality in the previous tidal incursion over the laid shellfish beds, where predicted geometric mean concentrations in each of the 14 studied tidal incursions, ranged from to 72 to 544 cfu/100ml.

Table 12 - Pearson correlation analysis between predicted mean log_{10} *E. coli* concentration in water during the tidal inundation immediately preceding each shellfish collection event in Table 2 and shellfish flesh concentrations of *E. coli* in each shellfish species.

		Log_{10} *E. coli* Mussels	Log_{10} *E. coli* Pacific oysters	Log_{10} *E. coli* Cockles
Modelled Log_{10} *E. coli* during	Pearson Correlation	.345	-.338	.325
the preceding tidal	Sig. (2-tailed)	.226	.237	.303
inundation period	N	14	14	12

In summary, the field component of this investigation has identified shellfish flesh *E. coli* accumulation patterns in broad accordance with the literature: i.e. considering the relative *E. coli* accumulation in the three species studied. However, literature accumulation rates, reported in Kershaw *et al.* (2012b), do exhibit very wide ranges. Both modelled and measured *E. coli* in the previous tidal incursion potentially impacting the laid shellfish in this investigation have not produced statistically significant correlations with the measured concentrations of *E. coli* in the

shellfish flesh. This lack of a clear linear relationship is in accordance with the past literature in this field which has not produced credible mathematical relationships linking water quality and shellfish flesh concentrations. The site chosen for this work may be imperfect given the degree of environmental variability in *E. coli* concentration during the study period but there is growing evidence of short term variability in faecal indicator concentrations in near-shore waters which may be more appropriately considered the 'normal' condition.

These observed diurnal and tidal patterns of *E. coli* concentration have been uncovered during microbial tracer investigations in the UK in which faecal indicators have been sampled on an hourly basis for 54 hours after tracer release (Wyer *et al.*, 2010). The empirical results, to date, suggest ubiquitous and surprisingly high 'natural' variability in *E. coli* (and enterococci) concentrations, commonly exceeding 2 $\log_{10}$ orders over a diurnal cycle, even when well characterised drivers, such as rainfall and river flow events, have not been operative. At the adjacent Swansea Bay bathing water designated sampling point (DSP) a separate, but complementary, sampling exercise was completed in 2011 to characterise this variability and provide accurate water quality data for 'black-box' model calibration to underpin real-time water quality prediction as recommended in WHO (1999; 2003) and Anon (2006) (see also: http://www.smartcoasts.eu/). Data from the 2011 summer sampling programme became available in November 2011 and results are illustrated in Figure 21 where every vertical line of symbols represents one sampling day and there are 60 days sampled in the 2012 bathing season. It is generally accepted that near-shore water quality can be impacted by riverine discharges, particularly after high flow events, and Figure 18 also shows the discharge record of a small urban stream discharging adjacent to the DSP. This diagram suggests a constant range in *E. coli* concentration each day approaching 2 $\log_{10}$ orders even during dry periods of river base-flow (e.g. after 1176 and around 2529 hours from monitoring start).

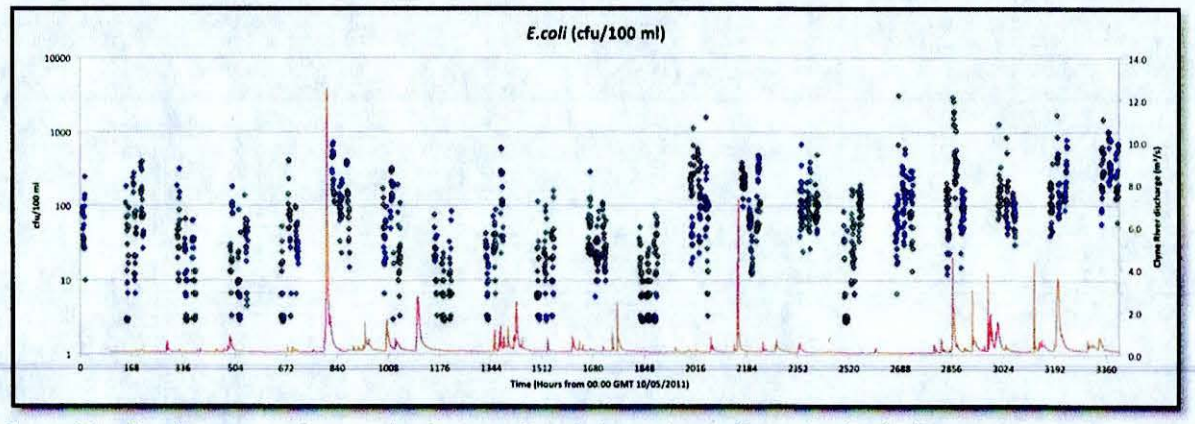


Figure 21 - *E. coli* concentrations at the Swansea Bay designated sampling point for bathing water compliance in approximately 30 minute samples for 60 bathing days during the 2011 bathing season. For comparison the flow in the Clyne river, a small urbanised stream discharging near the DSP is presented.

The affect of storm events, and higher river flows, is to elevate the daily mean values but the range of the data stay roughly constant. This is seen in Figure 22 where the daily data over the 60 runs, or days, of sampling are represented as mean $\log_{10}$ values and 95% confidence intervals on this mean value for each day (n≈20 samples/day).

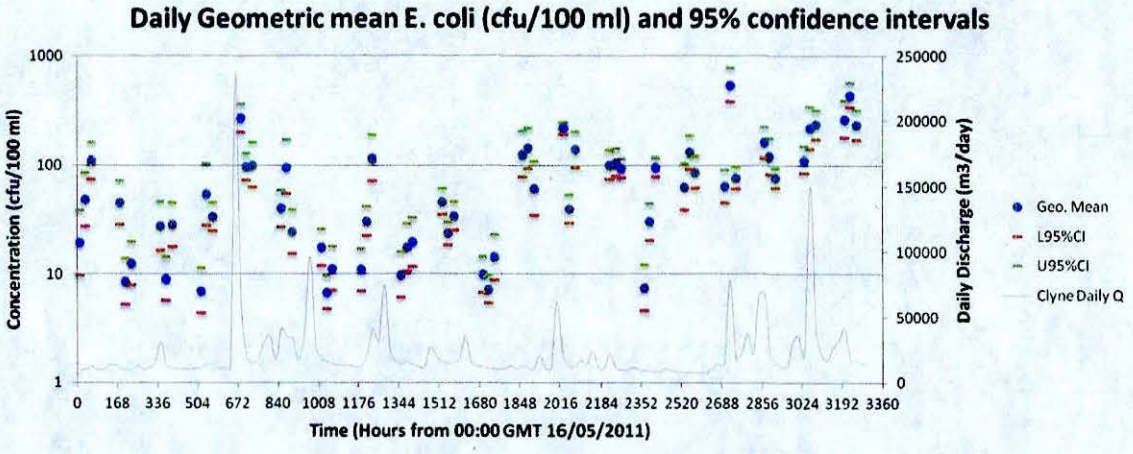


Figure 22 - Means and 95% confidence on the mean for 60 daily sampling runs in summer 2011 bathing season spanning 16th May to 29th September.

The pattern in Figure 22 suggests statistically significant between-day differences in *E. coli* concentrations, possibly driven by both: (i) variability in microbial input fluxes from rivers and intermittent discharges (Kay, *et al.*, 2009); and (ii) altered microbial decay rates in near-shore waters driven by solar irradiance and turbidity (Kay *et al.*, 2005). Furthermore, there is no obvious pattern suggesting lower or higher within-day variability where the geometric mean value is lower which might be expected during drier conditions. Figure 23 displays the weak statistical relationship between the daily mean value (horizontal axis) and the daily standard deviation (vertical axis).

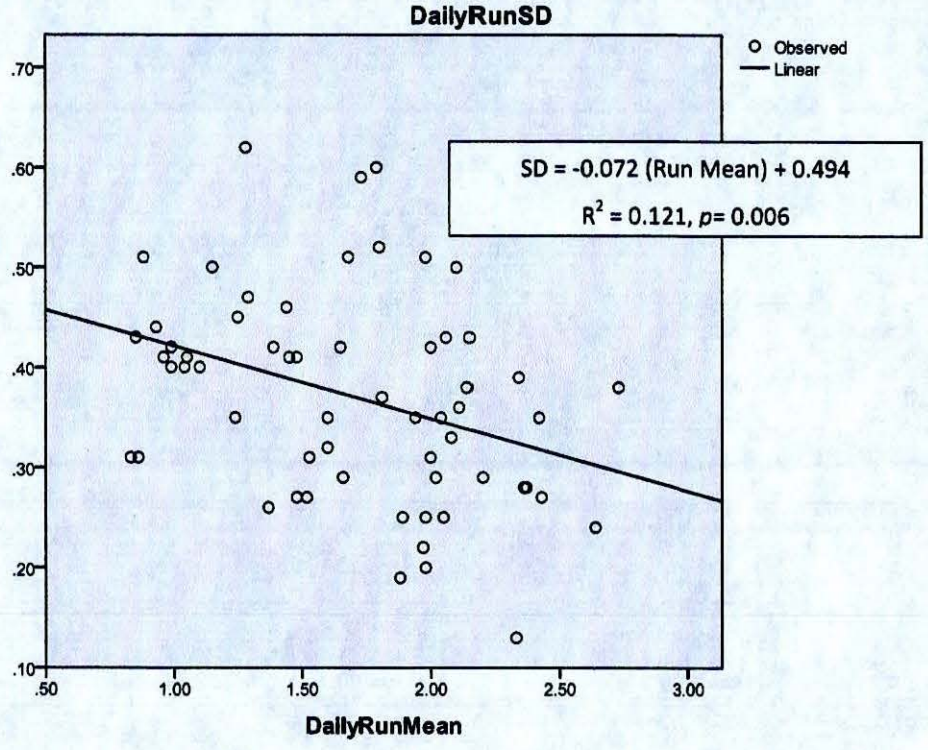


Figure 23 - The relationship between the mean $\log_{10}$ *E. coli* concentration during each day (horizontal axis) and the standard deviation of all samples collected on each day (vertical axis).

Whilst this relationship is statistically significant (i.e. $p<0.05$), the explained variance of only 12.1% which does not suggest a clear, and operationally useful, difference in the standard deviation

between days with low and high geometric mean values. The average $\log_{10}$ standard deviation value for the 60 days of sample collection was 0.37 and this is, therefore, used in the analysis below.

4 Discussion

4.1 Dynamics of *E. coli* accumulation and clearance in microcosms

The numbers of *E. coli* accumulated during the exposure period and the dynamics of shellfish cleansing during the post-exposure phase indicate that the microcosms were successful in simulating the proposed scenario of "low to moderate" levels of chronic contamination, which was identified as a gap in knowledge as highlighted in the first stage of this project (Kershaw *et al.*, 2012). In particular, the high intercepts observed in the regression models (Section 3.1.2) for the two highest target water concentrations (100 and 330 cfu/100ml) suggest the levels of the contaminant in the least contaminated tanks (1 to 100 cfu/100ml) are more representative of the lower feeding rates than those of the higher feeding rates.

The dynamics of *E. coli* accumulation and clearance in the tested shellfish populations evidenced an abrupt accumulation curve, followed by an equilibrium ("plateau") phase and a sharp clearance phase. The duration of the equilibrium phase is determined by the magnitude of the contaminant to which shellfish are exposed and the time of exposure. Despite differences in the dynamics of accumulation observed between species, the results show that all species were very efficient in reaching equilibrium across the whole range of water concentrations. In the literature review, we presented evidence that shellfish are able to accumulate enteric bacteria within 30 minutes of exposure to a contaminating source. We also estimated on the basis of regression models developed by Bernard (1989) that, depending on the water temperature, mussels and Pacific oysters could take 40min to 3h to accumulate 300 faecal coliforms (Kershaw *et al.*, 2012). Although the main purpose of our studies was not to quantify minimum accumulation times, the results obtained in the most contaminated tanks are consistent with this rapid accumulation phase and indicate that shellfish are able to accumulate *E. coli* to a plateau within 17h exposure to a contaminating source.

The results show that the level of bacteria in shellfish flesh appears to closely mirror the level in the ambient water. To our knowledge, this is the first study showing evidence of an equilibrium stage under 'chronic' pollution effects beyond 48h exposure to a contaminating source. This is of relevance when considering strategy for monitoring shellfish protected areas under Water Framework Directive and is discussed further below.

Mussels and Pacific oysters were able to clear all of the *E. coli* contamination accumulated in their tissues during the post-exposure period, which was designed to simulate the dynamics of shellfish cleansing during periods of good water quality. However, no results below the limit of detection of the enumeration method were detected in cockles exposed to the most contaminated tank (water target 300 cfu/100ml). This may be a due to the higher overall level of accumulation observed in cockles taking longer to clear despite the higher filtration rate by this species than by Pacific oysters or, to a lesser extent, mussels. It is likely that condition of the cockles and susceptibility to stress induced through the experiment also affected their physiological activity and ability to completely clear contamination.

In some experiments, shellfish cleared a proportion of the accumulated bacteria during the exposure phase. This is mainly attributable to occasional interruptions to dosing of specific experimental tanks caused by the occasional blockage of delivery tubes, to a lesser extent it may indicate that water processing and accumulation efficiency were not regulated by nutritional needs, which is consistent with the hypothesis that these processes are essentially autonomous, i.e. reflect physical characteristics of the filter pump as discussed in the first stage of this project (Kershaw *et al.*, 2012).

The extremely high variation of *E. coli* results between consecutive cockle samples (ex. >18,000 MPN/100g preceded or followed by 20 MPN/100g) during the exposure period is consistent with the evidence in the literature that cockles are one of the most efficient species at accumulating and clearing microbiological contaminants (Kershaw *et al.*, 2012).

4.2 Predicting levels in flesh from water

The regression models can be used to predict levels in shellfish flesh from that in the overlying water using real or simulated scenarios of sewage discharges. However, it should be noted that the explained variance in these models did not exceed 60%. This is high compared with observations made in field studies undertaken at actual shellfish growing sites (Kay *et al.*, 2007) but the policy community should be aware of this level of explanation prior to using these microcosm-derived data as the sole evidence-base for regulatory standards development. The accumulation factors indicate significant inter-species variations in predicted concentrations which are consistent with previous studies that analysed environmental, geographically dispersed (Campos *et al.*, 2011; Younger and Reese, 2011) and microcosm data (Kay *et al.*, 2007). These differences do not support the application of a single water quality standard for shellfish protected areas where more than one species is commercially harvested.

4.3 An approach to developing a water criteria for shellfish harvesting waters

The principal aim of this work is to suggest evidence-based criteria for *E. coli* concentrations in water which will result in compliance of shellfish with microbial standards for shellfish flesh. It is assumed in this analysis that the pattern of near-shore water *E. coli* variability, observed during the intensive sampling undertaken at the DSP site in 2011 is characteristic of UK 'chronic' pollution. The significance of this observation is that the likely 'dry weather' daily variability in near-shore water quality over UK shellfish beds approaches 2 $\log_{10}$ orders, or 100 fold. If this is, indeed, the 'normal' condition, it suggests: (i) the common assumption that relatively low level pollution can be characterised as a constant faecal indicator concentration does not reflect reality; and (ii) any sampling regime required for regulatory purposes should be designed to accommodate and characterise this variability. In the context of this investigation, access to the enhanced data set illustrated in Figures 18 and 19 has provided additional insight into natural patterns of faecal indicator concentration in near-shore waters and it may provide useful empirical evidence explaining why the international search by scientists and regulators for correlation between faecal indicators in shellfish flesh and overlying waters has proven so elusive to date. It may be prudent, therefore to accommodate the concept of variability by treating the 'chronic' water quality condition as a probability density function with a $\log_{10}$ standard deviation of 0.37 (as derived from the DSP data presented above). It would then be possible to use the observed concentration factors in Table 4 for the three species to derive shellfish flesh concentrations from any given water concentration.

An 'illustrative' analysis based on this approach is presented in Figures 24, 25 and 26 for oysters, mussels and cockles, respectively. This uses a modelled probability density function which assumes a $\log_{10}$-normal distribution for *E. coli* in water and shellfish flesh. Distributions are generated for geometric mean water concentrations ranging from 1–8 cfu/100ml, with initial $\log_{10}$ standard deviations of 0.37. For each distribution, the calculated accumulation factors for the three shellfish species are inserted from Table 6 and the shellfish flesh concentrations calculated as a probability density function (n=100).

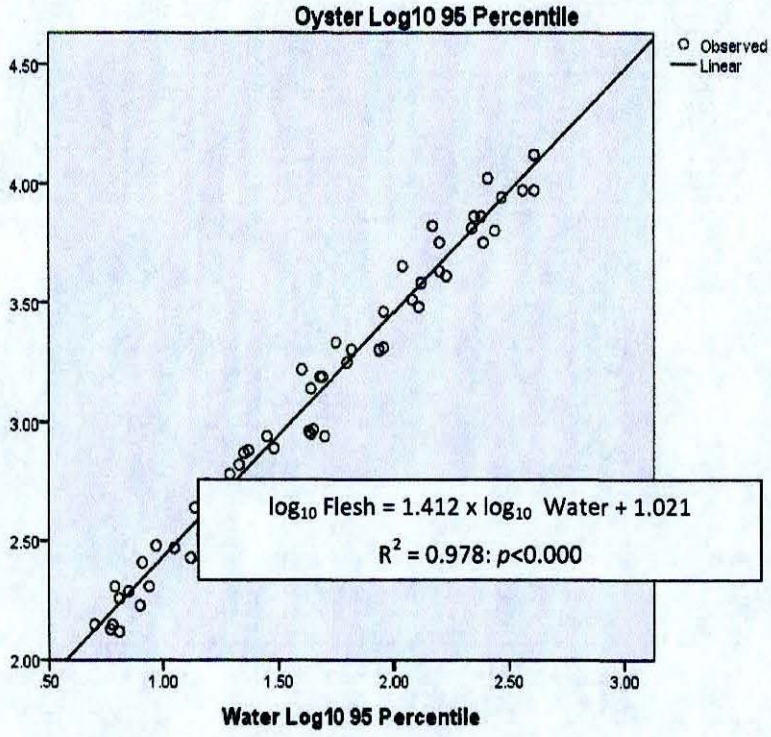


Figure 24 - Modelled oyster (vertical) and water (horizontal) $\log_{10}$ 95 percentile concentration values for *E. coli* assuming a probability density function with a standard deviation of 0.37 and geometric mean values from 1 to 100 cfu/100ml

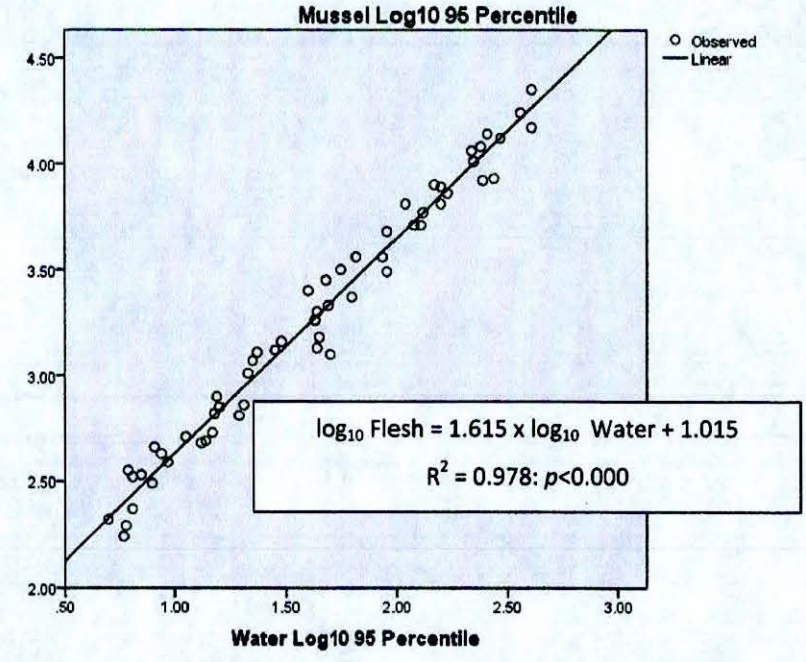


Figure 25 - Modelled mussels (vertical) and water (horizontal) $\log_{10}$ 95 percentile concentration values for *E. coli* assuming a probability density function with a standard deviation of 0.37 and geometric mean values from 1 to 100 cfu/100ml.

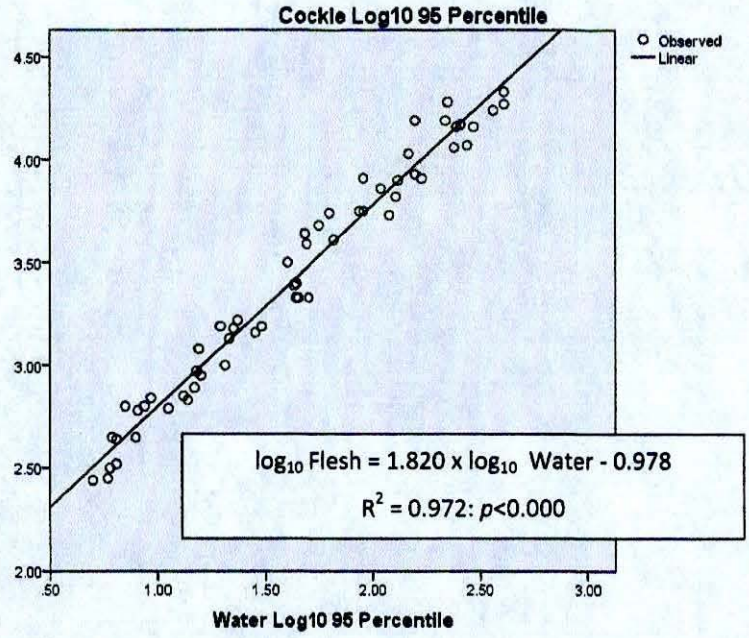


Figure 26 - Modelled cockle (vertical) and water (horizontal) $\log_{10}$ 95 percentile concentration values for E. coli assuming a probability density function with a standard deviation of 0.37 and geometric mean values from 1 to 100 cfu/100ml.

It is important to note that the high explained variances in Figures 24 to 26, as indicated by the R^2 terms in the regression models, do not indicate high correlation between empirically derived data from sea water and shellfish flesh. Rather, it is an exploratory analysis using semi-empirically derived pdfs of water and flesh *E. coli* concentration to investigate target water concentrations for a range of flesh *E. coli* concentrations. Using the regression equations in Figures 24 to 26, target water 95 percentile values are presented in Table 13 below.

Table 13 - Modelled water concentrations required to achieve shellfish flesh *E. coli* concentrations outlined, this analysis is derived from the regression equations defined in Figures 21 to 23 for the three shellfish species.
All values are expressed as 95 percentiles of flesh concentration as MPN/100g and water as cfu/100ml.

	Environmental investigations		Microcosm studies	
Shellfish Type	**Target Flesh 95 percentile *E. coli*/100g**	**Required Water 95 percentile *E. coli*/100ml**	**Target Flesh 95 percentile *E. coli*/100g**	**Required Water 95 percentile *E. coli*/100ml**
Pacific oysters (*C. gigas*)	500	18	500	36
	400	15	400	29
	300	11	300	22
	200	7	200	14
	100	4	100	7
Mussels (*Mytilus* spp.)	500	12	500	26
	400	9	400	20
	300	7	300	15
	200	5	200	10
	100	2	100	5
Cockles (*C. edule*)	500	8	500	1
	400	6	400	1
	300	5	300	1
	200	3	200	1
	100	2	100	0

The indicative water concentrations in Table 13 should be treated with extreme caution because the empirical investigations failed to quantify strong statistical association (and high explained variances) between water and flesh concentrations in this environment. They do, however, present a possible way forward in this area of environmental regulation recognising the inherent stochastic variability in microbial concentrations in natural waters which prevents identification of robust linear correlations.

It may not be the case that this stochastic variability is sufficiently well characterised for a range of sites to underpin derivation of UK-wide shellfish standards. If a relationship between 'chronic' (i.e. defined as low level and constant) microbial water quality and shellfish flesh concentrations of *E. coli* are required, then the laboratory microcosm tank systems provide the best available protocol.

It seems reasonable to conclude, therefore, that the emphasis of resource allocation to the tank microcosm experiments in this project has proven fully justified and this protocol offers the best approach for the definition of water limit values at shellfish harvesting sites. The challenge remaining will, of course, be to characterise the 'natural' variability with a defined 'standard' or compliance criterion.

4.4 Factors critical in controlling contaminant burden in shellfish exposed to chronic microbial pollution

Information on key factors controlling contaminant burden in shellfish both in relation to shellfish physiology and FIO survival have been recently reviewed by Campos *et. al.* (2012). The study highlights that the biological role of autonomous processes such as water pumping and filtration efficiency on FIO accumulation in bivalve molluscs has been insufficiently studied as have processes

affecting adsorption of FIOs to particles and their sedimentation/re-suspension and consequent availability to filter feeders.

4.5 Threshold concentration and exposure duration

Under favourable environmental conditions, FIO accumulation in shellfish may exceed that in the overlying waters within 30 minutes exposure to the pollution source (Kershaw et. al. 2012) and may reach a maximum concentration (Campos et. al. 2012) depending on the relative initial concentrations of FIOs in water and shellfish flesh. Time of exposure is therefore considered one factor that will affect maximum burden of FIOs accumulation in bivalve molluscs.

Over the range of concentrations studied in the microcosm experiments, maximum levels accumulated in shellfish during the exposure phase are shown to be proportional to the level of water contamination. In all experiments peak concentrations in mussels, Pacific oysters and cockles were observed at the first measurement interval which was between 12 and 18 hours. Further experimental work would be required to ascertain more detailed contamination profiles for differing FIO concentrations within this time period.

Tables 5, 6 and 13 set out indicative and modelled water concentrations to achieve shellfish flesh *E. coli* concentrations.

In the microcosm experiments, geometric mean concentrations in water of 83.7, 107.0 and 1.6 returned geometric mean levels of *E. coli* per 100ml in shellfish flesh above 300 for mussels, oysters and cockles respectively. Conversely geometric mean concentrations in water of 3.6, 17.2 and 0.8 returned geometric mean levels of *E. coli* per 100g in shellfish flesh below 300. Where above average values caused the 300 threshold to be exceeded in most cases it is observed that the flesh concentrations were closely in concert with overlying water concentrations (Figures 10 to 12). Further experiments could be undertaken for each species to target more closely the range giving flesh concentrations above and below this threshold or another threshold.

Campos *et. al.* 2011 (p.13) using logistic regression models found that a geometric mean and 90th percentile of *E. coli* of 10 and 55, respectively would be equivalent to the SWD G standard (300 faecal coliforms 100ml^{-1} FIL). The equivalent figures derived from the microcosm experiments for 'all species' under investigation were a geometric mean and 90th percentile of *E. coli* of 3 and 38, respectively.

4.6 Guidance on the role of environmental factors (water temperature, salinity)

In the microcosm experiments key environmental parameters of temperature and salinity were fixed within the tolerance range of all the three species tested.

Monitoring programmes should seek to avoid sampling strategies that overly represent periods when key water quality parameters may inhibit the optimum feeding and uptake of FIOs (and human pathogens) from the water column by the target species.

When considering use of surrogate species for monitoring care should be taken to ensure that the surrogate will be representative for the target species over the full range of environmental conditions (e.g. temperature and salinity variations) that shellfish are exposed to at a particular site.

This is important as surrogates must be able to properly reflect the limits for which the target species will function normally and maintain filtration activity in order to give representative results.

4.7 Guidance on discharge regimes to optimise shellfish quality

Options that may be considered in relation to discharges regimes include:

a) End of pipe standards for discharges direct to shellfish waters. Use of this approach could be considered further once a target shellfish flesh standard has been agreed for WFD monitoring purposes.

b) Developing new discharge scheme design standards to achieve shellfish water standards. Further development of existing Environment Agency technical design standards could be undertaken to provide water discharge permitting guidance relevant to any new water column standard or water column targets that would give an appropriate statistical probability of compliance with any new shellfish flesh standard.

c) Adopting a regime which includes the concept of identifying shellfish harvesting prohibition zones around discharges. These could for example be based upon a fixed distance proportional to size and level of treatment categories and receiving water criteria or determined on the basis of discharge specific dilution calculations or modelling.

4.8 Guidance on monitoring regimes for shellfish protected areas

Factors to be taken into consideration when considering any future change in the monitoring regime for shellfish waters/protected areas include:

a) The level of acceptable risk (for protection of the environment and or public health)

b) The choice of sample matrix i.e. water or shellfish flesh and if the latter the choice of a sentinel species or range of species. Sentinel species need to be protective of the species of prime public health significance see Younger and Reese (2011) on species equivalence factors.

c) The statistical structure of the compliance measure e.g. number of samples per year, the effect of this for sampling 4 and 12 times per year has been shown in Tables 5 & 6.

d) The physical location of samples e.g. whether it is more valid to target sampling to the point in water column that are occupied by the shellfish rather than defaulting to near surface water that may be of a differing quality.

e) Timing of sampling.

The results of both the microcosm and environmental studies have highlighted inter-species differences in levels of FIO accumulation. The use of shellfish flesh *E. coli* data derived from the shellfish hygiene monitoring programme in England and Wales is considered in Kershaw and Morgan (2013). Using a five year data set (2007-2011) statistical compliance for monitoring scenarios utilising one or more species, and single or multiple monitoring points are considered in this report.

5 Conclusions and Recommendations

1. The microcosm experiments were conducted at a single target temperature (10°C) and salinity (30psu) within the tolerance range of the three species used. The average sea water temperature and salinity (1970-2011) around the coast in England & Wales ranged between 2.4 to 21.6°C and 12.6 to 5.3 psu respectively. Further experiments to show the effect on accumulation factors of conditions towards both ends of these ranges for would further inform development of policy (30, 20 and 10 psu 5, 10 and 20°C).

2. Cockles harvested by mechanical dredging were used in the thirds experiment. It is thought that the cockles were adversely affected by the dredging process and that this was responsible for the high incidence of mortalities observed. It may also have affected the feeding regime of the animals and hence the observed accumulation factors. Cockles were also placed directly on the bottom of the tank in the third microcosm experiment which was also not ideal. Cockles are an in-faunal species and as such live within the sediment and filter-feed using an emergent siphon. A repeat microcosm experiment for cockles at two target water *E. coli* concentrations experiment utilising hand gathered cockles in microcosms with and without a suitable sediment substrate would further provide further confidence on accumulation in this commercially important species.

3. Linear regression models of *E. coli* levels in shellfish versus water were fitted for the six target water concentrations. These models show that 52% of the variance observed in *E. coli* levels in mussels and Pacific oysters and 60% of the variance in *E. coli* levels in cockles are explained by the variation of *E. coli* levels in the water. No direct association was apparent between the latter variable and the accumulation factor.

4. In this study it was not possible to define changes in, or maximum, species rate of *E. coli* accumulation for the sampling time intervals under consideration. However accumulation to peak levels was relatively rapid and generally within the period of the first sampling interval after dosing commenced (within 17 hours for all species). Shellfish are therefore likely to respond to quite short term stimulus such as high rainfall incidents that could be overlooked by occasional water sampling.

5. Over the range of conditions studied the relative ordering of inter-species *E. coli* accumulation was consistent between the results of the microcosm experiments those obtained in the field investigation and those reported in the literature. Cockles accumulate more than Pacific oysters which accumulate more than mussels.

6. Experiments to identify accumulation factors in a wider range of commercially important species could be undertaken to further inform the development and calibration of future water quality design standards for shellfish protected areas. It should only be necessary to undertake this for two or three target water concentrations over a contamination period of 96 hours.

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8 Appendices

APPENDIX I - DOSING DATA

Each experiment utilised two reservoirs of sewage each maintained at a particular but differing concentration of *E coli*. Refrigerated sewage stock was assayed for presumptive faecal coliforms and *E. coli* on a daily basis. A conversion factor of 0.85 was applied to the results of presumptive faecal coliforms to derive an assumed *E. coli* concentration. This concentration was then used to calculate the dilutions required for each sewage reservoir. Each reservoir served three dosing peristaltic pumps (6 pumps in total). Each pump served one microcosm tank and was calibrated and set to dose at a specific rate to achieve the target microcosm *E. coli* water concentration. Seawater flow rates to the experimental tanks were kept consistent throughout each experiment and between experiments.

Example sewage dosing data is given in Table A1 below.

Table A1: Dosing data at start of each experiment.

	Tank	Target *E.coli* No/100ml	Sewage stock *E.coli* No/100ml	Sewage volume L	Dechlorinated water volume L	Reservoir *E. coli* No/100ml	Dilution Required No	Tank flow L/min	Pump rate ml/min	Pump rate rpm	Calculated delivery *E.coli* No/100ml
Expt. 1	7	1	1,355,656	6.250	43.750	4,675	4,675	7	1.5	8.3	1.0
	4	3.3	1,355,656	6.250	43.750	4,675	1,417	7	5.0	27.8	3.3
	2	10	1,355,656	6.250	43.750	4,675	467	7	15.0	83.3	10.0
	1	33	1,355,656	0.172	49.828	169,457	5,135	7	1.4	7.8	33.9
	3	100	1,355,656	0.172	49.828	169,457	1,695	7	4.2	23.3	101.7
	6	330	1,355,656	0.172	49.828	169,457	514	7	13.5	75.0	326.8
Expt. 2	7	1	5,142,324	1.613	48.387	4,675	4,675	7	1.5	8.3	1.0
	4	3.3	5,142,324	1.613	48.387	4,675	1,417	7	5.0	27.8	3.3
	2	10	5,142,324	1.613	48.387	4,675	467	7	15.0	83.3	10.0
	1	33	5,142,324	0.045	49.955	165,881	5,027	7	1.4	7.8	33.2
	3	100	5,142,324	0.045	49.955	165,881	1,659	7	4.2	23.3	99.5
	6	330	5,142,324	0.045	49.955	165,881		7	13.5	75.0	319.9
Expt. 3	7	1	2,632,314	3.125	46.875	4,659	4,659	7	1.5	8.3	1.0
	4	3	2,632,314	3.125	46.875	4,659	1,553	7	5.0	27.8	3.3
	2	10	2,632,314	3.125	46.875	4,659	466	7	15.0	83.3	10.0
	1	32	2,632,314	0.088	49.912	164,520	5,141	7	1.4	7.8	32.9
	3	100	2,632,314	0.088	49.912	164,520	1,645	7	4.2	23.3	98.7
	6	316	2,632,314	0.088	49.912	164,520	521	7	13.5	75.0	317.3

APPENDIX II - SHELLFISH ACCLIMATION AND SURVIVAL.

The percentage of shellfish mortalities in relation to the total number of individuals in each experimental tank varied between 2–3.4% in mussels, 0–0.92% in Pacific oysters and 21–57% in cockles (Figure A2).

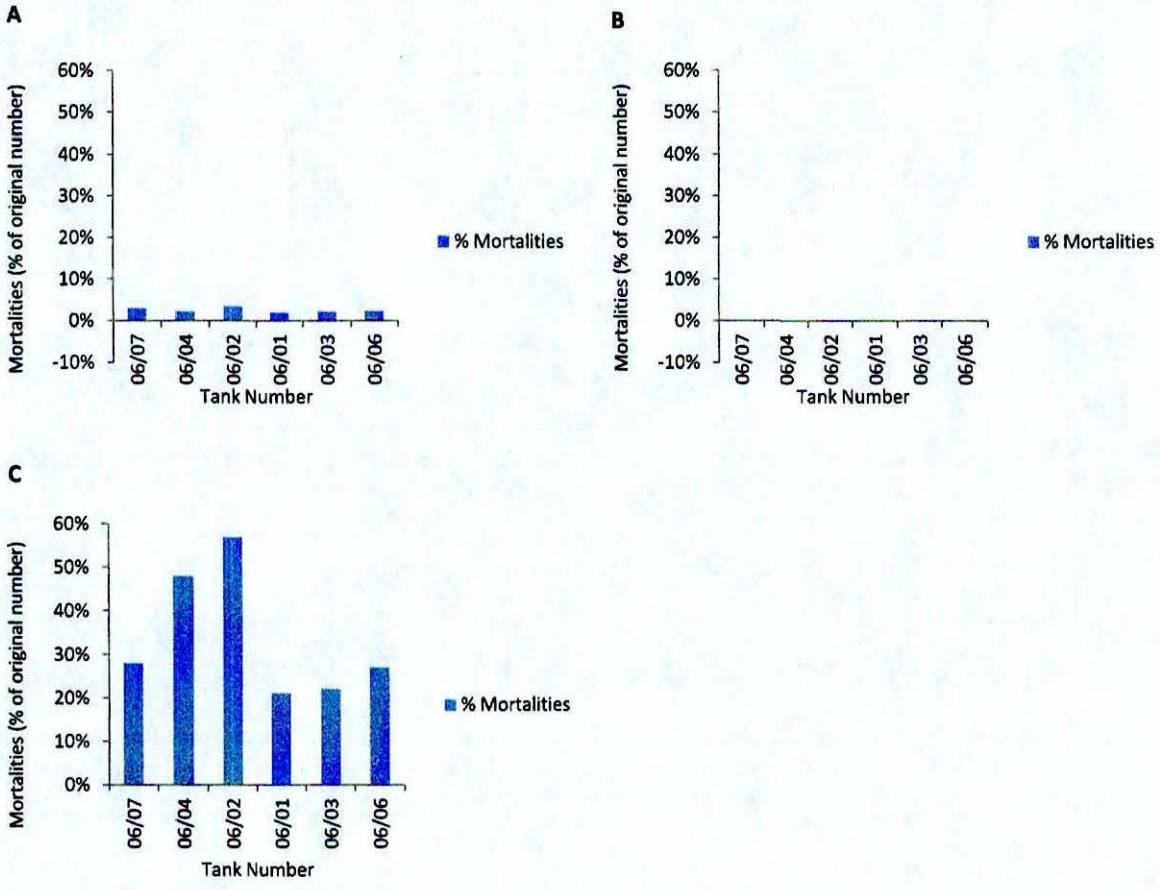


Figure A2. Shellfish mortalities (percentage) during the experimental work. A: mussels; B: Pacific oysters; C: cockles. Tank numbers and corresponding target water concentration (cfu *E. coli*/100ml) 06/07 = 1 ; 06/04 = 3.3; 06/02 = 10; 06/01 = 33; 06/03 = 100; 06/06 = 300.

Three quarters of the mortalities observed per species occurred during the first half of the experimental period. This suggests that some individuals underwent acclimatisation difficulties in the early stages of the experiment, leading to stress and eventually death which are typical in these sort of experiments. Furthermore, it is noted that the cockles were harvested by mechanical dredge and this is likely to have resulted in stress additional to that incurred from being transported and acclimatised to an experimental environment

The majority of mortalities in mussels and cockles occurred in the three least contaminated tanks (target water concentrations = 1, 3.3, 10 cfu *E. coli*/100ml). The more heavily contaminated tanks provided animals with food which may have contributed to their survival.

Cockles incurred the highest number of mortalities (34% of the total population), followed by mussels (2.3%) and oysters (0.1%). It is unknown whether the cause of such high death rates among the cockle population was stress or disease-related as no histological examination was undertaken.

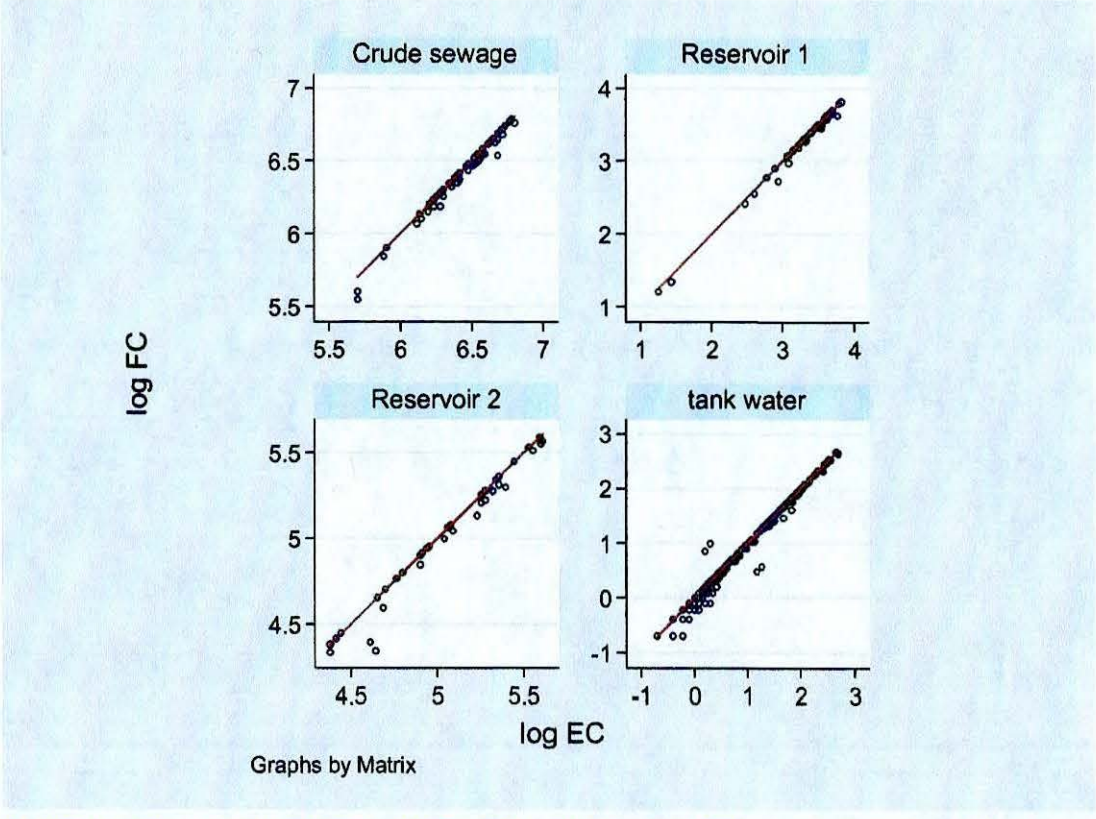
It is probable that, if disease was present, this would have spread rapidly to other individuals in the tanks. Observations of cockle and mussel mortalities were that they typically exhibited various stages of shell 'gaping'. Some individuals displayed a small gap between their shells that, when lightly pressed, did not close shut, while others showed wide, almost fully-open gapes. Other specimens were found dislodged from their shells. Specimens that perished showed no obvious common features of size or colour. Mortalities were counted and removed from tanks twice daily, immediately post-sampling. The health of individuals did not appear to improve following the removal of dead animals and, in fact, deteriorated at a constant rate throughout.

Bivalve molluscs are commonly air-stored during transportation and as a result can suffer from anoxia. It is estimated the animals used in these experiments were kept out of water for at least 6 hours prior to arrival at the laboratory. The temperature at which they were kept is unknown but it is likely to have been no greater than 15°C. Mussels have the ability to acclimatise to changes in temperature under laboratory conditions, adjusting their filtration rate until full acclimatisation is achieved (Kittner and Riisgard, 2005). Furthermore Angelidis (2007) found that mussels stored at lower temperatures (0-5°C) generally released less intra-valve water and had a higher rate of survival than those stored at higher temperatures (≈20°C). The ability to which each of the species used could acclimatise to the laboratory conditions would have been determined by the level of acclimatisation reached in the field and also the level of stress incurred during their collection and transportation. It is unlikely the primary factor inducing mortalities was related to either thermal or salinity stress as these variables were monitored throughout and kept at a constant rate within the tolerance thresholds for these species. Cockles experienced a much greater number of mortalities than mussels and oysters and this could be due to handling stress caused through collection, transportation and subsequent handling at the laboratory. In the event that the cause of death within the cockle population was disease related, stress through handling could have exacerbated the infection by reducing the immunity of individuals. Paul-Pont et al. (2010) found that the opportunistic bacteria *V. tapetis* took advantage of lowered immunity in cockle specimens that were already stressed due to handling and environmental factors. As filter feeders, bivalves are exposed to various pathogenic and/or opportunistic bacteria naturally present in the microflora of coastal environments. Specimens were not examined for pathogenic infection as part of this experiment however many different parasite species are known to use cockles as a host especially digenean trematodes which are the most common metazoan parasite in marine invertebrates (Lauckner, 1983). It is probable these animals were exposed to a variety of biotic and abiotic stressors (to varying degrees) in combination including; handling stress, salinity and thermal stress (less likely), starvation, disease, contaminant stress, restricted burrowing ability (cockles) and density stress.

APPENDIX III

RELATIONSHIP BETWEEN FAECAL COLIFORMS AND *E. COLI*

Linear regression models indicate a similarity between faecal coliform and *E. coli* results obtained in crude sewage, reservoir and tank water samples during the experiments as might be expected (Figure 8).



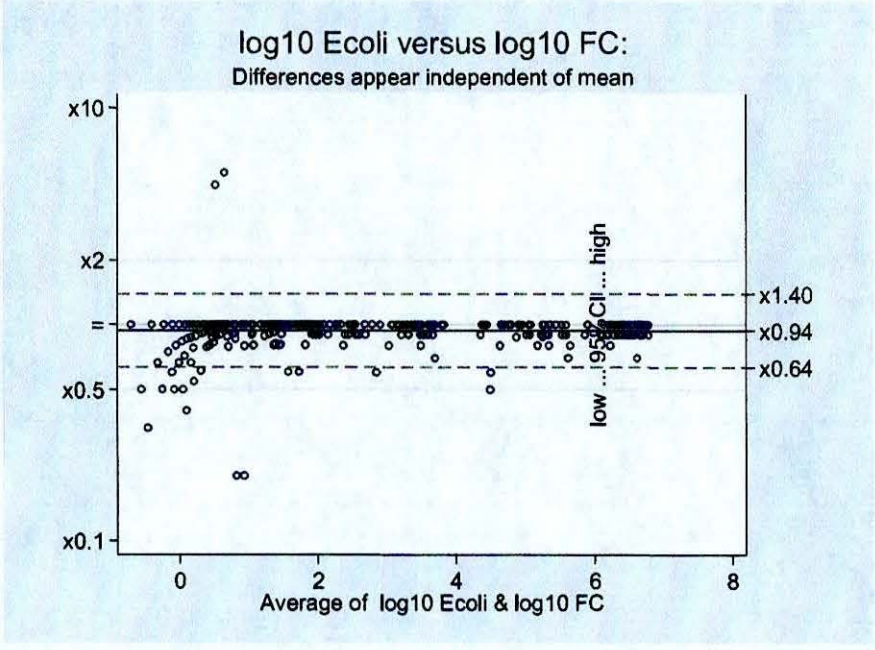
Relationship between levels of faecal coliforms and *E. coli* in sewage stock, sewage reservoirs and tank water, Line of equality shown in red.

The Bland-Altman method (Bland and Altman, 1986) was used to evaluate the agreement between the ratios of faecal coliforms:*E. coli* (Appendix IV). No significant differences were found over the range of water values indicating that either group of bacteria could be used in the analyses. However, *E. coli* is specifically associated with the intestines of warm-blooded animals and, for this reason, is considered a more reliable indicator of contamination of faecal origin. This bacterium was used in this study because it is the currently-used indicator of the risk of microbiological contamination in shellfish intended for human consumption (European Communities, 2004). It is also the indicator recommended by the UKTAG to replace the faecal coliform standard currently used to monitor shellfish waters under the Shellfish Water Directive, which will be revoked in the UK by the Water Framework Directive in 2013 (Warn *et al.*, 2010).

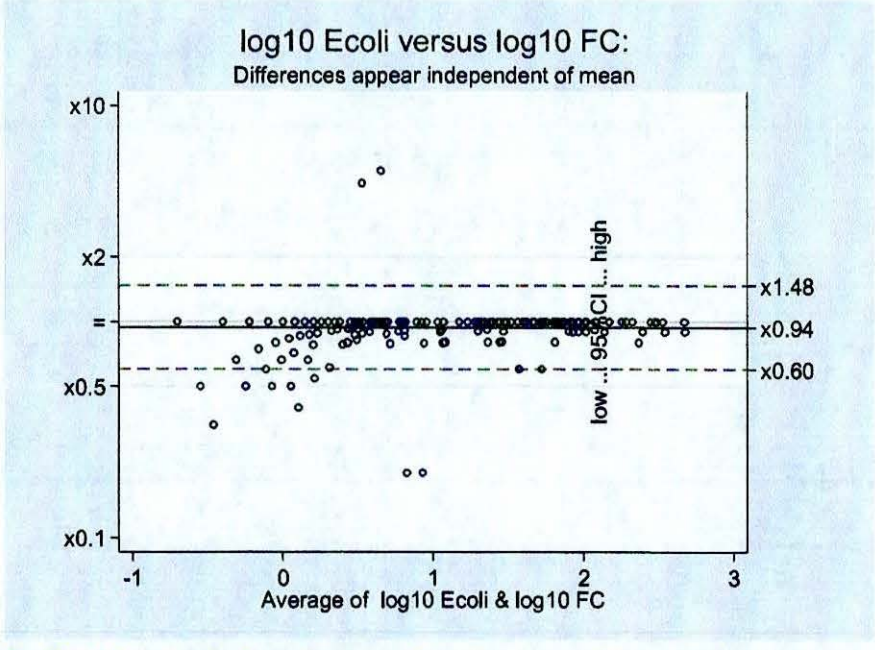
APPENDIX IV

BLAND-ALTMAN PLOTS FOR ALL SAMPLES IN WHICH FAECAL COLIFORMS AND *E. COLI* WERE ENUMERATED (A) AND FOR TANK WATER SAMPLES ONLY (B).

A



B

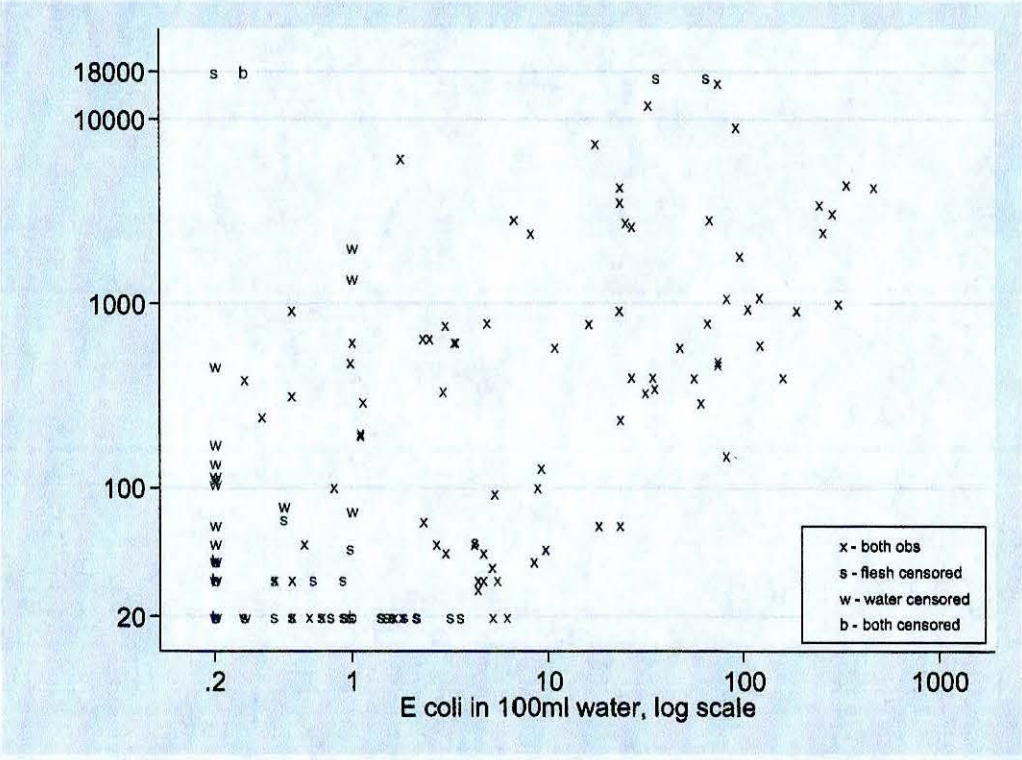


The Bland-Altman plot shows the ratios of the two enumerations against their averages. The horizontal line is drawn at the mean difference, and at the limits of agreement, which are defined as the mean difference plus and minus the standard deviation of the differences. Lines for 95% confidence interval of mean of differences are also shown.

Appendix V
Interpretation of the effect of censoring at LoD on the microcosm *E. coli* data.

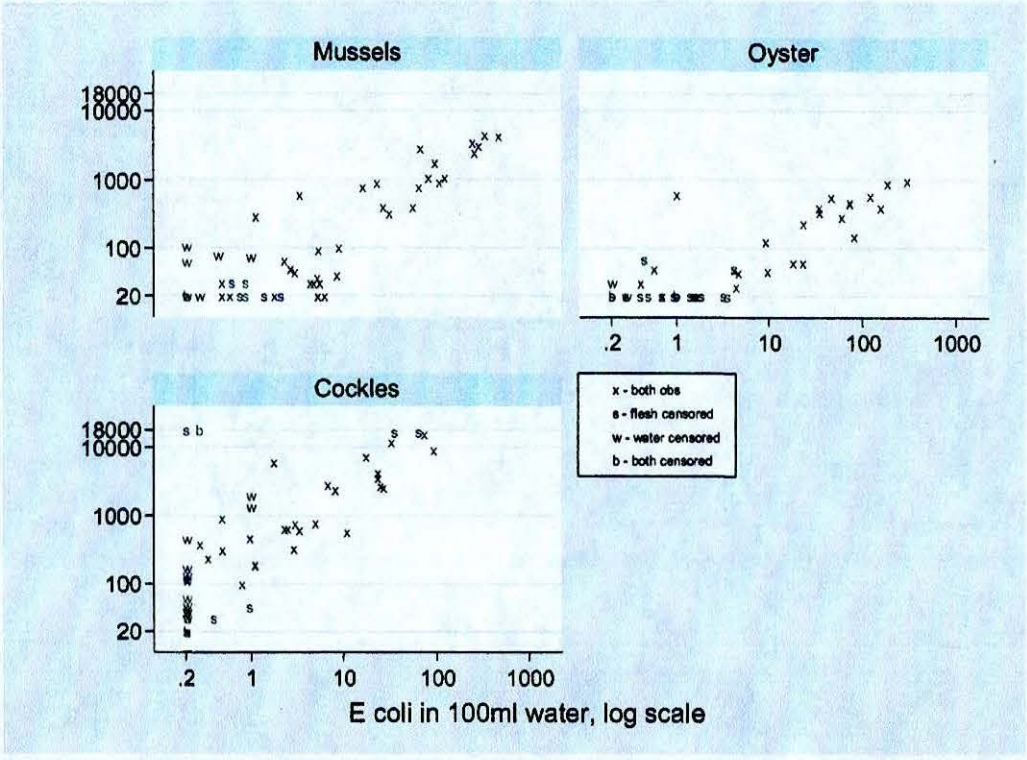
Newton and Rudel (2007) tested various correlation estimators using simulated data and compared these results with real laboratory data. For these real data, the authors noted that, because they do not know the true correlation for the pairs of data, their analysis was limited to considerations of (a) consistency among measures; (b) comparison with plotted data and (c) expected correlations based on knowledge about major sources of exposure. For similar reasons, a qualitative interpretation of the effects of censoring in the microcosm data was undertaken.

Overall, the levels of censoring for individual *E. coli* results were 152/396 (38%) in water samples and 144/395 (36%) in shellfish flesh samples. When paired replicate results were averaged and water and flesh *E. coli* results matched by the tank and closer sampling time proximity, only 87 of 198 pairs involved no censored *E. coli* result (56% censored). The vast majority were censored at the lower limit of detection (LoD) and 6 results were censored as >18,000 *E. coli* MPN/100g. A scatterplot of these 198 results shows a positive correlation between them and rows of results at the LoD. Although using only *E. coli* results where both water and shellfish flesh were measured would restrict the data range, there is no evidence that a correlation based on "*E. coli* detects only" would be biased in either direction.



Scatterplot of all paired *E. coli* results obtained in the microcosm experiments highlighting censored data.

The dataset was split into the three experiments with different species. In the figure below, it is clear that oysters show lower flesh values than the other species at comparable water exposure levels, and gravitate to the LoD in the flesh values. Cockle results are the opposite, and retain measurable numbers of bacteria in the flesh even when the water values are at LoD. Mussels are intermediate, giving rise to more LoD values on either variable. Using the assumption that the distributions and relationships continue past the LoD barrier for both variables, the relationship was estimated using Tobit regression. This technique gives unbiased estimates of the assumed directional link, i.e. that *E. coli* in flesh is the outcome of exposure to water bacteria.



Scatterplot of paired *E. coli* results obtained in the microcosm experiments in each species tested highlighting censored data.

The inherent variability of the MPN enumerations introduces a standard deviation of about 0.5 $\log_{10}$ to each enumeration. For the purposes of this study, the pragmatic approach adopted was to divide each recorded <LoD value by 1+No of <LoD values in the sample, where each sample comprises the enumeration in the same matrix taken within less than an hour, treating split samples as independent. Thus, if three shellfish flesh samples were each analysed as two replicates, and 4 of the measurements were recorded as <20, each <20 was replaced by 20/5 and then averaged on the logarithmic scale with the two other values (=$\log_{10}$ geometric mean).

APPENDIX VI

DESCRIPTIVE STATISTICS FOR FAECAL COLIFORMS AND *E. COLI* RESULTS IN SHELLFISH FLESH AND TANK WATER.

Experiment 1 (mussels): Water target = 1cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.92	2	10	0.0000	10	10
17.133	2	20	0.0000	20	20
28.967	2	20	0.0000	20	20
41	2	14	0.2130	10	20
66.367	2	22	0.4940	10	50
89.583	2	22	0.4940	10	50
112.58	2	14	0.2130	10	20
185.38	2	14	0.2130	10	20
196.93	2	10	0.0000	10	10
208.97	2	10	0.0000	10	10
232.75	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.53	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
16.658	2	0.5	0.1245	0.4	0.6	0.5	0.1245	0.4	0.6
28.508	2	0.6	0.0000	0.6	0.6	0.7	0.0883	0.6	0.8
40.342	2	0.8	0.1570	0.6	1.0	1.0	0.0000	1.0	1.0
65.45	2	0.9	0.0685	0.8	1.0	1.2	0.1033	1.0	1.4
88.525	2	0.6	0.2810	0.4	1.0	0.6	0.2810	0.4	1.0
112.52	2	1.4	0.0000	1.4	1.4	1.5	0.0410	1.4	1.6
184.71	2	0.9	0.0685	0.8	1.0	1.0	0.0000	1.0	1.0
196.67	2	0.1	0.2130	0.1	0.2	0.2	0.0000	0.2	0.2
208.7	2	0.1	0.0000	0.1	0.1	0.1	0.2130	0.1	0.2
232.56	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 1 (mussels): Water target = 3.3cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.83	2	10	0.0000	10	10
17.167	2	14	0.2130	10	20
29.033	2	40	0.4260	20	80
41.083	2	14	0.2130	10	20
66.533	2	32	0.2810	20	50
89.7	2	45	0.0685	40	50
112.73	2	66	0.3110	40	110
185.48	2	14	0.2130	10	20
197.08	2	10	0.0000	10	10
209.05	2	10	0.0000	10	10
232.85	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.49	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
16.742	2	2.1	0.3540	1.2	3.8	2.1	0.3540	1.2	3.8
28.608	2	1.7	0.1095	1.4	2.0	1.8	0.0685	1.6	2.0
40.425	2	2.1	0.1720	1.6	2.8	2.2	0.1930	1.6	3.0
65.525	2	4.4	0.0279	4.2	4.6	4.4	0.0279	4.2	4.6
88.592	2	3.0	0.0410	2.8	3.2	3.3	0.0186	3.2	3.4
112.62	2	2.3	0.0267	2.2	2.4	2.6	0.0952	2.2	3.0
184.79	2	1.8	0.0685	1.6	2.0	1.9	0.0324	1.8	2.0
196.79	2	0.1	0.0000	0.1	0.1	0.2	0.4260	0.1	0.4
208.82	2	0.1	0.2130	0.1	0.2	0.1	0.2130	0.1	0.2
232.66	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 1 (mussels): Water target = 10cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.75	2	10	0.0000	10	10
17.217	2	32	0.2810	20	50
29.117	2	102	0.1490	80	130
41.167	2	32	0.2810	20	50
66.583	2	50	0.0000	50	50
88.85	2	37	0.3850	20	70
112.83	2	14	0.2130	10	20
185.57	2	20	0.0000	20	20
197.17	2	20	0.0000	20	20
209.13	2	10	0.0000	10	10
232.93	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.36	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.10	0.10
16.825	2	4.7	0.0131	4.6	4.8	4.7	0.0131	4.60	4.80
28.675	2	8.1	0.1860	6.0	11.0	9.2	0.1660	7.00	11.99
40.525	2	5.5	0.1109	4.6	6.6	6.2	0.0397	5.80	6.60
65.625	2	2.7	0.1810	2.0	3.6	3.0	0.1840	2.20	4.00
88.717	2	5.2	0.0473	4.8	5.6	5.5	0.0786	4.80	6.20
112.76	2	6.2	0.0198	6.0	6.4	6.2	0.0198	6.00	6.40
184.92	2	5.2	0.1053	4.4	6.2	5.2	0.1053	4.40	6.20
197.12	2	0.1	0.0000	0.1	0.1	0.2	0.5500	0.10	0.60
208.94	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.10	0.10
232.72	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.10	0.10

Experiment 1 (mussels): Water target = 33cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.60	2	10	0.0000	10	10
17.25	2	402	0.1214	330	490
29.15	2	932	0.1017	790	1100
41.25	2	330	0.0000	330	330
66.65	2	275	0.1109	230	330
89.98	2	20	0.0000	20	20
112.98	2	20	0.0000	20	20
185.65	2	94	0.0978	80	110
197.28	2	20	0.0000	20	20
209.23	2	14	0.2130	10	20
233.02	2	14	0.2130	10	20

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.24	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
16.875	2	26.3	0.1388	21.0	33.0	29.3	0.0732	26.0	33.0
28.725	2	22.9	0.0535	21.0	25.0	25.5	0.1196	21.0	31.0
40.625	2	31.0	0.0000	31.0	31.0	31.0	0.0000	31.0	31.0
65.825	2	1.1	0.2130	0.8	1.6	1.7	0.0362	1.6	1.8
89	2	0.5	0.1245	0.4	0.6	0.6	0.2130	0.4	0.8
112.86	2	0.2	0.4260	0.1	0.4	0.2	0.4260	0.1	0.4
185.01	2	5.3	0.0917	4.6	6.2	5.9	0.0824	5.2	6.8
196.97	2	0.1	0.0000	0.1	0.1	0.1	0.2130	0.1	0.2
209.07	2	0.1	0.2130	0.1	0.2	0.1	0.2130	0.1	0.2
232.89	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 1 (mussels): Water target = 100cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.6	2	10	0.0000	10	10
17.3	2	1091	0.2720	700	1698
29.233	2	955	0.1900	700	1300
41.35	2	1100	0.0000	1100	1100
66.733	2	1832	0.1660	1400	2399
90.167	2	798	0.3000	490	1300
113.13	2	401	0.3420	230	700
185.72	2	622	0.1470	490	791
197.37	2	14	0.2130	10	20
209.3	2	63	0.1440	50	80
233.1	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.16	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
16.925	2	81.3	0.1570	63.0	105.0	81.3	0.1570	63.0	105.0
28.792	2	104.6	0.0790	92.0	119.0	104.6	0.0790	92.0	119.0
40.808	2	120.0	0.0051	119.0	121.0	120.0	0.0051	119.0	121.0
65.942	2	94.7	0.0453	88.0	102.0	100.0	0.0123	98.0	102.0
89.125	2	64.5	0.0760	57.0	73.0	64.5	0.0760	57.0	73.0
112.98	2	55.0	0.0112	54.0	56.0	55.0	0.0112	54.0	56.0
185.11	2	3.4	0.0726	3.0	3.8	16.9	0.0726	15.0	19.0
196.86	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
209.17	2	0.1	0.0000	0.1	0.1	0.5	0.1245	0.4	0.6
233.01	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 1 (mussels): Water target = 330cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-29.55	2	10	0.0000	10	10
17.333	2	2482	0.0741	2200	2800
29.3	2	4498	0.4390	2198	9204
41.433	2	2898	0.1159	2400	3500
66.783	2	4347	0.1332	3500	5400
90.333	2	3130	0.0685	2800	3500
113.27	2	3500	0.0000	3500	3500
186	2	790	0.0000	790	790
197.43	2	106	0.1720	80	140
209.07	2	75	0.0410	70	80
233.2	2	80	0.0000	80	80

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-30.08	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
17.008	2	253.9	0.1225	208.0	310.0	283.9	0.0540	260.0	310.0
28.675	2	333.0	0.0000	333.0	333.0	370.0	0.0000	370.0	370.0
41.092	2	66.0	0.0000	66.0	66.0	66.0	0.0000	66.0	66.0
66.075	2	459.9	0.0134	450.0	470.0	484.7	0.0190	470.0	500.0
89.242	2	282.0	0.1147	234.0	340.0	297.3	0.0824	260.0	340.0
113.07	2	242.5	0.0883	210.0	280.0	242.5	0.0883	210.0	280.0
185.17	2	79.9	0.0994	68.0	94.0	79.9	0.0994	68.0	94.0
197.24	2	1.0	0.0000	1.0	1.0	2.0	0.0000	2.0	2.0
209.28	2	1.0	0.0000	1.0	1.0	1.4	0.2130	1.0	2.0
233.06	2	0.5	0.0000	0.5	0.5	0.5	0.0000	0.5	0.5

Experiment 2 (Pacific oysters): Water target = 1 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.95	2	10	0.0000	10	10
10.25	2	10	0.0000	10	10
21.72	2	10	0.0000	10	10
45.55	2	10	0.0000	10	10
69.62	2	14	0.2130	10	20
93.57	2	10	0.0000	10	10
165.8	2	10	0.0000	10	10
190.8	2	20	0.0000	20	20
201.9	2	10	0.0000	10	10
213.9	2	10	0.0000	10	10
237.8	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
2.425	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.675	2	0.3	0.2130	0.2	0.4	0.3	0.2130	0.2	0.4
21.43	2	0.3	0.2130	0.2	0.4	0.3	0.2130	0.2	0.4
45.34	2	0.7	0.3370	0.4	1.2	0.7	0.3370	0.4	1.2
69.44	2	0.7	0.3370	0.4	1.2	0.7	0.3370	0.4	1.2
93.18	2	0.5	0.1245	0.4	0.6	0.6	0.2130	0.4	0.8
165.1	2	0.4	0.4260	0.2	0.8	0.6	0.2130	0.4	0.8
190.4	2	0.2	0.4260	0.1	0.4	0.3	0.3370	0.2	0.6
201.6	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
213.5	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.5	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 2 (Pacific oysters): Water target = 3.3 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.833	2	10	0.0000	10	10
10.383	2	48	0.9630	10	230
21.75	2	10	0.0000	10	10
45.633	2	14	0.2130	10	20
69.683	2	14	0.2130	10	20
93.583	2	20	0.0000	20	20
165.75	2	14	0.2130	10	20
190.87	2	20	0.0000	20	20
201.87	2	10	0.0000	10	10
214.02	2	10	0.0000	10	10
237.95	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-2.375	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.7417	2	0.4	0.4940	0.2	1.0	0.5	0.5500	0.2	1.2
21.475	2	0.9	0.2600	0.6	1.4	0.9	0.2600	0.6	1.4
45.392	2	1.4	0.0883	1.2	1.6	1.7	0.0362	1.6	1.8
69.508	2	1.5	0.0410	1.4	1.6	1.9	0.1900	1.4	2.6
93.225	2	1.6	0.1860	1.2	2.2	1.8	0.1660	1.4	2.4
165.18	2	0.3	0.2130	0.2	0.4	0.3	0.2130	0.2	0.4
190.44	2	0.7	0.0883	0.6	0.8	1.2	0.0000	1.2	1.2
201.66	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
213.58	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.56	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 2 (Pacific oysters): Water target = 10 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.783	2	14	0.2130	10	20
10.483	2	45	0.0685	40	50
21.867	2	14	0.2130	10	20
45.717	2	28	0.2130	20	40
69.767	2	14	0.2130	10	20
93.667	2	36	0.7880	10	130
165.43	2	14	0.2130	10	20
190.93	2	14	0.2130	10	20
201.93	2	10	0.0000	10	10
214	2	10	0.0000	10	10
237.98	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-2.308	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.8083	2	4.7	0.0393	4.4	5.0	4.7	0.0393	4.4	5.0
21.508	2	3.2	0.0772	2.8	3.6	3.5	0.0883	3.0	4.0
45.475	2	4.4	0.0279	4.2	4.6	4.9	0.0991	4.2	5.8
69.558	2	3.6	0.0685	3.2	4.0	3.8	0.0324	3.6	4.0
93.292	2	4.3	0.0717	3.8	4.8	4.3	0.0717	3.8	4.8
165.24	2	1.8	0.1660	1.4	2.4	1.8	0.1660	1.4	2.4
190.49	2	1.0	0.1245	0.8	1.2	2.1	0.0293	2.0	2.2
201.73	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
213.64	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.64	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 2 (Pacific oysters): Water target = 33cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.733	2	10	0.0000	10	10
10.583	2	152	0.4760	70	330
21.967	2	63	0.1440	50	80
45.8	2	63	0.1440	50	80
69.85	2	14	0.2130	10	20
93.75	2	10	0.0000	10	10
165.5	2	32	0.2810	20	50
190.93	2	10	0.0000	10	10
202	2	14	0.2130	10	20
214.07	2	10	0.0000	10	10
238.03	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-2.275	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.9583	2	80.9	0.0380	76.0	86.0	80.9	0.0380	76.0	86.0
21.558	2	23.0	0.0267	22.0	24.0	24.0	0.0000	24.0	24.0
45.542	2	18.0	0.0342	17.0	19.0	18.0	0.0342	17.0	19.0
69.625	2	0.5	0.0000	0.5	0.5	0.5	0.0000	0.5	0.5
93.358	2	1.6	0.0772	1.4	1.8	1.6	0.0772	1.4	1.8
165.33	2	0.4	0.0000	0.4	0.4	0.4	0.0000	0.4	0.4
190.54	2	0.2	0.0000	0.2	0.2	0.5	0.1245	0.4	0.6
201.79	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
213.71	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.71	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 2 (Pacific oysters): Water target = 100 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.683	2	10	0.0000	10	10
10.667	2	237	0.2040	170	330
22.017	2	490	0.0000	490	490
45.883	2	402	0.1214	330	490
69.933	2	586	0.1095	490	700
93.85	2	50	0.0000	50	50
165.58	2	47	0.5240	20	110
191	2	130	0.0000	130	130
202.05	2	50	0.0000	50	50
214.12	2	32	0.2810	20	50
238.07	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-2.225	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.8917	2	23.2	0.0922	20.0	27.0	23.2	0.0922	20.0	27.0
21.608	2	73.0	0.0713	65.0	82.0	73.0	0.0713	65.0	82.0
45.608	2	15.1	0.2300	10.4	22.0	16.9	0.1620	13.0	22.0
69.708	2	46.7	0.0066	46.2	47.2	46.7	0.0066	46.2	47.2
93.442	2	4.2	0.0293	4.0	4.4	4.2	0.0293	4.0	4.4
165.43	2	9.6	0.1271	7.8	11.8	9.6	0.1271	7.8	11.8
190.63	2	9.2	0.0999	7.8	10.8	11.5	0.0961	9.8	13.4
201.86	2	0.6	0.2130	0.4	0.8	0.6	0.2130	0.4	0.8
213.79	2	0.1	0.2130	0.1	0.2	0.1	0.2130	0.1	0.2
237.76	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 2 (Pacific oysters): Water target = 330 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-1.633	2	10	0.0000	10	10
10.783	2	932	0.1017	790	1100
22.083	2	1014	0.1530	791	1300
45.95	2	293	0.0732	260	330
70	2	603	0.1660	460	791
93.883	2	402	0.1214	330	490
165.7	2	470	0.6250	170	1300
191.07	2	350	0.2070	250	490
202.1	2	622	0.1470	490	791
214.17	2	20	0.0000	20	20
238.13	2	14	0.2130	10	20

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-2.192	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
10.042	2	184.9	0.0166	180.0	190.0	184.9	0.0166	180.0	190.0
21.708	2	303.0	0.0061	300.0	306.0	319.4	0.0384	300.0	340.0
45.692	2	59.5	0.0824	52.0	68.0	59.5	0.0824	52.0	68.0
69.775	2	121.0	0.1235	99.0	148.0	127.6	0.0911	110.0	148.0
93.508	2	157.8	0.1142	131.0	190.0	166.6	0.0809	146.0	190.0
165.51	2	73.0	0.0084	72.0	74.0	73.0	0.0084	72.0	74.0
190.69	2	34.5	0.1063	29.0	41.0	57.1	0.1070	48.0	68.0
201.94	2	1.0	0.0000	1.0	1.0	1.0	0.0000	1.0	1.0
213.88	2	0.5	0.0000	0.5	0.5	0.5	0.0000	0.5	0.5
237.83	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 1 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-0.45	2	10	0.0000	10	10
8.95	2	14	0.2130	10	20
20.45	2	28	0.6390	10	80
45.33	2	40	0.4260	20	80
69.93	2	22	0.4940	10	50
94.25	2	112	0.1357	90	140
165.1	2	36000	0.0000	36000	36000
189	2	20	0.0000	20	20
201.4	2	40	0.4260	20	80
213.3	2	10	0.0000	10	10
237.1	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
3.375	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
8.725	2	0.2	0.0000	0.2	0.2	0.2	0.0000	0.2	0.2
20.43	2	0.1	0.2130	0.1	0.2	0.1	0.2130	0.1	0.2
44.94	2	0.2	0.0000	0.2	0.2	0.2	0.0000	0.2	0.2
69.34	2	0.3	0.2130	0.2	0.4	0.6	0.2130	0.4	0.8
94.19	2	0.2	0.0000	0.2	0.2	0.2	0.0000	0.2	0.2
164.8	2	0.2	0.4260	0.1	0.4	0.2	0.4260	0.1	0.4
188.6	2	0.2	0.0000	0.2	0.2	0.2	0.0000	0.2	0.2
201	2	0.1	0.0000	0.1	0.1	0.1	0.2130	0.1	0.2
212.9	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 3.3 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-0.4	2	14	0.2130	10	20
9.2	2	102	0.1490	80	130
20.6	2	33	0.7360	10	110
45.48	2	193	0.0792	170	220
70.15	2	318	0.2270	220	460
94.43	2	481	0.2310	330	700
165.3	2	244	0.3880	130	460
189.1	2	199	0.8480	50	791
201.6	2	14	0.2130	10	20
213.3	2	63	0.1440	50	80
237.3	2	14	0.2130	10	20

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
3.325	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
8.825	2	0.8	0.0000	0.8	0.8	1.0	0.1245	0.8	1.2
20.48	2	1.0	0.3010	0.6	1.6	1.0	0.3010	0.6	1.6
44.99	2	1.1	0.0560	1.0	1.2	1.1	0.0560	1.0	1.2
69.06	2	0.5	0.1245	0.4	0.6	0.5	0.1245	0.4	0.6
94.29	2	1.0	0.3010	0.6	1.6	1.0	0.3370	0.6	1.8
164.9	2	0.3	0.3370	0.2	0.6	0.3	0.3370	0.2	0.6
188.6	2	1.1	0.0560	1.0	1.2	1.1	0.0560	1.0	1.2
201.1	2	0.1	0.0000	0.1	0.1	0.2	0.0000	0.2	0.2
213	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.1	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 10 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-0.35	2	10	0.0000	10	10
9.7	2	336	0.2320	230	490
20.717	2	622	0.1470	490	791
45.667	2	655	0.4210	330	1300
70.333	2	773	0.3190	460	1300
94.633	2	655	0.4210	330	1300
165.43	2	6194	0.5830	2399	15996
189.2	2	137	0.1337	110	170
201.68	2	173	0.1750	130	230
213.5	2	32	0.2810	20	50
237.52	2	10	0.0000	10	10

		Tank Water *E. coli* cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-3.258	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
8.9083	2	2.9	0.0212	2.8	3.0	3.1	0.0198	3.0	3.2
20.525	2	3.3	0.0186	3.2	3.4	3.3	0.0186	3.2	3.4
45.058	2	2.5	0.0741	2.2	2.8	2.8	0.0000	2.8	2.8
69.108	2	3.0	0.0410	2.8	3.2	3.0	0.0410	2.8	3.2
94.358	2	2.3	0.0806	2.0	2.6	2.4	0.1033	2.0	2.8
164.91	2	1.8	0.1388	1.4	2.2	2.1	0.0883	1.8	2.4
188.69	2	0.1	0.0000	0.1	0.1	0.1	0.2130	0.1	0.2
201.11	2	0.1	0.0000	0.1	0.1	0.2	0.0000	0.2	0.2
213.04	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.09	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 33 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-					
0.3167	2	10	0.0000	10	10
9.4	2	586	0.1095	490	700
20.85	2	2438	0.2220	1698	3499
45.85	2	798	0.3000	490	1300
70.533	2	390	0.1020	330	460
94.75	2	14	0.2130	10	20
165.22	2	36000	0.0000	36000	36000
189.32	2	32	0.2810	20	50
201.7	2	10	0.0000	10	10
213.5	2	14	0.2130	10	20
237.5	2	10	0.0000	10	10

		Tank Water E. coli cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
3.1917	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
8.9583	2	10.7	0.0057	10.6	10.8	11.3	0.0272	10.8	11.8
20.675	2	8.1	0.0228	7.8	8.4	8.1	0.0228	7.8	8.4
45.125	2	4.9	0.0125	4.8	5.0	4.9	0.0125	4.8	5.0
69.175	2	0.3	0.2130	0.2	0.4	0.3	0.2130	0.2	0.4
94.458	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
164.98	2	0.2	0.0000	0.2	0.2	0.2	0.0000	0.2	0.2
188.78	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
201.16	2	0.1	0.0000	0.1	0.1	0.2	0.0000	0.2	0.2
213.11	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.14	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 100 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-					
0.2833	2	10	0.0000	10	10
9.55	2	2649	0.4370	1300	5395
20.833	2	3597	0.2490	2399	5395
46.033	2	4347	0.1332	3500	5400
70.667	2	2898	0.1159	2400	3500
94.8	2	932	0.1017	790	1100
165.3	2	460	0.0000	460	460
189.48	2	28	0.6390	10	80
201.83	2	50	0.0000	50	50
213.67	2	10	0.0000	10	10
237.68	2	22	0.4940	10	50

		Tank Water E. coli cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
3.0917	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.1083	2	26.5	0.2050	19.0	37.0	26.5	0.2050	19.0	37.0
20.742	2	23.1	0.1191	19.0	28.0	24.2	0.0883	21.0	28.0
45.192	2	23.1	0.1191	19.0	28.0	25.5	0.1196	21.0	31.0
69.258	2	6.7	0.1810	5.0	9.0	6.7	0.1810	5.0	9.0
94.525	2	0.5	0.1245	0.4	0.6	0.6	0.2130	0.4	0.8
165.09	2	0.1	0.2130	0.1	0.2	0.1	0.2130	0.1	0.2
188.84	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
201.23	2	0.1	0.0000	0.1	0.1	0.2	0.0000	0.2	0.2
213.18	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
237.23	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

Experiment 3 (cockles): Water target = 330 cfu/100ml

		Shellfish MPN/100g FIL			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum
-					
0.2333	2	10	0.0000	10	10
9.75	2	15999	0.0000	15999	15999
21	2	9200	0.0000	9200	9200
46.183	2	23988	0.2490	15996	35975
70.85	2	23988	0.2490	15996	35975
94.833	2	12134	0.1700	9204	15996
165.38	2	7482	0.4670	3499	15996
189.33	2	2773	0.1430	2198	3499
201.92	2	2020	0.1059	1700	2400
213.68	2	1377	0.3410	791	2399
237.68	2	117	0.2310	80	170

		Tank Water E. coli cfu/100ml				Tank Water Faecal coliforms cfu/100ml			
Time	N	Geometric Mean	Standard Deviation	Minimum	Maximum	Geometric Mean	Standard Deviation	Minimum	Maximum
-									
3.0083	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1
9.175	2	73.1	0.1420	57.9	92.0	81.4	0.0753	72.0	92.0
20.825	2	90.9	0.0708	81.0	102.0	95.8	0.0384	90.0	102.0
45.258	2	64.1	0.1049	54.0	76.0	67.3	0.1357	54.0	84.0
68.975	2	35.5	0.1033	30.0	42.0	35.5	0.1033	30.0	42.0
94.592	2	32.2	0.1323	26.0	40.0	35.8	0.0685	32.0	40.0
165.18	2	17.3	0.0883	15.0	20.0	17.3	0.0883	15.0	20.0
188.91	2	24.5	0.0125	24.0	25.0	24.5	0.0125	24.0	25.0
201.31	2	0.5	0.0000	0.5	0.5	1.0	0.0000	1.0	1.0
213.24	2	0.7	0.2130	0.5	1.0	0.7	0.2130	0.5	1.0
237.28	2	0.1	0.0000	0.1	0.1	0.1	0.0000	0.1	0.1

APPENDIX VII

RESULTS OF ANALYSIS OF VARIANCE TESTING FOR DIFFERENCES IN THE ACCUMULATION RATES BETWEEN SPECIES.

Analysis of variance assuming equal variance (as indicated by the significance of the Levene homogeneity of variance statistic value which is >0.05), thus, using Tukey HSD as pairwise comparison test for group means. This indicates no significant difference between species and water concentration.

Dependent Variable: $\log_{10}$ *E. coli*

	(I) Matrix	(J) Matrix	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Equal Variance is evident from Levene's statistic, hence the appropriate ANOVA test statistic is Tukey HSD which is used here	Water	Mussels	-1.63929*	.12418	.000	-1.9693	-1.3093
		Oysters	-1.44929*	.12418	.000	-1.7793	-1.1193
		Cockles	-1.77988*	.12925	.000	-2.1234	-1.4364
	Mussels	Water	1.63929*	.12418	.000	1.3093	1.9693
		Oysters	.19000	.12418	.428	-.1400	.5200
		Cockles	-.14060	.12925	.699	-.4841	.2029
	Oysters	Water	1.44929*	.12418	.000	1.1193	1.7793
		Mussels	-.19000	.12418	.428	-.5200	.1400
		Cockles	-.33060	.12925	.063	-.6741	.0129
	Cockles	Water	1.77988*	.12925	.000	1.4364	2.1234
		Mussels	.14060	.12925	.699	-.2029	.4841
		Oysters	.33060	.12925	.063	-.0129	.6741

*. The mean difference is significant at the 0.05 level.

Test of Homogeneity of Variances

Log_{10} *E. coli*

Levene Statistic	df1	df2	Sig.
1.971	3	50	.130