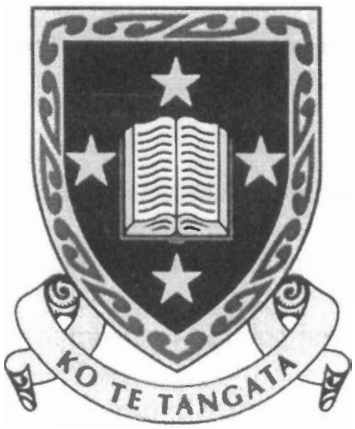
Freshwater Mussels as Bioindicators of Faecal Contamination



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Abstract

Mussels (*Hyridella menziesi)* were tested for their suitability as monitors of bacterial contamination of natural waters because of their ability to accumulate bacteria while filtering the environment for food. Several questions were addressed :

1. Can *E. coli* be recovered from mussels immersed in low-density suspensions of bacteria.
2. What is the optimum time required to immerse mussels in suspension of bacteria.
3. How long will the mussels retain bacteria once uptake had occurred.
4. Is it possible to estimate the numbers of bacteria present in contaminated waters, Using the numbers of bacteria recovered from mussels.

The Results of this study were :

1. It was possible to detect *E. coli* and *Salmonella* in mussels that had been immersed in water contaminated with these bacteria at densities as low as around 50 per 100 ml.

2. An experiment conducted to examine the ability of mussels to retain bacteria once uptake had occurred, showed there was rapid loss of *E. coli* (42.9%) over the first 4 hours and by 6 hours numbers had declined to very low levels (2.1%).

3. Although there was some evidence of a correlation between the concentration of *E. coli* present in surrounding waters and the numbers recovered from mussels it was not possible to predict the numbers of bacteria in the surrounding water from analysis of the mussels.

4. Similar experiments were done using diluted sewage instead of pure cultures of *E. coli* and *Salmonella.* Results were similar to those obtained using pure cultures. *E. coli* could be recovered from mussels when present in the diluted sewage at concentrations as low as 8 / ml.

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Chapter 1

Introduction

Human activities often introduce widespread contamination of many environments, including aquatic environments. Faecal contamination of natural waters may occur as a result of the discharge of sewage and animal processing wastes, and also at non-point sources due to runoff after rainfall. The present methods used to sample natural waters, collecting and analysing "grab" samples, may give misleading results that represent a particlar instant in time when the sample was taken. The development of a method that would give results more representative of the actual faecal contamination that is occurring in natural waterways would be useful.

Three methods may be used to quantitate the pollutants in aquatic systems. By studying the water itself, the sediment or the indigenous biota. The aim of this study is to use the latter, mussels (*Hyridella menziesi)*, to determine their suitability as biomonitors of faecal pollution.

Mussels filter large quantities of water while feeding and in this process accumulate microorganisms such as faecal coliforms and pathogenic bacteria that

may also be in the water. Analysis of mussels from faecally contaminated sites, should contain significant numbers of *E. coli,* confirming that a contamination event has occurred.

Because of the large volumes of water filtered, the mussels serve as the equivalent of filtering very large volumes of water through a membrane filter and thus may be able to detect low population densities of contaminating bacteria. In addition, the mussels filter water over long period of time, whereas samples of water taken for analysis are representative only of the moment in time at which the sample was taken.

Mussels have been analysed for their accumulation of eg chlorinated compounds Hickey (1997) and other studies have involved the examination of mussel tissue for bacteria and viruses (Collins and Power, 1997) and (Abad *et al*, 1990). Those studies were done in order to a) measure bioaccumulation of toxic contaminants or b) to look at effective ways of removing microbial contamination from mussels before consumption.

Little study has been done of the use of mussels as concentrators of indicator organisms for the monitoring of faecal contamination. The experiments described in this Thesis were designed with a view looking at mussels as potential monitors of bacteria contamination in freshwater.

Chapter 2

Literature Review

2.1 Introduction

The object of this research was to look at the possibility of using freshwater mussels as monitors of bacterial contamination of natural waters. In the event of filtering food from their environment, they bioaccumulate any pathogens that happen to be in the water suggesting they would be suitable biomonitors of faecal contamination.

The literature on water quality, microbial contamination, coliforms and other pathogens is very extensive. Consequently this Literature Review is selective and deals with water quality, microbial contamination and its monitoring and also a little on the *Hyridella menziesi.*

Microbial faecal contamination of freshwater environments is a concern because of the possible presence of pathogens. Faecal indicator bacteria are used to determine faecal contamination and thus the possible presence of these pathogens. However, methods for the detection of faecal indicator bacteria do not distinguish

from human or animal sources of contamination. Methods are required to determine where the contamination is coming from. If the source can be identified, then the problem can be managed effectively. The following Literature Review will highlight some of the current issues that face Water Quality Managers and will briefly discuss the microorganisms involved.

2.2 Water Quality and the Resource Management Act.

Principles and standards for the control of water quality are defined in the Resource Management Act (RMA) 1991. Most of the standards specified in the Act are narrative; that is, they are written in a way that emphasises the intent of the legislation, without specifying numerical criteria.

Section 30 of the RMA states that the functions of regional councils include the control of discharges of contaminants into water, and the management of land for the purpose of maintaining and enhancing the quality of water in waterbodies.

Two sections of the Act specify water quality standards. Section 70 (Rules about discharges) and 107 (Restrictions on grant of certain discharge permits), include standards relating to discharges acceptable levels of water or contaminants into receiving waters (after reasonable mixing). The Third Schedule of the Act lists water quality classes for the purposes of water body classification (section 69 Rules relating to water quality).

2.3 Sources of Water Contamination

Human activities have resulted in widespread contamination of many environments, including aquatic environments (Tanabe *et al*, 1994). Sources of microbial contamination come from inputs of faecal wastes at both point sources of discharge of sewage and animal processing wastes, and also at non-point

sources due to runoff after rainfall. Following natural drainage patterns or sewers, liquid waste discharges eventually enter natural bodies of water, such as groundwater, rivers, lakes, and oceans. These same water bodies are used by people in different ways, such as sources for drinking, household, industrial, and irrigation water, for fish and shellfish production and for recreational use. It is therefore important that the discharge of faecal contaminants into water be kept to minimal levels to prevent waterways from becoming vehicles of disease transmission.

Table 2.1 lists coliform concentrations in selected effluents. The data are from Close *et al* (1998) and Tipler and Borrie (1997).

Effluent Sample	Total Coliforms per 100 mL	Faecal Coliforms per 100 mL
Dairy shed	2,600,000	1,500,000
Chicken shed	470,000,000	240,000,000
Septic tank	15,000,000	7,300,000
Oxidation pond	Not analysed	2,500,000
Meat processing plant	Not analysed	2,0000000

Table 2.1. Coliform concentrations in selected effluents.

Apart from the effluent from the chicken shed, it can be seen (Table 2.1) that the discharges from a number of operations contain faecal coliforms at concentrations of a few million per 100 ml.

2.3.1 Runoff

Large numbers of sheep and cattle are farmed pastorally in New Zealand. Approximately 6000 dairy farms exist in the Waikato region, all having some form of effluent collection, treatment and disposal system. According to Environment Waikato Regional Council, (1994) the average herd size in the Waikato region is about 200 cows; an average dairy unit produces about 10 cubic metres per day of effluent, thus the total discharge of dairy shed effluent in the region is approximately 60,000 cubic metres per day. An estimated 70% of this effluent is discharged to surface water, with 30% discharged to land. 92% of the total dairy effluent is deposited on the pasture in the vicinity of the dairy shed. The runoff of this waste could have a major impact on the local water quality (surface and ground water), especially in the event that the effluent has not been adequately treated. When grazing cattle have access to a stream as their water source, they deposit a portion of their daily faecal matter directly into the stream (Biskie *et al*., 1988).

The runoff from these pastures results in faecal contamination of surface waters, this has recently become of concern in efforts to reduce pollution of surface waters. Several studies have shown that high levels of total coliforms are contained in agricultural runoff, regardless of whether the land has been grazed with subsequent contamination by either animal faecal materials (Doran and Linn, 1979; Robbins et al, 1971; McCaskey et al., 1971; Kunkle, 1979) or to the build up of stable bacterial populations in the soil (Smallbeck and Bromel, 1975; Faust, 1982). According to Gary and Adams, (1985); Sherer et al., (1988); and

Stephenson and Street, (1978), long term sampling has revealed that the presence of enteric bacteria persist in streams, even after the animals have been removed. There may also be bacterial contamination in runoff from land due to contamination by wild animals (wild goats and deer) (Schepers and Doran, 1980).

Effluent is applied to land as levels of microbial content and organic content are too high to permit direct discharge into waterways. Once animal manure is applied to the land it becomes a potential non-point source of pollution. The potential for excess bacterial pollution resulting from runoff from land used for effluent application has been shown by Janzen, (1974); Robbins et al, (1971); McCaskey et al, (1971). According to Robbins et al (1971), 2 to 23 % of the faecal coliforms deposited on fields by manure application or defecated directly by animals were lost in runoff from these areas on an annual basis (see Table 2.2).

Animal Type	Total Coliforms	Faecal Coliforms	Refernce
Dairy Cow	8.7 x 10^5/g	8.5 x 10^5/g	McCoy (1967)
Cattle manure	3.4-5.6 x x 10^5/g	3.2-5.3 x 10^5	Witzel *et al.* (1966)
Cattle feedlot waste	5 x 10^7/gdw	4.5 x 10^7/gdw	Hrubant *et al.* (1972)
Cow (r.w.) @17.9%TS	1.0 x 10^7/gdw	9.7 x 10^5gdw	Jones (1971)
Sheep		1.6 x 10^7/g	Geldreich *et al.* (1962)
Horse		1.26 x 10^4/g	Geldreich (1978)
Dog		2.3 x 10^7/g	Geldreich (1978)
Cat		7.9 x 10^6/g	Geldreich (1978)
Rabbit		20/g	Pavlova *et al.* (1972)
Chicken		1.3 x 10^6/g	Geldreich *et al.* (1962)
Duck		3.3 x 10^7/g	Geldreich *et al.* (1962)

r.w. = raw waste as collected, may include a short storage period.
%TS = % total solids.

Table 2.2. Bacterial indicator concentrations in animal manure.

Sherer et al (1992) have reported that sediment allows enteric bacteria to survive for months in an aquatic environment rather than the days as typically measured in water.

The results obtained by several researchers confirm that bacterial contamination is greater during high flow periods, even with the higher dilution rates, compared with base flow periods (Dudley and Karr, 1979; Kunkle, 1970; Robbins et al, 1971).

2.3.2 Animal Processing

Sheep and cattle are processed at meat plants. Wastes from these plants may also contaminate surface waters. The potential for environmental contamination through the indiscriminate handling of animal processing wastes is of concern due to the possible transmission of disease. According to Jack and Hepper (1969) salmonellosis mortality was traced to seepage from a slurry tank overflow.

Table 2.1 shows the numbers of coliforms characteristics of effluents. A typical meat processing plant wastewater with an annual through put of approximately 60,000 head of beef and 1,100,000 sheep and lambs contains in the vicinity of 20,000,000 faecal coliforms per ml Tipler and Borrie (1997). The wastewater from this plant (Canterbury Meat Packers Ltd (CMP)) is currently spread onto 305 ha of farmland owned by CMP, adjacent to the plant. The maximum annual quantity of wastewater produced by the processing operations is 1,143,000 m^3. This does not include irrigation and flushing water that may be added. In total there will be up to 1,887,000 m^3/year of water and wastewater applied to land.

According to Ray *et al* (1997) who investigated seepage from dairy farm effluent treatment ponds situated near Matamata, the most significant cause of excessive seepage was substandard pond floor construction (insufficient clay content and/or compaction).

2.4 Waste Treatment

Waste treatment serves to reduce the discharge of microorganisms to acceptable levels and reduce discharge levels of BOD, COD and nitrogen to levels which reduce effects of eutrophication.

2.4.1 Oxidation Ponds

Oxidation ponds are a very common method of treatment for organic wastewaters in New Zealand, (see water quality guideline references), including domestic sewage and animal wastes, e.g. from dairy sheds or piggeries. Effluents from the oxidation pond shown in Table 2.1 contain faecal coliforms present in numbers little different from that of the untreated dairy shed effluent which the pond was designed to treat. The effluent quality of dairy shed and municipal oxidation ponds in New Zealand is highly variable and sometimes poor (see water quality guidelines ref). This is thought to be due to variation in weather to which ponds are subjected. At least a 30 fold dilution of a pond effluent is desirable to avoid optical impacts in streams

.2.4.2 Wastewater Treatment Plants

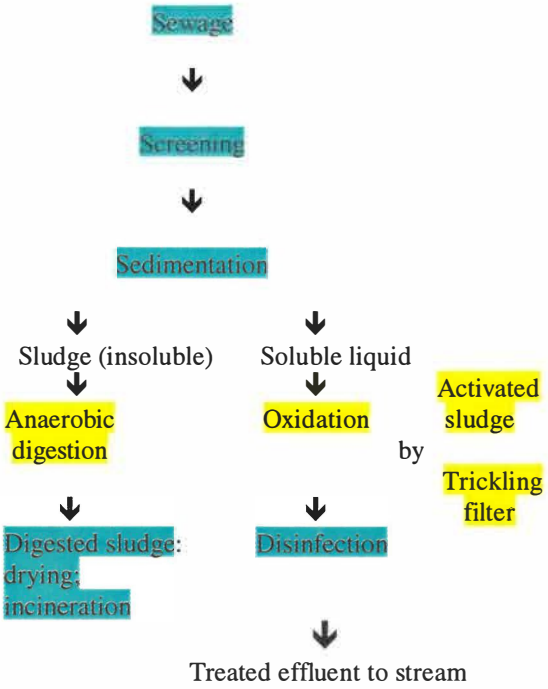

Figure 2.1. An overview of sewage treatment processes (Brock *et al,* 1994).

Wastewaters are derived from domestic sewage or industrial processes, and are too contaminated to discharge untreated into lakes or streams, for reasons of public health and recreational considerations.

Treatments are performed to remove pathogenic and potentially pathogenic microorganisms and also to decrease turbidity, eliminate taste and odour, reduce or eliminate nuisance chemicals such as iron or manganese, and soften water to make it useful for the laundry. See Figure 2.1 for an overview of sewage treatment processes. Sewage treatment is generally a multistep process of a mixture of biological and nonbiological treatment steps. These are discussed below.

Primary treatment

Primary treatment of sewage consists only of the physical removal of large objects and then the effluent is left to settle for a number of hours to allow suspended solids to sediment.

Secondary treatment

Secondary treatment processes reduce the biochemical oxygen demand (BOD) of the sewage to acceptable levels. Anaerobic decomposition is usually employed for the treatment of materials that have much *insoluble* organic matter, or for concentrated industrial wastes. Anaerobic decomposition processes operate semicontinuously in large enclosed tanks called **sludge digestors** or **bioreactors**, into which the untreated material is introduced and treated material is removed at intervals. The most common aerobic composition decomposition processes are the trickling filter and activated sludge methods.

Trickling filters contain a bed of crushed rocks on top of which the liquid containing organic matter is sprayed. The liquid slowly trickles through the bed, the organic matter adsorbs to the rocks, and a biofilm of microbial growth develops. The complete mineralization of organic matter to carbon dioxide, ammonia, nitrate, sulfate, and phosphate occurs.

In the **activated sludge** process, the wastewater is mixed and aerated in a large tank. Flocs (of bacteria) form. The effluent containing the flocs is pumped into a holding tank or clarifier, where the flocs settle. Some of the floc material is sent back to the aerator and the rest to the sludge digestor where the main process of

BOD reduction occurs. The main process occurring is the *adsorption* of soluble organic matter to the floc, and incorporation of some of the soluble materials into microbial cell material.

Tertiary treatment

Tertiary treatment is the final step in the treatment process. It is a physiochemical process employing precipitation, filtration, and chlorination to sharply reduce the levels of inorganic nutrients, especially phosphate and nitrate, from the final effluent. **Chlorination** is the most common method of ensuring microbiological safety in a water supply. In sufficient doses it causes the death of most microorganisms within 30 minutes. The chlorine also reacts with organic compounds, oxidising and effectively neutralizing them, thus improving the taste and smell of the water.

Bacterial Count	In samples (no/ml)		In biomass (no/g)		% of bacteria viable
	Total	Viable	Total	Viable	
Settled sewage	6.8×10^{8}	1.4×10^{7}	3.2×10^{12}	6.6×10^{10}	2.0
Activated sludge	6.6×10^{9}	5.6×10^{7}	1.4×10^{12}	1.2×10^{10}	0.85
Filter slimes	6.2×10^{10}	1.5×10^{9}	1.3×10^{12}	3.2×10^{10}	2.5
Secondary effluents	5.2×10^{7}	5.7×10^{5}	4.3×10^{12}	4.7×10^{10}	1.1
Tertiary effluents	3.4×10^{7}	4.1×10^{4}	3.4×10^{12}	4.1×10^{9}	0.12

Table 2.3. Some typical Total and viable bacterial counts at various stages of sewage treatment.

Table 2.3 illustrates some typical total and viable bacterial counts at various stages of sewage treatment (Atlas & Barthe, 1981).

2.5 Microorganisms of Concern

2.5.1 Bacteria

Vibrio cholerae

Cholera is a deadly disease caused by *Vibrio cholerae* which is a gram-negative, curved rod transmitted almost exclusively via water faecally contaminated.. Two major types have been identified, the *classic* and the *EI Tor* types. Following ingestion cholera vibrios attach firmly to small intestinal epithelium and grow and release enterotoxin. The enterotoxin causes large loss of fluids, 20 litres per day. Satisfactory sanitation, sewage treatment and the purification of drinking water is required to control cholera outbreaks. *V. cholerae* organisms can adhere to normal flora in freshwater and can survive for long period of time.

E. coli.

The genus *Escherichia* is a member of the Enterobacteriaceae that is usually found in the bowel of humans and animals, and is thought to be closely related to *Shigella* (Brenner, 1984). Colonisation takes place soon after birth (Escherich, 1885; Bettelheim *et al.,* 1974). *E. coli* has been found in the faeces of humans and domestic animals, in clinical material such as urine and blood (Farmer *et al.*, 1985).

Pathogenic Escherichia coli.

(I) Entero-toxigenic *E. coli* is the commonest cause of "travellers diarrhoea" and infant diarrhoea in developing countries. These strains produce a heat-labile toxin and/or a heat-stable toxin (Reed,1994).

(II) Entero-pathogenic *E. coli* is particularly associated with diarrhoea in newborn infants (Reed 1994).

(III) Entero-invasive *E. coli* acts like *Shigella* to produce a serious dysentery-like disease (Reed, 1994).

(IV) Entero-haemorrhagic *E. coli* causes hemorrhagic colitis and hemolytic uremic syndrome. Six verotoxins have been identified within this group, but only stx-1 and stx-2 seem to be important in human infections. *E. coli* 0157:H7 is the principle serotype of this group (Reed, 1994).

(V) Enteroadherent *E. coli* is a newly added category and has not yet been fully characterized (Hitchens et al.,1998).

Campylobacter

Campylobacter has been associated with the consumption of untreated water drinking water (particularly rain water from roof catchments) giving rise to campylobacteriosis. *Campylobacter* is a Gram-negative, curved rod which grows at reduced oxygen tensions and is found in the gut. Two major species are recognised, *C. jejuni* and *C. fetus.*

Salmonella species

A number of non-typhoid causing salmonellas including *Salmonella enteritidis* cause mild forms of gastrointestinal diseases. One of the most important pathogenic bacteria transmitted by water is *Salmonella typhi,* the organism causing typhoid fever.

2.5.2 Enteric viruses:

Enteric viruses include enteroviruses (such as polioviruses, coxsackieviruses A and B, echoviruses, enterovirus 68-71, and the hepatitis A virus), rotaviruses, hepatitis E, adenovirus, reoviruses, Norwalk and other small round gastrointestinal viruses. There are over 100 human viruses that can be present in sewage including many small round viruses that cause gastroenteritis.

Hepatitis A

Infectious hepatitis (hepatitis A) is a viral-mediated inflammation of the liver caused by a picornavirus (positive single-strand RNA virus).The virus is transmitted primarily through faecal contamination of water, food or milk. Shellfish (oysters, clams, mussels) harvested from waters polluted with human faeces provide a vehicle for the transmission of hepatitis A as they are filter feeders that tend to concentrate the hepatitis A virus (Brock, 1994).

2.5.3 Protozoa

Giardia lamblia are protozoans which are commonly found in surface waters. *G. lamblia* is a flagellated protozoan that is transmitted to human primarily by contaminated water in the form of a cyst. Cysts germinate in the gastrointestinal tract and bring about the symptoms of giardiasis. *Giardia* cysts are fairly resistant to chlorine and many out breaks have occurred from water sources only using chlorination as a means of water purification..

Cryptosporidium is a protozoan parasite found in man and many other animals The organism may be excreted in faeces as oocysts which may cause infection if digested.

Cryptosporidium is a waterborne parasite known to infect humans. In healthy individuals cryptosporidiosis is characterised by an acute self-limiting diarrhoeal illness lasting approximately 2-3 weeks. Cryptosporidiosis can be fatal for HIV sufferers. Water is an important vehicle in the transmission of this disease which was thought to be responsible for a disease outbreak in Milwaukee, Wisconsin, in April 1993. Up to 400,000 people became ill and 100 died, most of the people that died were AIDs patients.The 1995 edition of the NZDWS contains specific compliance criteria for protection against *Cryptosporidium.* A turbidity gaol of less than 0.1 NTU is desirable, although the use of stronger disinfectants and the physical removal of all particles the same size as *Cryptosporidium* may also be required in areas with a higher risk of disease.

2.6 Waterborne Disease

Animal faecal wastes are known to contain various pathogens, some of which can cause disease in humans (Geldreich 1972, Feachem et al. 1983, Bohm 1989). Waterborne pathogens infect around 250 million people each year resulting in 10-20 million deaths (Anon, 1996).

See Table 2.4 for a list of waterborne disease outbreaks due to microorganisms (in the USA). Note how nearly 60 % of both outbreaks and cases are of unknown etiology and of the enteric infections the majority are of Salmonellosis and Shigellosis. How many unreported outbreaks of mild infection by *E. coli* and *Campylobacter* is not known.

Although intensive research has been undertaken to treat pathogens in drinking water, less effort has been made to reduce pathogens in stormwater and wastewater. There are currently increasing concerns about waterborne disease caused by enteric viruses and parasitic pathogens and bacteria. This lack of stormwater treatment may result in significant pathogen loading to receiving waters. Chlorine disinfection does not inactivate viruses and Giardia lamblia in wastewater as effectively as it does in drinking water because of interference by dissolved organics and suspended particulates. Secondary treatment using activated sludge removes about 95% of viruses and a large percentage of *Cryptosporidium* oocysts and *Giardia* cysts. Because of the cost that would be involved in analysing the actual pathogens, wastewater monitors use pathogen indicators (faecal coliforms) to predict high pathogen levels. The US Environmental Protection Agency (EPA) in 1986 recommended that enterococci

be used as an indicators in marine water and *E. coli* in freshwater.(Water & Wastes, July 1999).

According to Wright (1996), the results of a study conducted in the Eastern Bay of Plenty found that *Giardia, Cryptosporidium,* and *Yersinia* were collectively causing more illness than salmonellosis, shigellosis, campylobacteriosis and amoebiasis. A 1994 national study of NZ laboratories showed that the only organisms routinely tested for in stool samples were *Salmonella, Shigella,* and *Campylobacter.*

Disease	Causal agent	Outbreaks[b] (% of total)	Cases[c] (% of total)
Bacteria			
Typhoid fever	*Salmonella typhi*	10	0.5
Shigellosis	*Shigella* species	9	9
Salmonellosis	*Salmonella paratyphi*, etc	3	12
Gastroenteritis	*Escherichia coli*	0.3	2.5
	Campylobacter species	0.3	2.5
Viruses			
Infectious hepatitis	Hepatitis A virus	11	1.6
Poliomyelitis	Poliovirus	0.2	0.01
Diarrhea	Norwalk virus	1.5	2
Protozoa			
Dysentery	*Entamoeba histolytica*	0.1	0.05
Giardiasis	*Giardia lamblia*	7	13
Unknown etiology			
Gastroenteritis		57	58

[a] *Compiled from data provided by the Centres for Disease Control.*
[b] *Of 650 outbreaks in recent decades.*
[C] *Of 150,000 cases over the same period.*

Table 2.4. Waterborne disease outbreaks due to microorganisms (USA)[a]

2.6.1 Campylobacteriosis.

One of the ways in which *Campylobacter* is transmitted to humans is via a water route where the surface waters are not subjected to chlorination. They are thought to account for many cases of bacterial diarrhea in children. Infection includes a high fever (usually greater than 104^{o}C), nausea, abdominal cramps, and a watery, frequently bloody, stool.

Between 10 and 18th February 1996, 19 residents of the Ashburton town area were notified as cases of campylobacteriosis. The outbreak was linked to the failure of a chlorination plant and high rainfall which would have increased run-off from surrounding agricultural areas. *C. fetus* is of economic importance because it is a major cause of sterility and spontaneous abortion in cattle and sheep.

2.6.2 Cholera.

Cholera enterotoxin catalyzes a life-threatening diarrhea which can result in dehydration and death. In 1992 hundreds died of cholera in Ecuador and several other Latin American countries. This disease is rare in N.Z.

2.6.3 Typhoid fever.

Although more advanced treatment methods have almost eliminated typhoid fever from many parts in the world, occasional epidemics of typhoid occur due to a breakdown on water treatment methods, floods, earthquakes, contamination of

water pipes and leaking sewage.The most serious means of transmission is the water route or via food that has come into contact with contaminated water. Typhoid fever disease is also rare in N.Z.

2.6.4 Hepatitis.

Hepatitis A infection can be subclinical in mild cases, or can lead to severe liver damage in chronic infections. The type A virus spreads from the intestine via the bloodstream to the liver and usually results in jaundice, a yellowing of the skin and eyes, and a browning of the urine due to stimulation of bile pigment production by infected liver cells. In severe cases the permanent loss of a portion of liver function can occur.

2.6.5 Giardiasis

Giardiasis is a severe form of gastroenteritis, caused by protozoan parasite *Giardia lamblia,* the antidote is a powerful chemotheraphy drug. High chlorine contact times and dose rates can protect against *Giardia.*

The symptoms are an explosive, foul-smelling, watery diarrhea, and intestinal cramps, flatulence, nausea, and malaise. Between 1965 and 1982, 53 waterborne outbreaks of giardiasis affecting over 20,000 people were reported in the United States, most outbreaks occurring in undeveloped or mountainous regions.

Table 2.5 lists the minimal infective doses required for some pathogens Bitton, 1980; Bryan, 1977; Gunnerson *et al.*, 1984; Schiff *et al.*, 1984a,b).

Organism	Minimal infective Dose
Salmonella spp.	10^4-10^7
Shigella spp.	10^1-10^2
Escherchia coli	10^6-10^8
Vibrio cholerae	10^3
Campylobacter jejuni	10^2-10^6
Giardia lamblia	10^1-10^2 cysts
Cryptosporidium	10^1 cysts
Hepatitis A virus	1-10 PFU

Table 2.5. Minimal infective doses for some pathogens and parasites.

2.7 Monitoring of Bacterial discharge

According to (Brock, 1994) one of the main tasks of water microbiology is the development of laboratory methods which can be used to detect microbiological contaminants that may be present in drinking water. It is not practical to examine water directly for the wide variety of organisms, in particular pathogenic organisms, that may be present. This task would be difficult and time consuming and pathogens will be present in wastewater only from time to time. Therefore indicator organisms are used to determine the presence of pathogenic organisms Pathogens of serious concern are rare, their chances of detection small. Indicator bacteria are used to detect faecal contamination and hence the possibility of occurrence of pathogens. Coliforms are the indicator of choice.

Several methods are available for the detection of indicator microorganisms in environmental samples, including wastewater (Ericksen and Dufour, 1986; Seidler and Evans, 1983). Environment Waikato is the organization that routinely (once a

month) samples the rivers and streams in the Waikato region for the detection of total and faecal coliforms. The procedure routinely used to detect coliforms is the membrane filtration (MF) method described in *Standard Methods for the Examination of Water and Wastewater* (ALPHA, 1989). Concentration of bacteria on membrane filters is a practical way of analysing waters with low bacterial density. Large amounts of water may be filtered through a membrane filter, but particles other than bacteria will also be kept on the filter surface; membrane filtration therefore cannot be used for waters of low bacterial density compared with the density of other particles (WHO, 1982).

2.7.1 Membrane Filtration

The Membrane Filtration (MF) method is based on the principle that certain cellulose esters can form uniform, porous membranes which permit diffusion of aqueous liquids but which retain particles such as bacteria (Pyle, 1980). Microorganisms deposited on one surface of such membranes can use nutrients which diffuse through the pores when the opposite surface of the membrane is placed in contact with such a solution (Goetz & Tsuneishi, 1951). For counting, the optimum number of colonies on a membrane filter of diameter 45-50mm is about 100 colonies. Thus, the membrane filter method may be used without dilution of the laboratory sample for waters with a bacterial density less than 1000 per ml, and is highly recommended for bacterial densities less than 10 bacteria per ml.

Advantages of using MF Method

- small input of time (compared with MPN method).
- results obtained rapidly, except when confirmation of colonies is required.
- saves on the quantity of media required and materials used.
- procedure may be carried out in the field
- ability to resuscitate stressed bacteria.

2.7.2 Most Probable Number Method (MPN).

The MPN method is applicable to all kinds of sample : clean and turbid water, sewage and sewage sludge, muds and other sediments, provided the bacteria may be evenly distributed in the prepared test samples. The technique may cover samples with low bacterial densities (0.02 bacteria per ml and above). The following is a list of the disadvantages of using the MF method. These disadvantages can be overcome using freshwater mussels, which are able to concentrate bacteria over time, in the MPN method using selective media.

Disadvantages of using MF method

- The results obtained from water samples reflect only the point in time in which the water sample was taken and not a period of time.
- is not suitable for samples containing few coliform organisms in the presence of many non-coliform organisms which are capable of growing on the media used.
- this method does not detect the production of gas by the bacteria but relies on the products of lactose fermentation.

- different MF media for coliform bacteria support the growth of slightly different coliform bacteria.
- Inconsistencies in MF determinations related to the characteristics of the filters.

2.7.3 Indicator Organisms

The coliform bacteria are a collection of relatively harmless microorganisms that live in large numbers in the intestines of man and warm- and cold-blooded animals. A specific subgroup of this collection is the faecal coliforms, the most common member being *Escherichia coli. E. coli* is the only coliform species exclusively of faecal origin, although not always directly linked to a health risk (Hazen, 1988). Therefore the detected presence of *E. coli* in water systems is a positive indication that faecal contamination of that water system has occurred.

E. coli is present in the lower human gut at concentrations of around 10^7-10^8 organisms per g (O'Leary, 1989; Lederberg, 1992), and of the order of 10^9 per g in faeces (McCoy, 1991).

These organisms may be separated by their ability to grow at elevated temperatures and are only associated with the faecal material of warm-blooded animals.

The most widely used indicator is the coliform group of organisms. This group of bacteria is defined as all those aerobic and facultative aerobic, Gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas formation

within 48 hours at 35°C. The coliform group of organisms are suitable as indicators because they are common inhabitants of the intestinal tract, both of humans and warm-blooded animals. They are present in large numbers and can be used a an indicator of pathogenic bacteria which occur at lower concentrations. One concern with monitoring using indicator bacteria is their survival following discharge. If survival rates are low, underestimates of true levels of discharge will result. For many years investigations have been made on the successful survival of *Salmonella* spp. and *E. coli* in water and ecosystems (Andre et al, 1967; Beard, 1940; Geldreich et al, 1968; Hood and Ness, 1982; Rudolfs et al, 1950). According to Burton et al (1986) *E. coli* has usually been observed to survive as long as or longer than *Salmonella* spp. supporting the majority of previous investigations.

Results of a survey carried out by the United States Environmental Protection Agency indicated that *E. coli* was a good bacterial indicator for freshwater (Dufour, 1984).

2.8 Microbial contamination and Water Quality

2.8.1 Drinking Water

Due to the efficiency of water purification treatment practices the incidence of disease from the consumption of polluted water is rare in New Zealand. Filtration plays a significant role in the reduced microbial load of water. Chlorine disinfection also plays a role in the successful reduction of microorganisms associated with diseases. See Table 2.6 for some of the faecally sourced microorganisms implicated in drinking water related illness.

Bacterial	Viral	Parasites
Salmonella typhi	Enteroviruses (71 types)	**Protozoa**
Salmonella spp.	Adenoviruses (31 types)	*Giardia intestinalis*
Shigella spp.	Hepatitis A	*Cryptosporidium parvum*
Pathogenic *E. coli*	Reovirus	
Campylobacter spp	Rotavirus	**Helminths**
Vibrio cholerae	Norwalk virus	*Ascaris lumbricoides*
Yersinia enterocolitica	Cocksackie virus	*Taenia spp.*
		Enchinococcus granulosis

Table 2.6. Some of the faecally sourced microorganisms implicated in drinking water related illness.

2.8.2 Recreational Water

Organism	Infection
Bacteria	
Aeromonas hydrophila	Wound infection
Leptospira	Leptosprirosis
Mycobacterium spp.	Skin or subcutaneous lesions
Legionellae	Legionaire's disease or Pontiac fever
Pseudomonas spp.	Dermatitis, ear infection
Vibrio spp.	Wound infection, septicemia
Staphylococcus aureus	Wound infection
Viruses	
Adenoviruses	Conjunctivitis, pharyngitis
Protozoa	
Naegleria fowleri	Meningoencephalitis
Acanthamoeba	Subcutaneous abcesses; conjunctivitis.

Table 2.7. Non-enteric infections resulting from contact with recreational water.

Appearance and odour are two important characteristics which influence the recreational use of a water. Many inland fresh waters are used for recreational purposes ranging from activities such as boating (where occasional occasional immersion may result) to swimming (where intentional immersion occurs). The health-related risks associated with these activities are of current concern (Anon. 1991a,b; Phillip 1991; Fewtrell *et al*. 1992). In these activities there is a reasonable risk that water could be swallowed, inhaled (Harrington *et al*. 1993), or come into contact with ears or nasal passages, mucous membranes and cuts in the skin; allowing pathogens to enter the body (Till *et al*. 1998). Table 2.7 lists infections non-enteric infections acquired by contact with recreational water.

Most exposure to contaminated water is brief but diseases such as Hepatitis A, discussed earlier, may occur. Some Gastrointestinal infections associated with the

accidental ingestion of pathogens and parasites in waste-water-contaminated recreational waters are shown in Table 2.8 (Cabelli, 1989).

Etiologic Agent	No. of Cases	Source (Location)
Giardia	9	Wading pool, day care centre
Giardia	7	Wading pool, day care centre
Cryptosporidium	26	Water slide (park)
Giardia	14	Swimming pool (park)
AGI	15	Creek (private home)
AGI	15	Dunking booth (fair)
E. coli 0157:H7[a]	80	Lake (park)
Cryptosporidium	500	Wave pool (park)
Shigella sonnei	203	Lake (park)
Shigella sonnei	23	Lake (swimming area)
Giardia	4	Lake (campground)

AGI, Acute gastrointestinal illness of unknown etiology.
[a]Mixed outbreak of *E. coli* 0157:H7 and *Shigella sonnei.*
Adapted from Moore et al. (1994).

Table 2.8. Outbreaks of gastroenteritis associated with recreational water.

2.8.3 Monitoring Recreational Water

Guidelines by (Till *et al,* 1998) were produced to provide guidance for water managers implementing the Resource Management Act, 1991 and the Health Act, 1956 for shellfish-gathering or contact recreation. The guidelines use "acceptable" swimming-associated illness risks of 8 per 1,000 bathers for freshwater, these values are used by the EPA. In New Zealand recreational waters are managed by the regional councils, territorial local authorities and health authorities. These guidelines are set out in Table 2.9 (Till *et al,* 1998).

Running median (estimated monthly) < **126 *E. coli* /100ml.** **ACCEPTABLE/GREEN MODE:** • Continue routine (eg. Weekly) monitoring.
Running median between **126 and 273 *E. coli*/100ml.** **ALERT/AMBER MODE I.** • Increase sampling to at least twice weekly, in order to improve the information base. • Prepare a report on potential health risks and causes of elevated bacteriological levels.
Single sample > **273 *E. coli* / 100ml** (irrespective of the running median) **ALERT/AMBER MODE II:** • Increase sampling to daily. • Undertake a sanitary survey, report on sources of contamination.
Single sample > **410 *E. coli*/100ml** (irrespective of running median) **ACTION MODE** • Erect warning signs. • inform public, through the media that a public health problem exists.

Table 2.9. Guidelines for water quality suitable for freshwater bathing.

The guidelines shown in the Table 2.9. are interim and are intended as guidelines only, due to the small number of studies done.

2.9 Sentinel Mussels

2.9.1 Why use freshwater mussels as Bioindicators?

There are three basic methods that can be used to quantitate pollutants in aquatic systems Phillips (1977, 1978). Study of the pollutant levels in :

1. The water itself,
2. In the sediment,
3. In a member of the indigenous biota.

- According to Phillips (1980) the basic pre-requisites for a suitable organism are:

1. The organism should accumulate the pollutant without being killed by the levels encountered in the environment.
2. The organism should be sedentary in order to representative of the study area.
3. The organism should be abundant throughout the study area.
4. The organism should be sufficiently long-lived to allow the sampling of more than one year-class, if desired.
5. The organism should be of reasonable size, giving adequate tissue for analysis.
6. The organism should be easy to sample and hardy enough to survive in the laboratory, allowing defecation before analysis (if desired) and laboratory studies of pollutant uptake.

- According to Haug *et al.* (1974) pre-requisites should also include :

7. The organism should tolerate brackish water.
8. A simple correlation should exist between the pollutant content of the organism and the average pollutant concentration in the surrounding water.

- According to Phillips (1976, 1977) a further requirement is :

9. All organisms of a given species used in a survey should exhibit the same correlation between their pollutant content and the average pollutant concentration in the surrounding water, at all locations studied, under all condition.

Freshwater mussels have the qualities described above and are therefore a potentially useful tool for environmental monitoring and impact assessment, especially because of their ability to concentrate environmental contaminants in their flesh. Thus, pathogenic microorganisms may be accumulated by mussels to levels far above those found in the surrounding environment. This ability to retain faecal microorganisms makes shellfish a potentially useful tool for determining faecal contamination of natural waters (Webber, D. Phil thesis-reported in Trollope & Al-salihi, 1984).

2.9.2 The advantages of using mussels to quantitate pollution (Phillips, 1980).

1. The biological availability of the pollutant is measured directly.
2. Gives a time-averaged index of pollutant availability.
3. There are higher concentrations of the pollutant in the mussel than ın the environment.

2.9.3 Factors that need to be considered when using mussels as Bioindicators.

1. Seasonal changes.
2. Species.
3. Body lipid.
4. Age/Size/Weight.
5. Sex of mussel.

6. Sexual cycle.
7. The position at which mussels are collected, e.g. depth.
8. Successful depuration of mussels.
9. Gradients in food.
10. Pollutant interactions at uptake.
11. Storage and or excretion.
12. Gradients in suspended silt.
13. Salinity, temperature, etc

The degree to which both organic and inorganic contaminants are accumulated can be influenced by biotic factors, including pumping activity, growth, biochemical composition, reproductive condition and metabolism (Widdows & Donkin, 1992).

2.9.4 *Hyridella menziesi*

The species of *Hyridella menziesi* are members of the Phyllum Mollusca, Class of Bivalvia. Molluscs are found in a great range of habitats all over the world increasing their usefulness as a bioindicator.

Mussels are laterally compressed and their two shells function largely for protection. The visceral mass is suspended from the dorsal midline, and the muscular foot is attached to the visceral mass anteroventrally. The gills hang down on each side, covered by a fold of the mantle.The posterior edges of the mantle

folds are modified to form dorsal excurrent and ventral incurrent openings. Cilia on the gills and inner surface of the mantle direct the flow of water over the gills.

Mussels are sedentary filter-feeders. The respiratory currents bring both oxygen and organic material to the gills, where ciliary tracts direct them to the tiny pores of the gills (See Figures 2.2 and 2.3). Gland cells on the gills and labial palps secrete copious amounts of mucus, which entangles particles suspended in the water going through gill pores. Ciliary tracts move the particle-laden mucus to the mouth. In the stomach the mucus and food particles are kept whirling by a rotating gelatinous rod, called a crystalline style. Solution of layers of the rotating style frees digestive enzymes for extracellular digestion. Ciliated ridges of the stomach sort food particles and direct suitable particles to the digestive gland for intracellular digestion (Hickman, 1995).

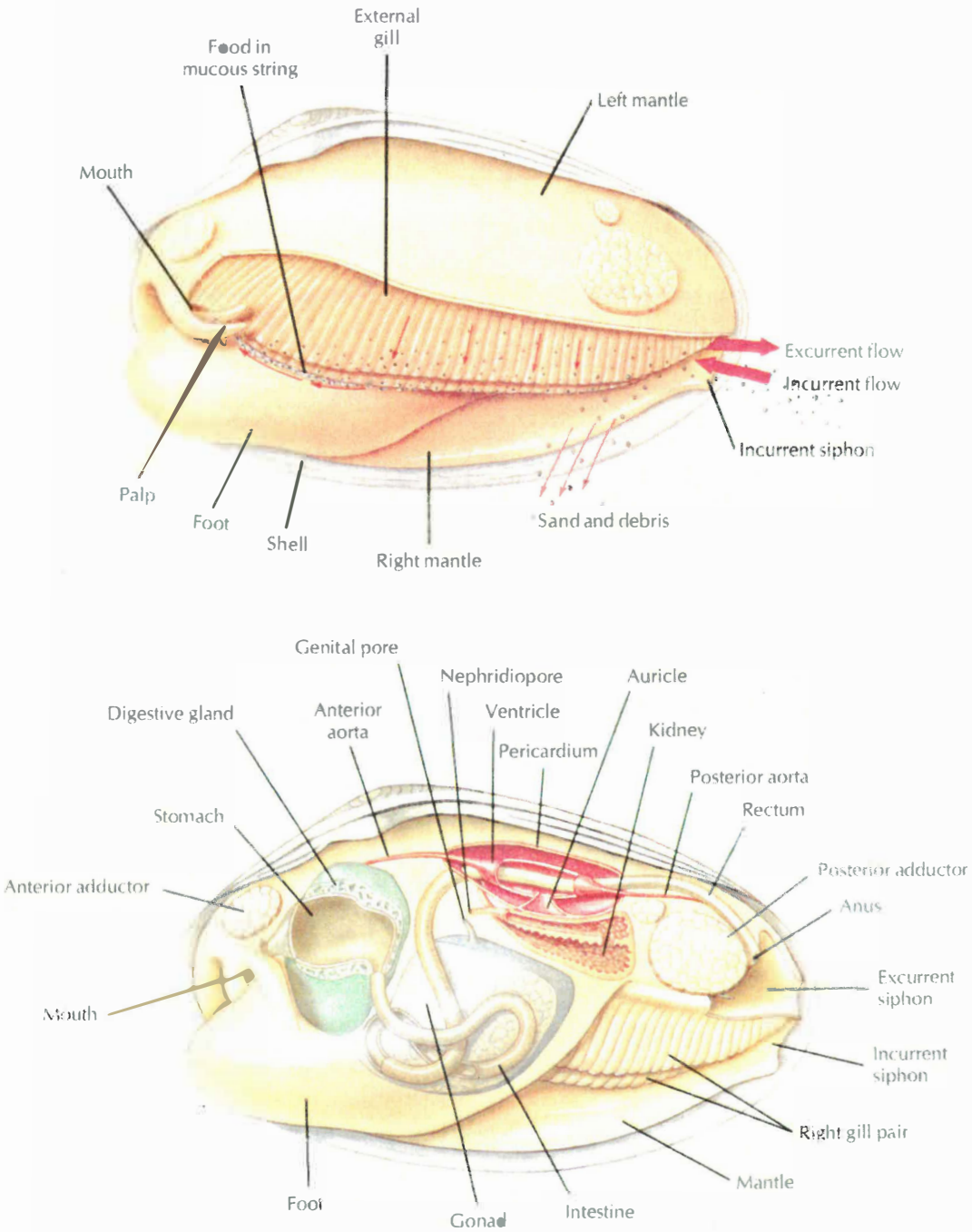

Figure 2.2 Illustration of the feeding mechanism of Bivalves

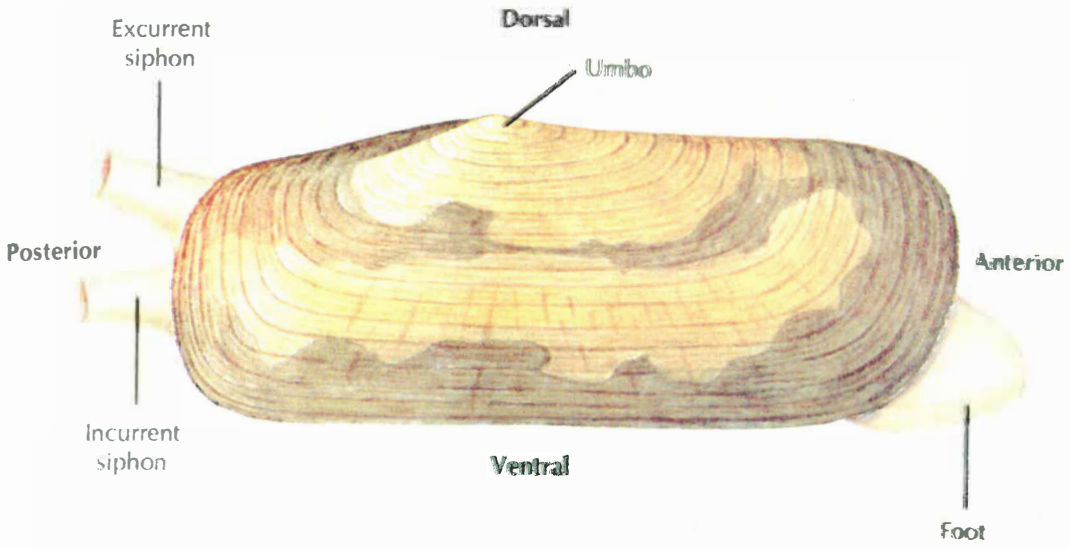

Figure 2.3 The external view of the right valve (of a Bivalve)

Species of *Hyridella* are found throughout Australasia (Walker, 1981). The New Zealand species *Hyridella menziesi* has been recognised as having potential for environmental monitoring (Roper & Hickey, 1993) and was the species of freshwater mussel used in the set of experiments for this thesis.

Chapter 3

General Experimental Methods

3.1 Introduction

This chapter provides a general overview of the materials and methods common to this study. Methodologies used for specific experiments are described in the relevant chapters.

3.2 Collection and storage of mussels

Freshwater mussels (*Hydridella menziesi)* were collected from the same site at Lake Rotoiti each time and were transported from the site of collection in chilly bins containing wet sacks that were wrapped around the mussels. Each bin contained approximately 100 mussels. After collection the mussels were stored immersed in the Waikato River until needed. Up to 15 mussels were contained within a plastic mesh cage and tied suspended under a private jetty.

3.3 Mussel depuration

Depuration was performed over periods between 5 and 6 days. Mussels were collected from a jetty on the Waikato River and transported to fish tanks containing sand and slow flowing, dechlorinated freshwater previously exposed to ultraviolet light. Approximately 15 - 30 mussels were added to each tank. Mussels were kept in these tanks until 5 days before the experiment. They were then transferred to 3.5L beakers or buckets, without sand, with slow flowing freshwater. The elimination of entrained bacteria, including faecal coliforms, from shellfish is due to the high rate of water exchange that is characteristic of shellfish (Mesquita *et al.,* 1991) and is generally complete within a few days.

Before mussels were used in the experiment described here, a control step was included in the methods to determine successful depuration of mussels, a set of uncontaminated mussels were analysed to determine the natural faecal coliforms present before contamination.

3.4 Culture and preparation of *E.coli*

- **Moderate level contamination**

TSB that had been inoculated with *E. coli* and incubated overnight was diluted by 10^5. Then 3.5ml of this was added by pipette to 3500ml of water. The original suspension was able to be determined using an MPN count. It was therefore possible to estimate the concentration of *E. coli* added to each beaker.

- **High level contamination**

TSB that had been inoculated with *E. coli* and incubated overnight was diluted by 10^4. Then 3.5ml of this was added by pipette to 3500ml of water.

3.5 Preparation of mussels for contamination

3500 ml of water was added to a 5000ml Beaker containing a perforated sheet of plastic on which immersed mussels sat in order to keep them from the magnetic stirrer, that was operated at slow speed to ensure thorough aeration of the water.

The beakers were then contaminated with the appropriate diluted suspension described in Section 3.4. Once the *E. coli* was added to the water a 10 minute interval was allowed to ensure the bacteria were evenly dispersed throughout the water. A subsample of 100 mls of water was removed and transferred into a sterile capped bottle and placed in a refrigerator at 4^oC prior to counting. Depurified mussels were a observed for gaping. Mussels showing gaping, with their shells partly opened and siphons extended, were assumed to be actively filtering. Any mussels not showing these features were not selected for experimental use. Five mussels were then added to each beaker. The time was simultaneously recorded to ensure the mussels were contaminated for exactly two hours or the preset time required for contamination. When the appropriate time had elapsed, 100 mls of water was collected from the beaker in order to determine the extent of removal of bacteria. The mussels were removed from the beaker to determine the numbers of bacteria retained by them.

3.6 Mussel preparation after contamination

Mussels were prepared according to APHA Standard Methods (1989).

3.6.1 Cleaning the Shells

Hands were washed with soap and water than rinsed with 70 % alcohol. Mussel shells were then scrubbed to remove growth, loose material and byssal threads using a brush under running water.

3.6.2 Removal of mussel tissue

The mussels were held in the hand over a sterile beaker. A sterile knife was placed in the byssal opening. The shells were levered apart with a twisting motion, allowing the draining of the shell liquor. Adductor mussels were cut and the mussel allowed to fall into the sterile beaker.

3.6.3 Preparation of mussel homogenate.

The mussel was weighed to the nearest gram. An equal weight of 0.5% sterile peptone water was added. The beaker contents were transferred to a sterile blender and homogenised for 60 to 120 seconds. Two ml of the homogenate contained 1 gram of shellfish meat. A grinding time of 60 to 90 seconds was found to be optimum.

3.7 Enumeration of Bacteria

3.7.1 Media

All media were prepared as per manufacturers instructions. Media ingredients are listed in Appendix A.

General diluent.

Bacto Peptone was used as a general diluent. Peptone was used to mix dilutions of bacterial cultures (0.1%), mussel slurries (0.5%) and, in the MF method (0.1%), to analyse water samples.

Media for culturing and storing E.coli.

TSA was used to make up plates and slopes (for culture and storage of *E.coli*). TSA was added to the appropriate volume of distilled water, heated until boiling to dissolve the agar, and sterilised at 121°C for 15 minutes. The agar was left to cool before either dispensing into universal bottles for slopes or pouring into Petri plates for culture. Plates were stored at 4°C for up to 2 weeks. Before each experiment, plates were streaked to recover colonies that had been stored in the fridge at 4°C, the recovered colonies were then used to inoculate TSB.

Slopes of E. coli for storage

100 ml of TSA agar was prepared, melted, and then 10 ml dispensed to each of 10 Universal bottles. The bottles were then autoclaved and while still molten were left to set on an angle leaving the agar half an inch below the cap of the bottle.

Once the agar had set each slope was inoculated with a colony of *E. coli,* spread over the slope surface. The slopes were then incubated for 24 hours at 35°C. Following incubation the slopes were stored in the cool room at 4°C until required. 48 hours before experimental work was to start *E. coli* was recovered by streaking a TSA plate and incubating for 24 hours at 35°C. A gram stain of the *E. coli* was conducted to inspect the culture was pure.

Media for MPN determination

Lauryl Tryptose Broth was used as the confirmatory medium for faecal coliforms and was dispensed into 10 ml tubes for the MPN tests. The ingredients of the broth were mixed with distilled water in amounts depending on whether a single strength or double strength solution was required before being dispensed into 10ml, screw-capped bottles, and then sterilized at 121°C for 15 minutes. Tubes were stored in a cool cupboard for no more than a week.

Each tube also included 0.01 g/L of Bromocresol Purple a pH indicator (Ajax Laboratory Chemicals). Tubes that remained purple were scored as negative, tubes that showed a colour change to yellow were scored as positive for the presence of faecal coliforms.

Preparation of mFC plates for Spread-Plate technique.

The mFC agar (Difco, USA) was mixed with distilled water and brought to the boil to dissolve the agar. The agar was then left to cool and then was poured into Petri dishes. The plates were left to dry before being stored at 4°C until required.

Preparation of mFC plates for Membrane Filtration technique.

mFC agar (Difco, USA) was used to culture and enumerate *E. coli* in water samples. The commercial mFC Agar was mixed with distilled water and brought to the boil to dissolve agar. 10 ml of Rosolic acid solution was then added and the agar boiled for 1 minute. The pH was measured to ensure it was 7.4 ± 0.2 at 25°C. The agar was then cooled to 50°C and then poured into standard Petri dishes to a thickness of about 2-5 mm. The plates were left on a flat surface to set before they were stored, at 4°C, until needed.

Preparation of Nutrient Agar (NA) + Mug for the Membrane Filtration technique.

Nutrient Agar + Mug (Difco) was mixed with distilled water and then sterilized at 121°C for 15 minutes. Once the solution had cooled, it was poured into Petri dishes and left to set on a flat surface before they were stored at 4°C until needed.

A majority of *E. coli* produce the enzyme β-glucuronidase (GUD). The presence of GUD in *E. coli* cleaves the MUG substrate to release 4-methylumbelliferone (MU). When exposed to longwave (365 nm) UV light in the dark, MU exhibits a bluish fluorescence which is easily seen and *E. coli* colonies are presumptively identified.

3.7.2 Enumeration of experimental water using the Spread-plate technique

This technique may only be used for waters with bacterial density higher than 250 bacteria per ml, using portions of 0.1 ml (WHO, 1982). Samples of 1ml may be tested if the agar plates have been predried to lose approximately 2 g of water.

Media

Plates of mFC agar were used to culture and enumerate *E. coli* in water samples. Preparation of these plates are described in Section 3.7.1. Before each experiment had commenced, the plates were removed from the refrigerator and allowed to dry thoroughly before use.

Storage of test samples

100 ml water samples were collected from the experimental beakers into sterile plastic containers, before and after the experimental time required for contamination or control, and were promptly placed in a refrigerator at 4°C until required. Then they were used for counting by spread-plate and membrane filtration within 6 hrs of their collection.

Drying of plates

It was important to ensure that plates had a sufficiently dry surface and that no condensation had formed on the inside of the lid. Plates were always removed from the refrigerator and dried prior to use (inverted with lids off for 1-2 hours at 44°C).

Pretreatment of the water sample before plating

To ensure even distribution of the bacteria in the sample, the sample was shaken thoroughly in its container immediately prior to removing a 1ml sample by pipette for plating.

Plating of the bacteria

0.1 or 1 ml of the sample was added with a pipette to the mFC agar. A sterilized glass rod was used to spread the sample evenly over the agar. The volume added was recorded and the plates were then added to an incubator at 44°C for 24 hours.

Counting the colonies

The plates selected for counting were those with between 25-300 colonies. Plates were colonies too numerous to count were reported as TNTC.

The formula for expressing the Bacterial Density (BDT) of the sample was :

$$BDT = \frac{\text{Colony Count}}{\text{Volume}} = \text{bacteria per ml.}$$

3.7.3 Enumeration of experimental water using Membrane Filtration

The method used was that described in (ALPHA, 1998). The concentration of bacteria on membrane filters is a practical way of analysing water with low bacterial density (WHO, 1982) but cannot be used for waters of low bacterial density compared with the density of other particles that may be present in the sample. This technique is highly recommended for bacterial densities less than 10 bacteria per ml. See Figure 3.1.

Samples of water of a known volume were passed through a sterile membrane filter, and the filter then placed on a culture medium highly selective for coliform

organisms (in this case mFC agar) and incubated for 24 hours at 44°C. See Section 3.7.1 for the preparation of mFC media.

Incubation at 44.5°C is the essential step in this method, since the ability to grow and ferment lactose at 44.5°C is the key distinguishing feature of the faecal coliform group. In order to obtain accurate counts, the temperature was held absolutely steady; if the temperature is too high the faecal coliforms cannot grow, if the temperature is too low the non-faecal bacteria start to grow. The mFC plates were placed in water-tight containers and immersed in a waterbath, which gave much more accurate temperature control than did use of an incubator. After 24 hours blue colonies were counted and the membrane filter then transferred to a plate of Na + Mug for 6 hours at 35°C. This step was used to distinguish colonies of *E. coli* from those of other faecal bacteria. Colonies that were fluorescent under UV light were counted. See Section 3.7.1 for the method used to prepare Na + Mug.

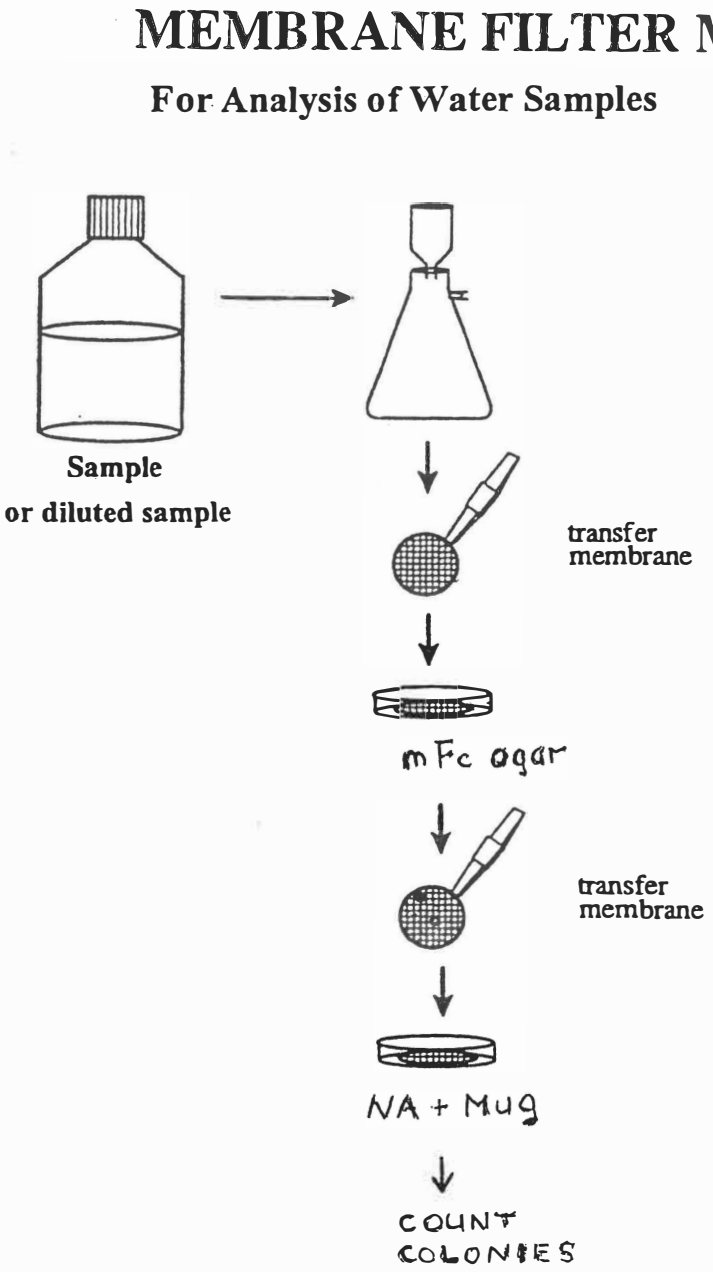

Figure 3.1 The Membrane Filtration Method

3.7.4 Most Probable Number Method (MPN)

The enumeration of *E. coli*. in shellfish was performed by the MPN method described in (WHO, 1982). The MPN technique is designed to give the results with a defined and relatively good precision, but requires a large number of tubes and incubator space. According to (WHO, 1982) the multiple tube technique is applicable to both clean and turbid water provided that the bacteria are evenly distributed in the samples. All samples taken here were mixed thoroughly to ensure even distribution. It was assumed that on incubation each tube that received one or more viable organisms in the inoculum will show growth on incubation.This method gives results which are expressed as the *most probable number* (abbreviated to MPN) for each stage of the coliform test. See Figure 3.2.

Procedure

The MPN test was done as follows. A test sample (prepared as in Section 3.4) was progressively diluted, in 10-fold steps, and multiple aliquots from each dilution step were inoculated into growth medium. To make up dilutions of a sample, 1 ml of the sample was added to a tube containing 9 ml of sterile peptone. This was mixed thoroughly before 1 ml was removed and transferred to another tube containing 9 ml of sterile peptone. This was continued until the required dilutions were accomplished. For each dilution step (4 or 5 steps), 5 tubes of medium were inoculated. The volume of the test sample added to the medium was recorded on MPN Sheets, along with the dilution factor for each step (see Appendix B for a copy of the MPN sheets used).

If there was growth in some, but not all of the tubes in three successive serial dilutions, standard MPN Probability Tables can be used to estimate the bacterial concentration in the sample (see Appendix B for a copy of the MPN Probability Table).

The test consists of two stages :

Stage 1 **Presumptive**

The medium used was Lauryl Tryptose Both (LT) , which contains lactose as fermentable carbohydrate and a surface active agent, sodium lauryl sulphate, to suppress Gram positive bacteria. This medium was prepared beforehand and sterilized according to manufacturer instructions. Double strength medium in volumes of 10 ml were used for sample volumes of 10 ml. Double strength medium was only used for the control samples and the low concentration samples. All the remaining samples were examined using single strength medium. Single strength medium in volumes of 10 ml were used for sample volumes of 1 ml to keep the nutrient concentration at the same level for both samples (1 ml and 10 ml). Tubes were marked from 1 - 25 and a record of the corresponding dilution was kept on MPN sheets.

The medium also contained bromocresol purple . Coliforms can ferment lactose and the associated production of acid is detected by the colour change of the medium from purple to yellow. Those tubes which changed colour were assumed to contain coliform bacteria and were recorded as positive for coliform bacteria. Control tubes were also included, which were 1 x LT inoculated with 1ml of peptone, and 1 x LT inoculated with a loopful of TSB.

Stage 2 **Confirmation**

In order to confirm the presence of *E. coli,* a loopful of growth from the positive tubes was used to inoculate Eppendorf tubes containing 1 ml of EC Mug. Tubes were incubated in a water bath at 44.5^{0}C. This temperature is close to the upper limit of the growth range for *E. coli.* Although there are a few other related species that grow at this temperature, in most circumstances 80% or more of the bacteria recovered are *E. coli*. The inoculated EC Mug Eppendorf tubes were left in the waterbath for 24 hours before being examined under an ultra violet light. Those tubes which showed fluorescence were assumed to contain *E. coli* and were recorded as positive for *E. coli*. It is important not to transfer mussel tissue from LT to EC Mug eppendorf tubes because the mussel tissue will fluoresce. A negative control consisting of an uninoculated Eppendorf containing EC + Mug and a positive control consisting of an Eppendorf containing EC + Mug and inoculated with the suspension used to contaminate mussels were also included. The results from the MPN method were used to determine the most probable number of *E. coli* present in 100g (wet weight of mussels) by reference to the MPN probability table.

3.7.5 MPN Probability Table

According to (WHO, 1982) this estimate tends to be greater than the actual number, and the error tends to diminish with increasing numbers of tubes in each dilution examined. (See Appendix B for the MPN Probablity Table).

FAECAL COLIFORMS
MPN METHOD
For Analysis of Shellfish

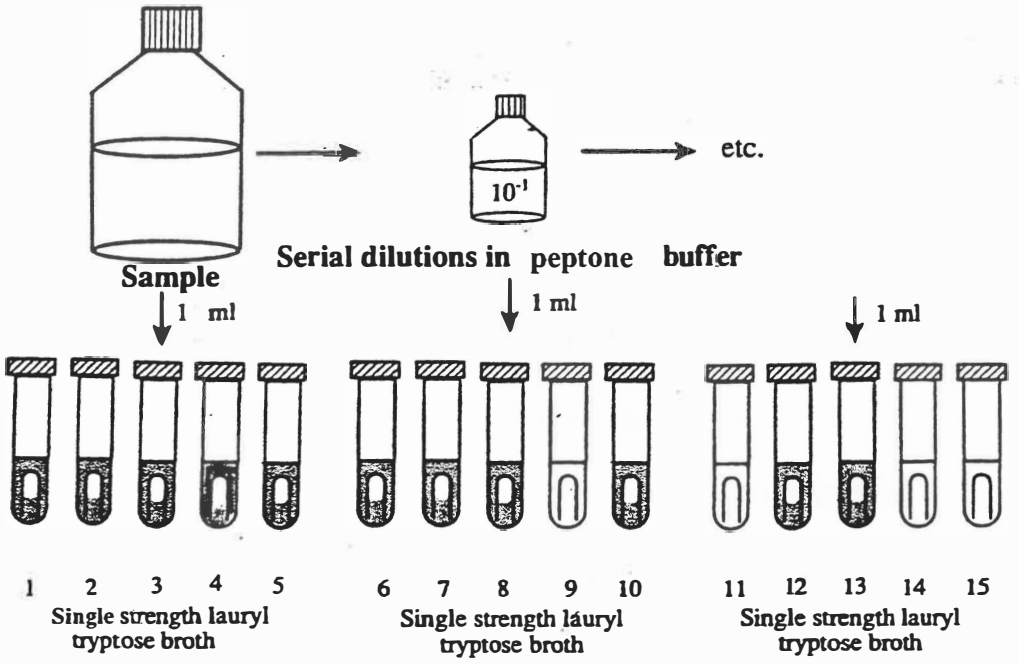

Figure 3.2
The MPN method for faecal coliforms

3.8 Identification of Bacteria

3.8.1 Use of Microbact Identification Kits

The Microbact kit was used to identify the *E. coli* used in these experiments. The kit reduced the media preparation required and gave quick results.

A pure culture of *E. coli* was used to inoculate the kit (see Section 3.7.1 for preparation of the pure culture). 4 colonies of the *E. coli*, obtained using a toothpick, were added to a saline solution, and mixed well to form an evenly distributed bacterial suspension. 100 ml aliquots of the bacterial suspension was added to each of the 12 wells of the Microbact kit. The rows were checked to ensure each was half-filled. 2 drops of sterile mineral oil were added on top of wells 1-3, (lysine, ornithine, H_2S). The plastic strip covering the wells was replaced and the kit incubated at 35°C for 24 hours.

After 24 hours, a Pasteur pipette was used to add :

1. 2 drops of Kovac's indole reagent to Well 8 (indole well). The reaction was read after 2 minutes and the presence of indole was indicated by a dark red colour change giving a positive result). *E. coli* indole-positive
2. 1 drop each of the 2 separate Voges Proskauer reagents I and II were added to well 10. The reaction was read after 15 minutes and the presence of acetoin was indicated by a bright red colour. *E. coli* is Voges-Proskauer negative.

3. Add one drop of TDA reagent. The reaction was read immediately and subsequent colour change ignored. The presence of Indolepyruvic acid was detected by a reaction resulting in a cherry red colour (TDA positive). *E. coli* is TDA negative, giving a straw or brown colour.

The reactions from the Microbact kit were determined by comparing the colours observed with the "Master" chart provided with the kit. Each well was scored either +ve or -ve and the score was calculated using the reaction index given. The results were entered into a laptop computer to obtain a Microbact Identification of the *E. coli* culture.

3.8.2 Gram Stain Method

The technique was also used to identify the *E. coli* grown to use in experiments, and to ensure that a pure culture was present.

The *E. coli* cells were collected by brushing an asceptically flamed loop over an *E. coli* colony growing on one of the slopes prepared and stored in Section 3.7.1. A dried smear of *E. coli* was stained with ammonium crystal violet (0.5g/100ml H_2O) for 30 seconds, washed with Gram's Iodine (1g I in 2% KI) for 30 seconds and washed with ethanol (95%) until the washings were pale violet.

Under the microscope *E. coli* was identified by Gram-reaction and morphology. *E. coli* are Gram-negative rods that tend to be short in actively growing cultures, they are about 1 x 3 μm, and stain red.

3.8.3 Identification of Salmonella.

Salmonella are Gram-negative, usually motile, non-sporing rods. They are facultative anaerobes, catalase positive and oxidase negative. Characteristic biochemical reactions include : H_2S production, lysine decarboxylation, use of citrate as a carbon source, negative urease reaction, and the ability to ferment glucose with the production of gas and an inability to ferment either lactose or sucrose.

To identify *Salmonella* colonies, a Tryptic Soy Agar (TSA) slope was inoculated with *Salmonella* and left overnight at 37°C in an incubator. After 24 hours five colonies were selected to streak a plate of Triple Sugar Iron Agar (TSI) agar, using a sterile loop. The plate was then incubated for 24 hours at 37°C. After 24 hours the plate was examined for a typical *Salmonella* reaction of alkaline (red) slants and acid (yellow) butts, with or without H_2S production (blackening).

Chapter 4

Preliminary Experiments

4.1 Introduction

The objective of these preliminary experiments was to :

1. Determine how to eliminate the *E. coli* naturally present in the mussels.

2. Determine if 2 hours was a sufficient time to detect a substantial number of *E. coli* in mussels.

3. Determine how repeatable the results are using 5 mussels in each experiment.

4.2 Materials and Methods

4.2.1 Media

The media used for enumeration of *E. coli* were TSA, TSB, LT, peptone and EC + Mug. Methods for their preparation are described in Section 3.7.1.

4.2.2 General Methods.

The methods used for the collection, and storage of mussels is described in 3.2.
The methods used for depuration are described in Section 3.3

The preparation of the mussels for contamination is described in Section 3.5 and of their sampling and homogenisation in Section 3.6.

The Pour- plate method

This technique is only suitable for bacteria that are not inactivated by exposure to temperatures of 43-45^{o}C, therefore is suitable for the enumeration of *E. coli.*
mFC agar was prepared as described in Section 3.7.1. and left in a water bath preheated to 44.2^{o}C for cooling and tempering the culture medium after boiling to melt the agar. For each water sample, the sample was shaken before 1 ml was removed by pipette and added to a sterile Petri dish. The bottle containing molten mFC was quickly removed from the waterbath, the lid removed and the opening flamed before pouring into the Petri dish. The agar was then left to set before being transferred to an incubator set at 44.2^{o}C. Plates were incubated in an inverted fashion. After 24 hours of incubation the colonies were counted and recorded.

4.2.3 Preparation of mussels for contamination

1 x 5000mL sterile beaker, containing 3500mL of UV treated dechlorintated freshwater, was contaminated with a 10^4 dilution of *E. coli* culture grown overnight in TSB. (See the raw data in Appendix C for the concentration of *E. coli* added to each beaker). The beaker (on a magnetic stirrer) was left for 10 minutes before a 100ml water sample was taken for analysis by using a spread-plate method using 0.1 ml per sample and pour plate method using 1 ml per sample, or the membrane filtration method (WHO, 1982).

The spread-plate method is described in Section 3.7.2. and the pour-plate method in Section 4.2.2.

Water samples were stored in the cool room at 4°C for approximately 2 hours before being analysed. 5 mussels were then added to each beaker. The mussels in beakers were kept in a temperature controlled room at approximately 15°C, on a magnetic stirrer, until removed for analysis. At Time 0, 5 control mussels (uncontaminated) were analysed using the MPN method to determine the level of *E. coli* naturally present in the mussels before they were contaminated. Mussels were left for 2 hours after which 100 ml of water was collected for analysis using either the spread plate method, the pour plate method or the membrane filtration method. Immediately after removing the water sample, the mussels were removed for analysis.

4.2.4 Enumeration of *E. coli* in Mussels

The mussels were analysed using the MPN method described in Section 3.7.4.

4.3 Results and Discussion

An aim of the preliminary experiments (1-7) was to establish a protocol to reduce the natural *E. coli* (background) in the mussels before adding mussels to beakers of water contaminated with *E. coli.* The results obtained from the first experiment and experiment 3 did not show that uptake of *E. coli* added to the beakers had occurred. This may have been a) due to the high numbers of background *E. coli* already present in the mussels b) an equilibrium may have occurred between the natural *E. coli* and the additional *E. coli,* and c) Experiment 1 had an MPN count of $>3.1 \times 10^5$, therefore uptake may have occurred but the dilutions used to inoculate the MPN Series were not extended enough to determine this. Note that on the bar graph a definite number was used, 3.1×10^5 for Experiment 1.

(For Experiment 2 the *E. coli* count in the control mussels was <400, the control counts for Experiments 4,6 and 7 were <4).

It was assumed that the high numbers of natural *E. coli* present was due to an insufficient depuration process. In Experiment 1 the mussels had been depurated in running water for 2 days, but not all of the sand had been removed from the tank. For the remaining experiments, all sand was removed and the depuration time was increased to 5 days.

Using this small modification, *E. coli* counts in the control mussels, for all experiments but two did not exceed 100 *E. coli* per 100g of mussel tissue. The high numbers of *E. coli* (1.6×10^3 / 100g mussel) present in the control mussels for the anomalous experiment (experiment 8) were due to the larger number of mussels used in this experiment (55) as opposed to the usual 10 mussels. An extra depuration tank was needed for those experiments using larger numbers of mussels. See Appendix C for the raw data for these experiments.

Preliminary experiments (2,4,5,6,7) indicate that after 2 hours of immersing mussels in water contaminated with *E. coli,* it was possible to detect a substantial number of *E. coli* in the analysed mussels. This suggests that 2 hours is sufficient time to enable mussels to pick up enough *E. coli* to notice the difference.

After 2 hours of immersing mussels in *E. coli,* the mussel slurry analysed gave results of around 10^4 *E. coli* per 100g of mussel, except when the control mussels contained 80 *E. coli* or more. This suggests there is good repeatability using 5 mussels.

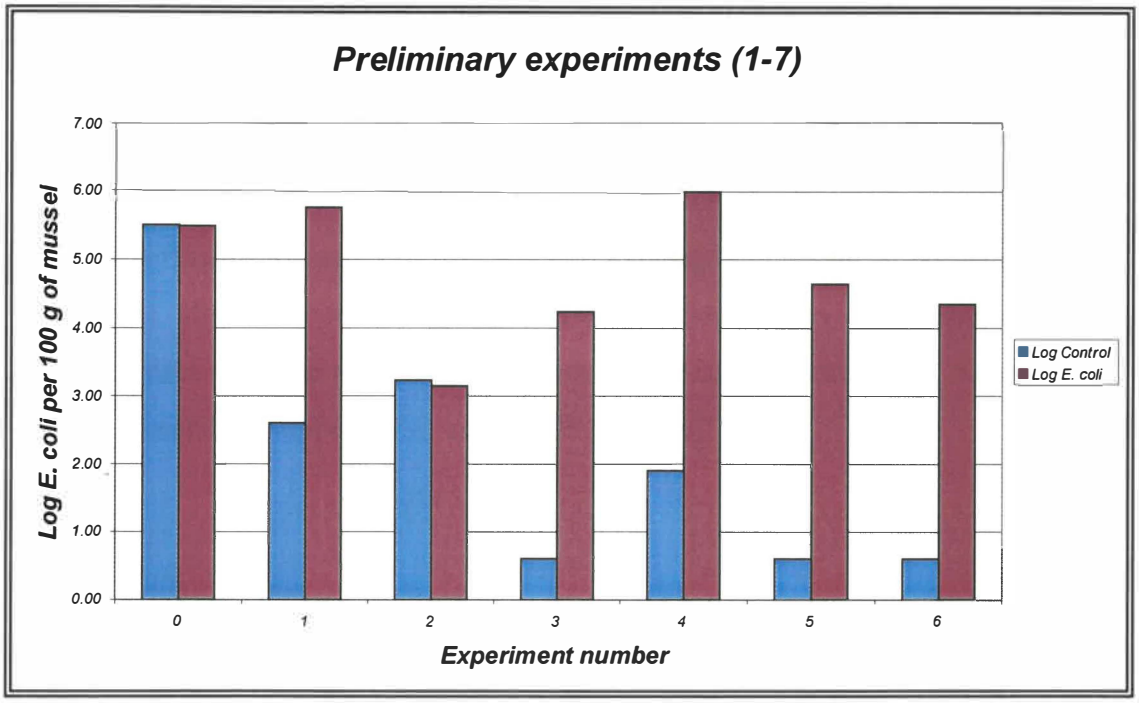

Figure 4.1 The results from Preliminary Experiments (1-7)

4.4 Summary

1. Depuration of experimental mussels for 5 days was successful at lowering natural *E. coli* present in mussels 12 out of 14 times (for all experiments).

2. 2 hours contamination was sufficient time to detect significant *E. coli* uptake in mussels.

3. Repeatable results were obtained using 5 mussels per MPN count.

4.5 Future Experiments

These experiments showed that it is possible to detect significant *E. coli* numbers in mussels after 2 hours of contamination. Further studies to determine the optimum time required to immerse mussels in faecally contaminated water and recover detectable numbers of *E. coli* would be useful.

Chapter 5

Determination of the Optimum Time required to immerse Mussels in a contaminated sample

5.1 Introduction

The overall aim of the study described in this thesis is to investigate the potential use of mussels for bacterial monitoring. Before studying the uptake of *E. coli* and *Salmonella* by mussels, it was necessary to determine the optimum time required to immerse mussels in a contaminated sample before analysis. The previous preliminary experiments indicated that 2 hours was a sufficient time to detect significant uptake of *E. coli* in the mussels. The aim of these experiments is to investigate how long it takes for the bacterial numbers in the mussels to reach a maximum. After this time depurification will result in a decrease in numbers and thus decreasing sensitivity of detection. Therefore these results will give an indication of the time frame required to immerse mussels in a waterway that is thought to be faecally contaminated.

Three experiments were completed, described below as experiments 8, 9, and 10, the raw data for these experiments are listed in Appendix C.

5.2 Materials and Methods

5.2.1 Media

The media used for enumeration of *E. coli* were TSA, TSB, LT, peptone and EC + Mug. Methods for their preparation are described in Section 3.7.1.

5.2.2 General Methods

The methods used for the collection, and storage of mussels is described in 3.2. The methods used for depuration are described in Section 3.3.

Preparation of the *E. coli* suspension is described in Section 3.4. Suspension concentrations of between (1.8×10^4 - 3.2×10^5), (4.2×10^5 - 8.1×10^5) and (1.3×10^5 - 2.0×10^5) of total *E. coli* per 3500 ml were used to contaminate mussels in experiments 8,9 and 10 respectively. See Appendix C for the concentration used for individual beakers (beakers representing different time intervals in each experiment).

The preparation of the mussels for contamination is described in Section 3.4 and for their sampling and homogenisation in Section 3.5.

The MPN method used is described in Section 3.7.4.

5.2.3 Enumeration of *E. coli* in suspending water

The pour plate method is a convenient method but is only suitable for bacteria that are not inactivated by exposure to temperatures of 43-45°C. It was therefore used here in experiment 8 for the enumeration of *E. coli* numbers in water samples taken from experimental beakers (in experiments 9 and 10 the membrane filter method was used to analyse water samples, this method has been previously described in Section 3.6.3).

The pour plate method used mFC agar which was prepared as described in Section 3.7.1. and left in a water bath preheated to 44.2°C for cooling and tempering the culture medium after boiling to melt the agar.
For each water sample, 100 mls was taken from the experimental beaker and shaken before 1 ml was removed by pipette and added to a sterile Petri dish. The bottle containing molten mFC was quickly removed from the waterbath, the lid removed and the opening flamed before pouring into the Petri dish. The agar was then left to set before being transferred to an incubator set at 44.2°C. Plates were incubated in an inverted fashion. After 24 hours of incubation the colonies were counted and recorded.

5.2.4 Enumeration of *E. coli* in Mussels

Five Mussels taken from beakers at different time intervals (see experiments 8-10, below) were prepared as described in Section 3.5 and counted using the MPN method as described in Section 3.7.4.

5.3 Experiment 8

10 x 5000mL sterile beakers, each containing 3500mL of UV treated dechlorintated freshwater, were each contaminated with total *E. coli* concentrations of between ($1.8 \times 10^4 - 3.2 \times 10^5$ / 3500ml). Beakers (on magnetic

stirrers) were left for 10 minutes before 100ml water samples were taken for analysis by using the spread-plate (0.1 ml used per sample) and pour plate (1 ml used per sample) methods (WHO, 1982). The spread-plate method is described in Section 3.7.2. and the pour-plate method in Section 5.2.3.
Water samples were stored in the cool room at 4°C for approximately 2 hours until being analysed.

5 mussels were then added to each beaker, and mussels were left for various times before being analysed. The mussels in their beakers were kept in a temperature controlled room (at 15°C), on a magnetic stirrer, until being removed for analysis. 5 control mussels (uncontaminated) were analysed, at Time 0, using the MPN method to determine the level of *E. coli* naturally present in the mussels before they were contaminated.

At 0.25, 0.5, 1.0, 1.5, 2.0, 4.0, 6.0, 8.0, 24.0 hours, 100ml water samples were collected from each beaker. Immediately after removing the water sample at each time, the 5 mussels were removed and the *E. coli* enumerated by MPN. Counts were expressed as *E. coli* / 100g mussel. Five mussels kept in a beaker to which no *E. coli* had been added were analysed after 24 hours for *E. coli,* using the MPN method. This served as the negative (background) control.

5.4 Experiment 9

5.4.1 Enumeration of *E. coli* in mussels

Experiment 9 was a repeat of Experiment 8 described above. It differed from Experiment 8 in two respects.

1. The beakers were each contaminated with total *E. coli* concentration of between $4.2 \times 10^5 - 8.1 \times 10^5$ / 3500ml.
2. The sampling times were 1.0, 2.0, 4.0, 6.0, 8.0, and 24 hours.

All other experimental conditions and experimental procedures were described in Experiment 8 (Section 5.3).

5.5 Experiment 10

5.5.1 Enumeration of *E. coli* in mussels

Experiment 10 was done in exactly the same way as experiments 8 and 9 except that :

1. The beakers were each contaminated with total *E. coli* concentrations of between $1.3 \times 10^5 - 2.0 \times 10^5$ / 3500ml.
2. The sampling times were 4.0, 6.0, 24.0, 48.0, and 72.0 hours.

5.6 Results and Discussion

The results of experiments 8, 9 and 10 are shown below. See Appendix C for Raw Data.

The purpose of these experiments was to determine the time when maximum numbers of *E. coli* could be recovered from mussels following exposure.

Experiment 8

In Experiment 8 the background numbers of *E. coli* present in the control mussels were high, 1.6×10^3 per 100g of mussel, as to prevent any accurate and precise determination of the extent of *E. coli* uptake by the experimental mussels. Counts recovered after 24 hours of contamination, appear to exceed those *E. coli* initially added. Although it appears that the *E. coli* are multiplying in the mussels, the

numbers may also represent an incomplete homogeneous distribution of mussel tissue in the slurry.

However, these results do show sampling between 15 minutes of exposure and 6 hours is sufficient time to detect *E. coli* in waters containing an *E. coli* concentration of 10 per ml or higher, using mussels with high background numbers of *E. coli* (1.6 x 10^3 *E. coli* per 100g of mussel tissue), possibly mussels that have been sitting in the waterway, before the contamination event, that have not been depurated. These conditions would not give any quantitative information.

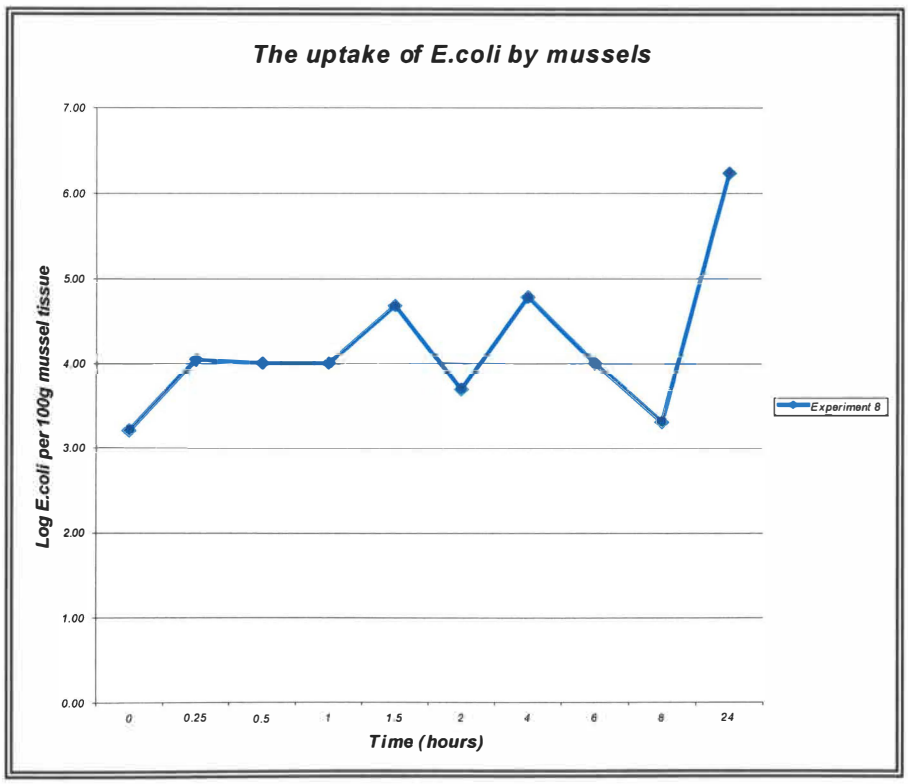

Figure 5.1 Uptake of *E. coli* by mussels (Experiment 8)

Experiment 9

By 1 hour of contamination rapid uptake had taken place, 6 x 10^4 *E. coli* per 100g of mussel tissue was recovered, 4.6 x 10^5 being the total *E. coli* initially added to the beaker water. Between 1 and 2 hours of exposure there was little change. Between 2 and 6 hours of exposure there was a steady increase of *E. coli* counted. The 6 hour MPN count apparently contained more *E. coli* (3.3 x 10^6 per 100g of mussel) than added to the beaker at the start of the experiment (4.9 x 10^5) as in the 24 hour result in Experiment 8. Between 6 and 8 hours of exposure numbers decreased, the mussels contaminated for 8 and 24 hours gave concentrations similar.

These results suggest sampling mussels from contaminated waters between 1 hour of exposure and 6 hours would enable the detection of *E. coli* in waters containing an *E. coli* concentration of 120 per ml or higher.

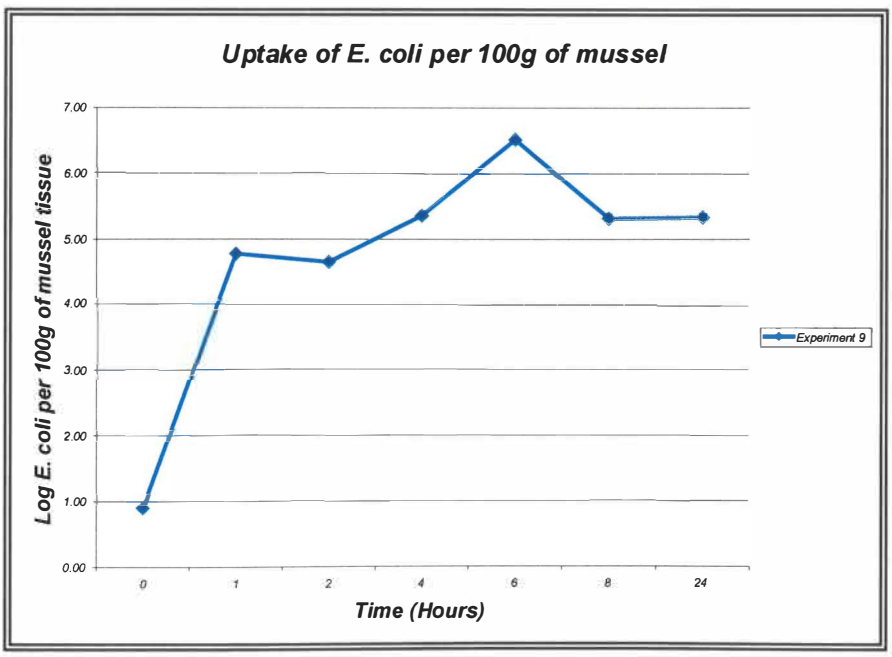

Figure 5.2 Uptake of *E. coli* by mussels (Experiment 9)

Experiment 10

The beaker waters used in Experiment 10 initially contained between 1.3 x 10^5 – 2.0 x 10^5 total *E. coli* / 3500 ml, less than the concentrations used to contaminate in Experiment 9. The *E. coli* numbers recovered per 100g mussel tissue ranged from 1.4 x 10^3 – 2.5 x 10^3 between 4 and 6 hours. The 24, 48, and 72 hour samples all recovered more bacteria than were intially added as in the 24 hour result obtained in Experiment 8. After 48 and 72 hours it was not possible to recover *E. coli* from the water samples.

The mussels in Experiment 10 were exposed to lower concentrations of *E. coli* (approximately 50 per ml between 4 – 6 hours) than Experiment 9 (approximately 120 - 180 per ml between 1 – 6 hours). This may explain the lower numbers recovered between 4 and 6 hours in Experiment 10 compared to Experiment 9.

Comparing the results of Experiments 9 and 10, it would seem that *E. coli* trapped by the mussels multiply in the mussel, however, another explanation may be that there may have been mussel tissue, probably from the gut, that had not been completely homogeneously distributed in the slurry.

These results suggest that sampling mussels from contaminated water (containing approximately 50 *E. coli* per ml) after 4 to 6 hours of exposure is enough time to detect the uptake of *E. coli* by the mussels and therefore faecal contamination.

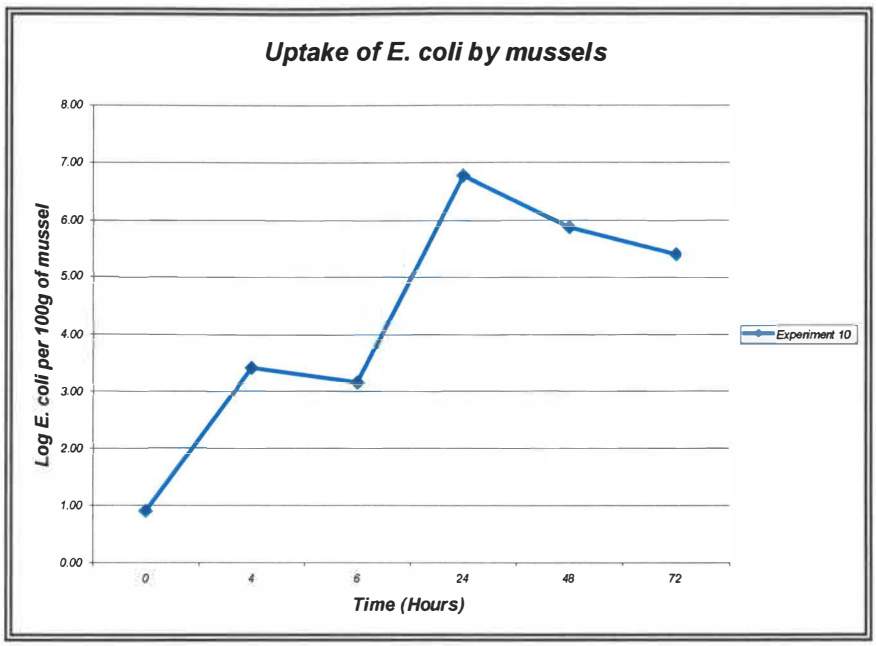

Figure 5.3 Uptake of *E. coli* by mussels (Experiment 10)

5.7 Summary

1. In Experiment 9 the mussels were exposed to higher concentrations of *E. coli* than Experiment 10. This may explain the higher initial uptake of *E. coli* per 100g of mussel tissue in Experiment 9 than 10.

2. All 3 experiments eventually recovered numbers of *E. coli* that exceeded those added initially to the beakers. No *E. coli* could be recovered from water samples after 48 hours.

3. These results suggest that for use as bacterial monitors, mussels analysed after exposure to contaminated waters (containing *E. coli* concentrations of 50 / ml or higher) between 4 to 6 hours will detect the presence of *E. coli* and therefore faecal contamination.

4. Although these results indicate that mussels could be used successfully as indicators of faecal pollution, the mussels did not take up the *E. coli* in a quantifiable way. From the MPN results of the mussels it was not possible to predict the numbers present in the water.

5.8 Suggestions for further work

It would be useful to know how long mussels retain the bacteria once uptake has occurred. This would inform samplers of the time frame they have to collect immersed mussels after a contamination event has occurred.

Chapter 6

The Retention of *E. coli* by Mussels.

6.1 Introduction

Mussels are filter feeders, trapping food particles in mucus covering their gills which is then drawn in to the mouth and eventually accumulated in the gut. Along with food particles, pathogens that may be present due to faecal contamination of the surrounding environment, may also be accumulated. Tissue localisation of contaminants have been determined for a number of mussel species and invariably the highest contaminant concentrations have been found in the digestive tracts of all species studied. Thus it has been deducted that contaminant ingestion is a function of shellfish feeding physiology (Power & Collins, 1989). This study has been conducted to determine how long mussels retain *E. coli,* or how rapidly they lose them, when put through a depurification process.

If mussels are to be used as bioindicators information on *E. coli* retention is required to allow environmental samplers to determine the time frame needed to collect mussels from sites that are thought to have been affected by a faecal contamination event. Sampling waterways for faecal contamination using grab samples is not always possible due to the distance samplers often have to travel to get to contamination sites. By the time they arrive they may have missed the contamination by hours. The use of mussels as bioindicators allows the detection of a faecal contamination event some time after it has passed.

The aim of this experiment is to determine how long mussels retain *E. coli* at detectable levels and thus giving an indication of the time frame samplers have to collect mussels from faecally contaminated sites. Mussels will be depurified employing the "closed" system with ultraviolet sterilisation, and using the MPN method.

Inital depuration of 40 mussels for 5 days.

↓

5 gaping mussels were retained as control mussels and were analysed by MPN. (Control 1)

↓

35 mussels were exposed to water containing *E. coli* and left for 24 hours in a temperature controlled room.

↓

After 24 hours of exposure 5 mussels were retained as an undepurated control zero time sample (Control 2).

↓

The remaining 30 mussels were then placed in the depuration tank where mussels were removed at various times and the retained *E. coli* counted by the MPN method.

Figure 6.1 The Retention experiment.

6.2 Materials and Methods

6.2.1 Collection and Storage of mussels.

The methods used for the collection, and storage of mussels is described in Section 3.2.

6.2.2. Initial mussel depuration.

The initial method used to depurify mussels for experimental use is described in Section 3.3.

6.2.3 Preparation of the Depuration tank.

The tank used to depurify mussels was thoroughly washed with hot soapy water, before being left to soak overnight in a detergent solution. On the day of the experiment the tank was given a thorough rinse with dechlorinated, UV treated water.

The depuration tank was a recirculating UV system. Water was circulated at 1 litre/30 seconds. The water was pumped to an elevated system which contained the UV system. The efficiency of the ultraviolet light was evaluated by taking regular samples of water that had passed through the light, before entering the tank and testing it using the membrane filter method.

6.2.4 Culture and Preparation of *E. coli.*

Preparation of E. coli.

E. coli NZRM 916 was sourced from the New Zealand Reference Medical Collection (Porirua, N.Z.).

For more detail on preparation of TSA slopes, TSA plates, TSB broth, see Section 3.7.1.

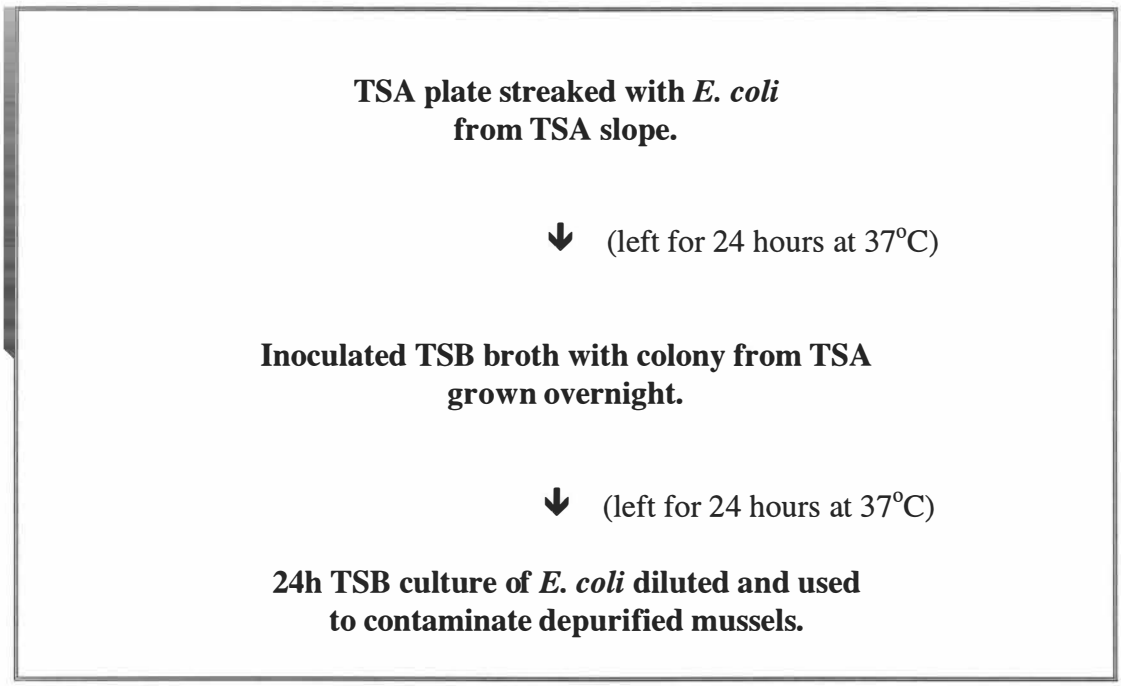

Figure 6.2 Preparation of *E. coli* suspension.

Dilutions used to inoculate mussels and MPN of suspension.

A dilution of 10^4 (2.4 x 10^6 *E. coli* per ml) of the *E. coli* grown overnight in TSB was used to contaminate the initial depurified mussels. The next step was to pipette 3.5 ml of this to each 5000ml beaker containing 3500 ml of dechlorinated, UV treated water. Therefore the total *E. coli* added to each beaker was 8.4 x 10^6 per 3500 ml of water. One ml dilutions 10^7 - 10^{11} were used to inoculate an MPN

series to determine the *E. coli* concentration in the original suspension and thus estimate the *E. coli* concentration of the diluted supension.

6.2.5 Preparation of Mussels.

The preparation of the mussels for the experiment are described in Sections 3.5.

6.2.6 Enumeration of Bacteria.

6.2.6.1 Media

The media used for the enumeration of *E. coli* were TSA, TSB, LT, peptone and EC + Mug. The method used to prepare these media is described in 3.7.1

6.2.6.2 Water samples

A sample of the water entering the depuration tank was analysed with the Membrane Filtration method described in Section 3.7.3. This water had passed through a UV steriliser. Samples were taken immediately before the experiment commenced and everyday until the experiment was completed.

6.2.6.3 Exposure of mussels to *E. coli*.

Once the 30 mussels had depurified for 5 days, 5 mussels were analysed by MPN to serve as a negative control to determine whether or not any *E. coli* were naturally present.

The remaining 25 depurified mussels were divided into 5 x 5000ml beakers containing 3500ml of UV treated water, dechlorinated water. Then 3.5 m1s of the 10^4 dilution were added to all of the 9 beakers and were placed in a temperature controlled room (at 15°C) for exactly 24 hours.

Following 24 hours of contamination :

- One set of 5 contaminated mussels were analysed by the MPN method, to determine the *E. coli* concentration in mussels after contamination, but before depuration (Control 2).

- The *E. coli* suspension was diluted in the range of 10^7 - 10^{11} and used to inoculate a MPN series to enumerate the *E. coli* concentration in the original suspension.

6.2.6.4 Final Depuration process

The remaining 30 contaminated mussels were removed for depuration and evenly spread on the bottom of the depuration tank which had UV-treated, dechlorinated water steadily flowing in and out at a rate of 1 litre/30 seconds.

The time at which the mussels were added to the depuration tank was recorded so that mussels could be removed and analysed by the MPN method after 4, 6, 24, 48, and 72 hours of depuration. 5 mussels were removed at a time for analysis. These mussels were selected from different parts of the tank to get a good

representative sampling. Mussels were prepared for the MPN method as explained in Section 3.5.

6.2.6.5 Enumeration of *E. coli* in mussels

Preparation of the mussels for homogenisation after depuration is described in Section 3.6.3.

The MPN method was used to enumerate the *E.coli* per 100g wet weight of mussels. This method is described in Section 3.7.4 and the following is a flowchart of the procedure (Figure 6.3).

At each time interval 5 mussels were removed from depuration tank for analysis by MPN.

↓

MPN tubes were left for 24 hrs at 37°C in an incubator.

↓

After 24 hours positive tubes were identified and a loopful from each transferred to 1 ml of EC + Mug.

↓

The EC + Mug was left for 24 hours in a waterbath at 44°C before being observed under a UV light for fluorescence, to determine the presence of *E. coli*.

↓

After 48 hours positive tubes from the remaining MPN tubes were transferred to EC + Mug.

Figure 6.3 The enumeration of *E. coli*.

6.3 Results and Discussion

The raw data for this experiment are listed in Appendix C.

The MPN results counted 2.4 x 10^{10} *E. coli* per ml in the original suspension grown up overnight in TSB. Therefore a 10^4 dilution of this suspension added to beakers had an *E. coli* concentration of around 2.4 x 10^6 per ml (3.5 ml of this dilution was added to the beakers therefore the total number of *E. coli* added to each beaker (containing 3500ml volumes of water) was 8.4 x 10^6).

The results of the MPN count for the Control 2 mussels (the mussels analysed immediately after 24 hours of contamination and before the final depuration process) was 2.6 x 10^4 *E. coli* per 100g of mussel tissue. This is the figure that was used to compare the MPN counts of the mussels undergoing final depuration. See Table 6.1.

The results show a greater than 50 % loss of *E. coli* by 4 hours of depuration.
(See Figure 6.4, a line graph of the results from this experiment, note, points have been connected for ease of reading).

	4hr	6hr	24hr	48hr	72hr
***E. coli* in Control 2 mussel /100g**	2.6 x 10^4	2.6 x 10^4	2.6 x 10^4	2.6 x 10^4	2.6 x 10^4
mussel weight (g)	74.3	76	72.2	70.2	67.6
***E. coli* in mussel per 100g**	1.5 x 10^4	7.3 x 10^2	6.1 x 10^2	6.6 x 10^2	1.6 x 10^2
% of Total *E. coli* retained	42.9	2.1	1.7	1.8	.42

Table 6.1 The figures used to calculate the percent of *E. coli* retained by Mussels.

By 6 hours of depuration a mere 2.1 % of *E. coli* had been retained by the mussels.

After 72 hours of depuration it was still possible to detect *E. coli* in the mussels by MPN since the *E. coli* count had not declined below that of the initial control mussels analysed, therefore in this experiment successful depuration was not achieved.

The water entering the depuration tank counted zero *E. coli* present per ml over the duration of the experiment.

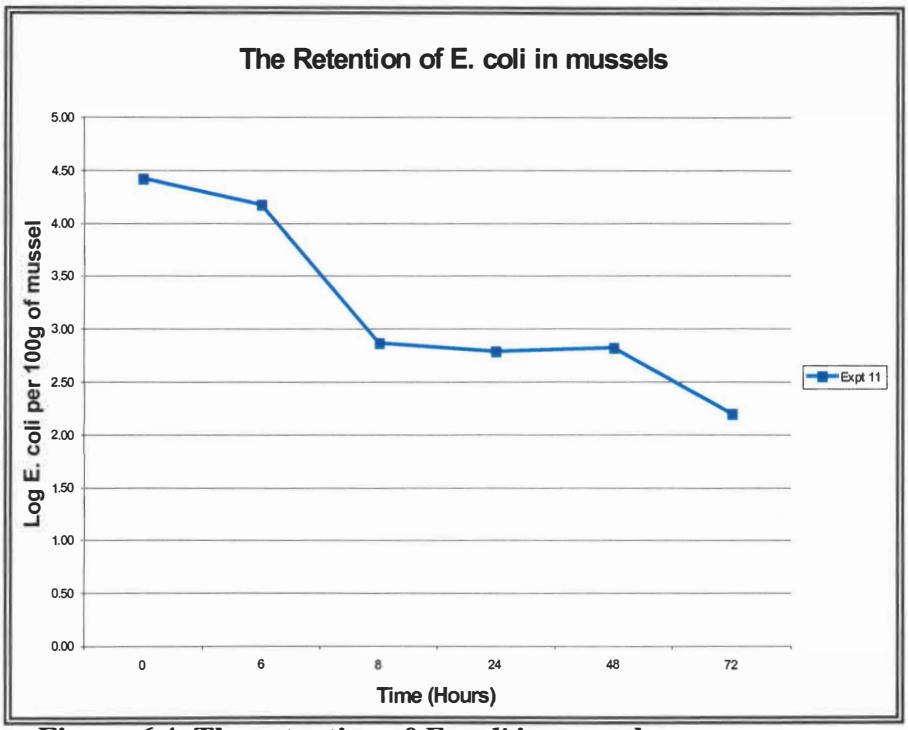

Figure 6.4 The retention of *E. coli* in mussels

In this experiment each beaker contained 2.4 x 10^3 *E coli* per ml, which would represent heavily contaminated water, this concentration of *E. coli* is approximately 33% higher than that found in primary treated raw sewage (800 *E. coli* per ml).

6.4 Summary

This study suggests that mussels placed at a site of intermittent contamination should be sampled no longer than 4 hours after the contamination event (in very dirty water).

By 4 hours the mussels will have retained 42.9 % of the *E. coli.* However, 2 hours later the mussels may only have retained 2 %.

6.5 Future Experiments

Identical experiments using lower concentrations of *E. coli* would provide a comparison for this experiment, to determine if the retention rate is the same.

Chapter 7

The uptake of *E. coli* versus the uptake of *Salmonella* by Mussels

7.1 Introduction

The genus *Salmonella* belongs to the family *Enterobacteriaceae. Salmonellae* are gram-negative, usually motile, non-sporing rods. They are facultative anaerobes, catalase positive and oxidase negative. Members of this genus are 1 to 2 μ in length, free growing, and oxidize amino acids aerobically, ferment carbohydrates to acid and gas anaerobically, to acquire energy. They are high-temperature mesophiles normally residing in human and animal intestinal tracts. They tolerate a wide range of acidity, temperature, and moisture conditions and therefore survive or grow in foods that do not support more fastidious bacteria.

Factors contributing to the spread of Salmonella.

1. Increasing populations with potential for contaminating the environment,
2. A mobile society,
3. Large centrally located food preparatory establishments,
4. Large mills processing animal feeds that are then widely distributed.
5. Proliferation of nonsterile but susceptible convenience foods,
6. The employment of subclinically ill and asymptomatic workers, and
7. Contamination of soils and waterways.

The interrelationship between these is shown in Figure 7.1. It can be seen that a major input of *Salmonella* into the aquatic environment results from effluent, sewage sludge and from farm livestock (including run off from pasture).
The salmonellae have been recovered from water originating with a sewage treatment plant that flowed many miles downstream under ice.

Definitions attributed to *Salmonella*

Salmonella infection is via the oral route and may result in either clinical or subclinical infection. Salmonellae may produce 3 main types of disease, but mixed forms are frequent

A. The "Enteric Fevers": Typhoid (*S typhi)* and paratyphoid (*S. paratyphi, S schottmulleri,* etc). Organisms ingested with contaminated food or drink reach the small intestine, from which they enter the intestinal lymphatics. They then travel via the thoracic duct into the blood stream and are thus disseminated into many organs, including the kidney and the intestines, where organisms multiply in lymphoid tissue and are excreted in the stool.

B. Septicemias: This is frequently due to *S choleraesuis*. Early invasion of the blood stream follows infection by the oral route, although intestinal involvement is often absent. The organisms are widely disseminated and tend to cause focal suppuration, abscesses, meningitis, osteomyelitis, pneumonia, and endocarditis, especially in debilitated hosts.

C. Gastroenteritis: (Often called "food poisoning") Several *Salmonella* are responsible including *S typhimurium* and *S enteritidis,* or *S derby*. Symptoms appear after only 1-3 days' incubation.
Serotypes *S Typhi* and *S paratyphi* A and B, constitute at severe hazard and are normally transmitted via contaminated water supplies and poor sanitary conditions (Lowry and Bates, 1989). After manifest or subclinincal infection,

some individuals continue to harbour organisms in their tissues for variable lengths of time. Three percent of survivors of typhoid become permanent carriers, harboring the organisms in the gallbladder, the intestine, or, the urinary tract.

The intention of this study was to compare the uptake of *E. col* , with the uptake of *Salmonella* by mussels. The *E. coli* and *Salmonella* experiments were conducted at the same time.

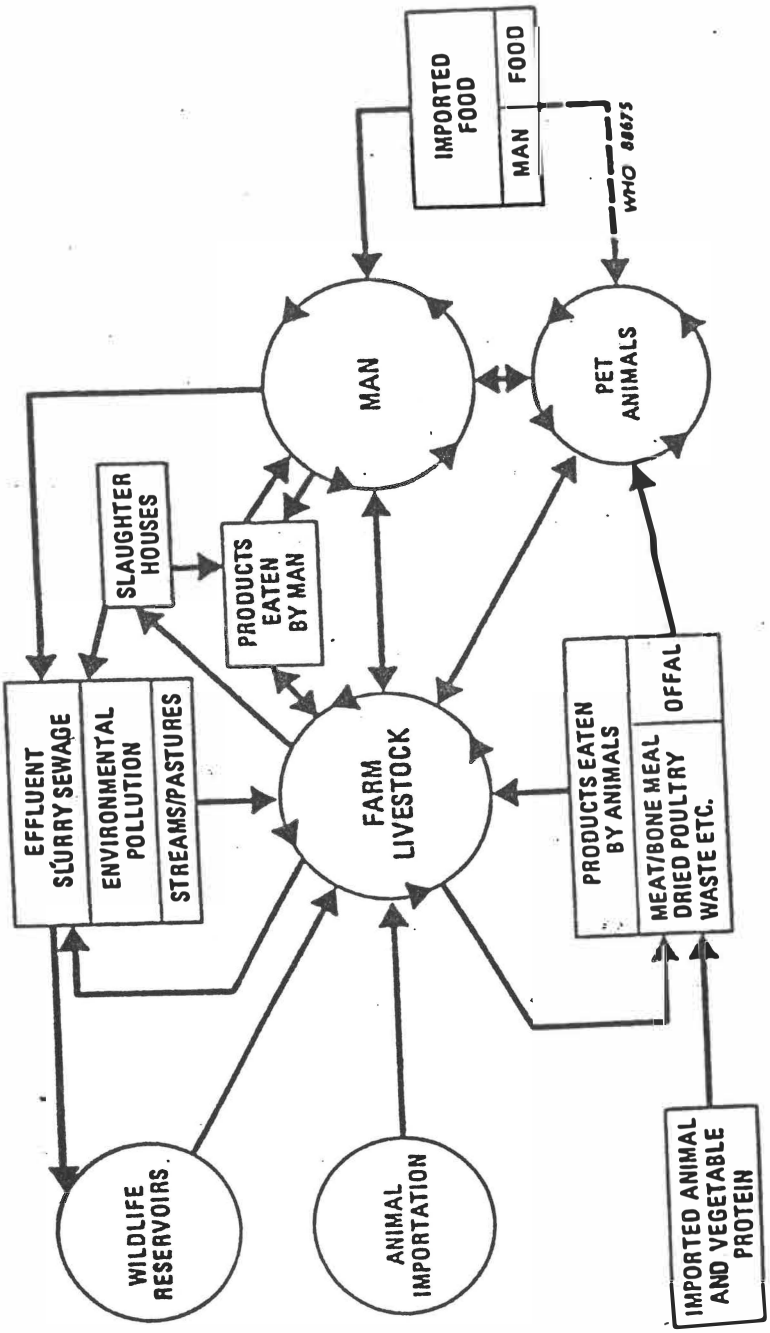

Figure 7.1 The *Salmonella* Cycle

7.2 Materials and Methods

7.2.1 Collection, and Storage of mussels.

The methods used for the collection, and storage of mussels is described in Section 3.2.

7.2.2 Mussel Depuration.

See Section 3.3 for information on mussel depuration method.

7.2.3 Preparation of Mussels for contamination.

The 60 mussels used in this experiment were collected, depurified, and prepared for contamination or control by the methods described in Sections 3.2, 3.3, and 3.5. Mussels to be homogenised were prepared using the methods in Sections 3.6.

7.3 Enumeration of *E. coli.*

7.3.1 Media.

Media used for the enumeration of *E. coli* was TSA, TSB, LT, peptone, EC + Mug. The method used to prepare these media is described in 3.7.1

7.3.2 Culture and Preparation of *E. coli*

The method used to culture and prepare the *E. coli* suspension for this experiment is described in Sections 3.4 and 3.7.1. 1 ml dilutions of 10^7 - 10^{11} were used to inoculate an MPN series to determine the *E. coli* concentration in the original suspension. A dilution of 10^5 (approximately 50 – 75 *E. coli* per ml) was added to each experimental beaker See Appendix C for the concentrations of *E. coli* detected in the waters of individual beakers.

7.3.3 Contamination of mussels by *E. coli*.

5 x 5000mL sterile beakers, containing 3500mL of UV treated dechlorintated freshwater, were each contaminated with a 10^5 dilution of *E. coli* culture grown overnight in TSB, see Sections 3.4 and 3.7.1 for preparation of *E. coli* culture.
Beakers (on magnetic stirrers) were left for 5 minutes before 100ml water samples were taken for analysis by the Membrane Filtration, see Section 3.7.3.

5 mussels were then added to each beaker, and mussels were left for various times before being analysed. 5 control mussels (uncontaminated) were analysed using the MPN method to determine the level of *E. coli* present in the mussels before they were contaminated

7.3.4 Enumeration of *E. coli* in mussels

At 2, 4, 6, and 24 hours, mussels were removed from their various beakers and examined by the MPN method for *E coli* per 100 g of mussel tissue. The MPN method is described in Section 3.7.4.

7.4 Enumeration of *Salmonella.*

7.4.1 Media.

The media used to enumerate *Salmonella* was 2% Buffered Peptone water, Rappaport-Vassiliadis soya peptone (RVS) broth, Xylose lysine desoxycholate (XLD) agar, 0.1 and 0.5% peptone to make up suspension dilutions and to make up mussel slurry respectively (See Appendix for media ingredients).

2% Buffered Peptone water.

Buffered peptone water, purchased from, was dissolved in distilled water to make up 1500 ml. This solution was then dispensed to 150 screw capped McCartney bottles. The bottles were then sterilised at 121^0C for 15 minutes. The solution had a pH of 7.2. This media was used in the preliminary resuscitation enrichment step.

Rappaport-Vassiliadis Soya Peptone (RVS) Broth.

RVS media, commercially prepared by Oxoid, was mixed with distilled water to make up a 1500 ml solution. The solution was heated gently, with frequent agitation before being dispensed in 10 ml volumes into screw-capped McCartney bottles. The bottles were then sterilised at 115^0C for 15 minutes. The pH of the RVS was 5.2.This media was used in the selective enrichment step.

Xylose Lysine Desoxycholate (XLD) Agar.

XLD media, from , was dissolved in distilled water. The solution was heated gently, with frequent agitation, to dissolve the agar. It was then brought to the boil before cooling to 55^0C before pouring plates. The plates were left to dry. The final pH of the XLD agar was 7.4. This agar was used in the selection/differentiation step.

Peptone.

The preparation of the 0.1 and 0.5 % peptone used to make up suspension dilutions and mussel slurry is described in Section 3.7.1

7.4.2 Culture and Preparation of *Salmonella*

Cultures of *Salmonella* were supplied by MIRINZ Food Technology and Research. A plate of TSA was streaked with *Salmonella* and incubated for 24 hours at 37^0C. A colony from the TSA plate was then used to inoculate 10 ml of Tryptic Soy Broth (TSB). The TSB was incubated overnight for 24 hours at 37^0C and was then used to make up diluted suspensions of *Salmonella*.

10-fold dilution series were prepared by initially taking 1 ml of inoculated TSB and transferring to 9 ml of 0.1% peptone to produce a dilution of 10^1. Thereafter the dilutions were repeated using the peptone only. A dilution of 10^5 (approximately 50 *Salmonella* per ml) was used to contaminate the mussels and 1 ml of dilutions 10^7 - 10^{11} were used to inoculate an MPN series to determine the *Salmonella* concentration in the original suspension.

The results of this MPN series allowed an estimation of the concentration of *Salmonella* in the 10^5 dilution used to inoculate beakers (5 x 10^4 *Salmonella* per ml) 3.5 ml of this dilution was added to each beaker giving a final concentration of about 50 *Salmonella* per ml in each beaker.

7.4.3 Contamination of mussels.

5 x 5000mL sterile beakers, containing 3500mL of UV treated, dechlorintated freshwater, were each contaminated with 3.5 ml of a 10^5 dilution of *Salmonella* culture grown overnight in TSB (1.8 x 10^5 total *Salmonella* added to each beaker, or approximately 50 per ml according to the MPN of suspension) see Section 7.2.3 for preparation of *Salmonella* culture. The beaker water was not analysed.

5 mussels were then added to each beaker, and mussels were left for various times before being analysed. 5 control mussels (uncontaminated) were analysed using the MPN method to determine if there were any *Salmonella* present in the mussels before they were contaminated.

7.4.4 Enumeration of *Salmonella* in mussels.

Preparation of mussels for MPN method.

The MPN test was completed as follows. A sample (5 mussels that had been homogenised in 0.5% peptone) was progressively diluted, in 10-fold steps, and multiple aliquots from each dilution step were inoculated to 2% buffered peptone water (Double and single strength). It is assumed that on incubation each tube that received one or more viable *Salmonella* in the inoculum will show growth (this was difficult to determine therefore all tubes were used to inoculate RVS Broth).

To make up dilutions of the sample, 1 ml of the sample was added to a tube containing 9 ml of sterile buffered peptone water. This was continued until the required dilutions were accomplished to inoculate MPN. Dilutions were mixed thoroughly before 1 ml was removed and transferred to another tube containing

9 ml of sterile buffered peptone water, see Figure 7.2 for an illustration of the MPN method for *Salmonella*.

For each dilution step (5 steps, 5 tubes of medium were inoculated. The volume of the test sample added to the buffered peptone water was recorded on MPN Sheets (see Appendix B), along with the dilution factor for each step,

SALMONELLA QUANTITATIVE MPN METHOD

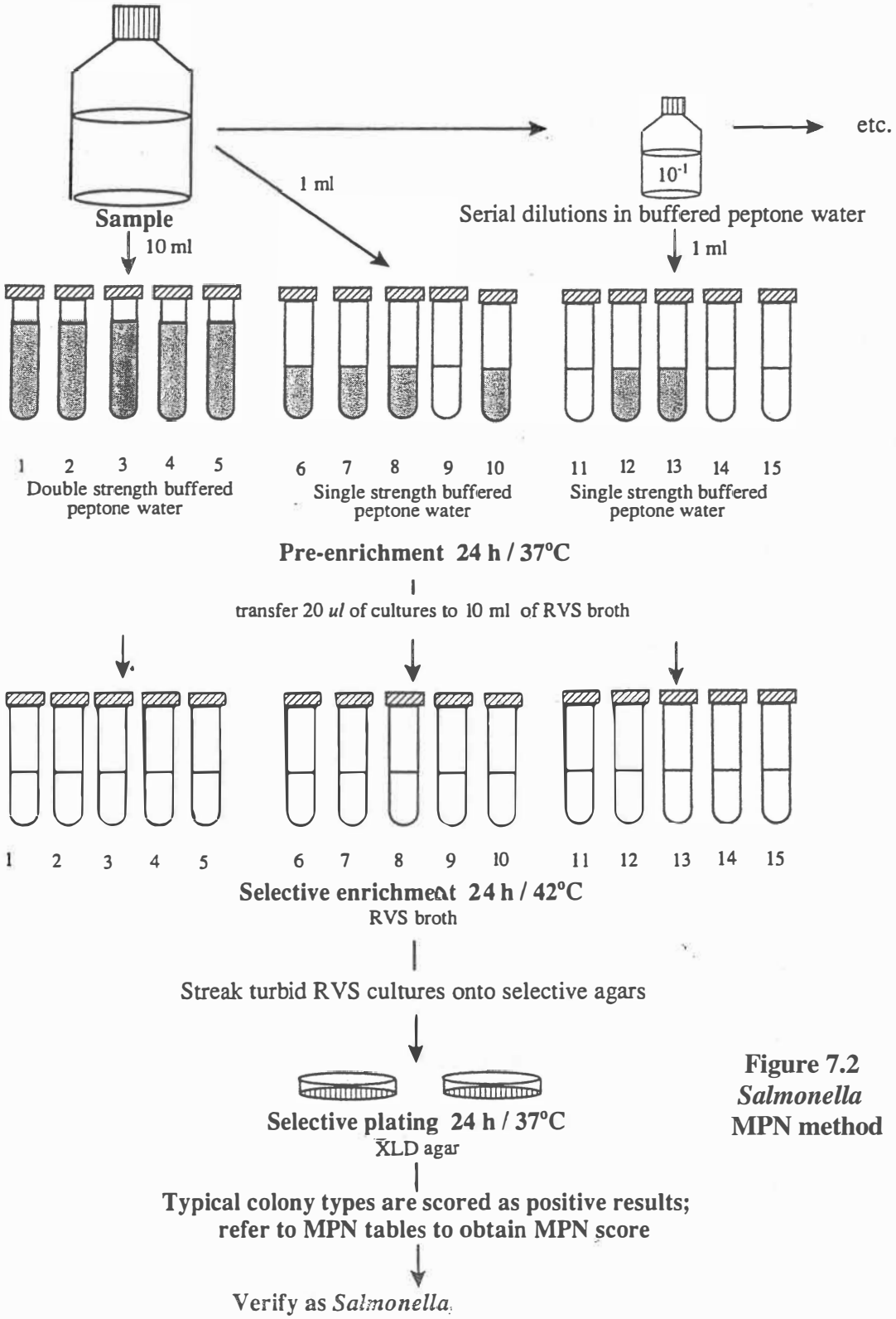

Figure 7.2
Salmonella
MPN method

Salmonella Quantitative MPN Method.

This test consisted of three stages :

Stage 1 **Preliminary Resusitative Enrichment.**

The non-selective medium used was buffered peptone water , which allows the resuscitation of injured cells that may have been sub-lethally damaged during heating, freezing and drying processes or by physical changes in the bacterial environment (ICMSF, 1978; MIRINZ, 1980; ISO, 1990). The pre-enrichment in BPW should not be too short since injured cells have an extended lag phase and during pre-enrichment these cells should not only have time to resuscitate but also to multiply (van Schothorst & Kampelmacher, 1968).

This medium was prepared beforehand and sterilized according to manufacturer instructions. Double strength medium in volumes of 10 ml were used for sample volumes of 10 ml. Double strength medium was only used for the control samples and the low concentration samples. All of the remaining samples were examined using single strength medium. Single strength medium in volumes of 10 ml were used for sample volumes of 1 ml to keep the nutrient concentration at the same level for both samples (1 ml and 10 ml). Dilution of the sample with buffered peptone water was also to prevent sample-induced changes in pH, nutrient composition or other characteristics of the medium that may affect recovery of injured bacteria. Tubes were marked from 1 - 25 and a record of the corresponding dilution was kept on MPN sheets. Once all the buffered peptone water tubes had been inoculated with the relevant *Salmonella* dilutions, the tubes were placed in an incubator for 20 hours at 37^0C.

Stage 2 **Selective Enrichment.**

0.1 ml from all of the buffered peptone water tubes were transferred to 10 ml of RVS broth (pre-warmed to 42^0C) this temperature is important for successful enrichment. When this temperature is too low many competitive organisms can multiply. When it is too high salmonellas may not multiply sufficiently or may even die. Therefore only several tubes should be removed from the waterbath at a time. RVS broth encourages the multiplication of salmonellae, while reducing and.or inhibiting the growth of competing organisms such as coliforms. Once all RVS tubes had been inoculated they were left in waterbaths at 42^0C for 24 hours.

Stage 3 **Selection / Differentiation**

The last step consists of transferring a loopful of the RVS culture to one plate of XLD agar. The RVS was streaked to obtain single, well isolated colonies. The plates were incubated at 37^0C for 24 hours. *Salmonella* colonies were the red colonies with black centres. The results from this MPN method were used determine the most probable number of *Salmonella* present in 100g (wet weight of mussels) by referencing the MPN probability table

7.5 Results and Discussion

See below for the results of the uptake of *E. coli* by mussels compared with the uptake of *Salmonella*.

(See Appendix C for the raw data and see Figure 7.3 for a line graph of the experiment. Points have been connected for ease of reading).

E. coli

These results show a rapid uptake of *E. coli* over the first 2 hours, increasing at a slower rate between 2 and 6 hours . However, those mussels exposed for 24 hours apparently contained more *E. coli* (1.1×10^6) than was added to the beaker at the start of the experiment (2.9×10^5). Although it seemed that *E. coli* could be multiplying in the mussel, it is also likely that mussel tissue, probably from the gut, may not have been completely homogeneously distributed in the slurry.

These results compliment those from experiments 9 and 10 (those results suggest that sufficent uptake of *E. coli,* by the mussels, for detection, had occurred between 4 and 6 hours of exposure to water containing *E. coli* concentrations of 50 per ml or higher). These results also suggest that exposing mussels to waters containing *E. coli* concentrations of approximately 50 per ml or higher, for between 2 and 6 hours, is sufficient time to detect the presence of *E. coli* and therefore faecal contamination.

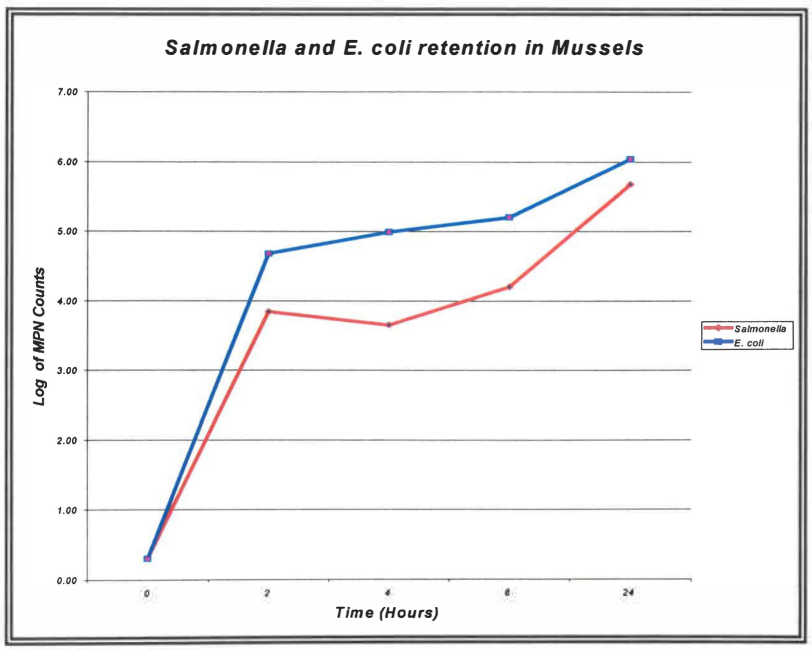

Figure 7. 3 The uptake of *Salmonella* and *E. coli* by mussels

Salmonella

Lower numbers of *Salmonella* were recovered at all time intervals compared to *E. coli*. However, Figure 7.3 shows the uptake pattern by mussels of both *E. coli* and *Salmonella* is similar. The *Salmonella* results after 24 hours of contamination have also shown a marked increase, with numbers exceeding those estimated to have been added to the beakers. Which may be due to incomplete homogeneous distribution of mussel tissue in the slurry.

These results show that mussels immersed in freshwater containing *Salmonella* (at concentrations of approximately 50 per ml) will accumulate sufficient *Salmonella* to allow direct counting using the *Salmonella* MPN method after exposure for only 4-6 hours.

Water samples collected.

No data was obtained for samples collected from *Salmonella* contaminated waters. XLD was not a suitable media to analyse water samples so analysis was abandoned.Therefore, the *Salmonella* count in the water, was estimated from the MPN suspension count, taking into account the inital dilution of 10^5 and multipling by the 3.5 ml added to each beaker.

7.6 Summary

1. These results suggest that the mussels bioaccumulate *E. coli* and *Salmonella* in a similar pattern.

2. These results show that mussels immersed in freshwater containing *Salmonella* or *E. coli* (at concentrations of approximately 50 per ml or higher) will accumulate sufficient *Salmonella* or *E.coli* to allow direct counting using the appropriate MPN method, after exposure for only 4-6 hours.

7.7 Future Experiments

Identical experiments using a variety of different pathogens and lower concentrations would provide a comparison for this experiment.

Chapter 8

The Immersion of Mussels in Raw sewage.

8.1 Introduction

Increasing population densities have increased the amount of liquid wastes now being produced. Safe disposal systems are required to prevent the contamination of waterways. Faecal contamination through untreated or inadequately treated sewage effluents entering lakes, rivers, or groundwater that serve as municipal water supplies, creates conditions for a rapid dissemination of the pathogens.

Waterways may be faecally contaminated by :

- Runoff
- Discharge from the meat industry
- Discharge from human wastewater
- Discharge from sewage treatment plants.

See Table 2.1 for numbers of bacteria in various industrial effluents.

This is of significance because of the many uses waterways have for the human population including :

- Drinking Water
- Swimming
- Fishing
- Boating

The degree of faecal contamination of a waterway may be established by demonstrating the presence of "indicator organisms". The "indicator organism" *E. coli* is frequently used for freshwater systems and was used in this experiment. The presence of *E. coli* can only establish the possibile presence of enteropathogenic organisms such as *Salmonella* and *Shigella*. Due to the presence of low numbers of these enteropathogenic organisms, direct detection is difficult. However, mussels filter large amounts of water while feeding, bacteria or pathogens in this water accumulate in the mussel and as a result become concentrated over time, increasing the ability of these microorganisms to be directly detected.

The intention of this study was to examine the immersion of depurified mussels in a sample of raw sewage (primary treated) before removing the mussels for analysis. The raw sewage was used to simulate natural conditions of contamination and was obtained from the Hamilton City Council Wastewater Treatment Plant. (See Table 2.3 for the numbers of total and viable bacteria in samples from different stages of sewage treatment and in the suspended biomass).

8.2 Materials and Methods

8.2.1 Media

The media used for enumeration of *E. coli* were LT, peptone and EC + Mug. Methos for their preparation are described in Section 3.7.1.

8.2.2 General Methods.

The methods used for the collection, and storage of mussels is described ın Section 3.2. The methods used for depuration are described in Section 3.3.

Dilutions 10^0 , 10^1 and 10^2 of the raw sewage were used to contaminate the mussels. These dilutions had an estimated concentration of 800, 80, and 8 *E. coli* per ml respectively.

The preparation of the mussels for contamination is described in Section 3.5 and of their sampling and homogenisation in Section 3.6.

8.3 EXPERIMENT 13

8.3.1 Preparation of Mussels for contamination

3 x 5000mL sterile beakers, containing 3500mL of UV treated dechlorintated freshwater, were each contaminated with a 10^2 dilution of the raw sewage (primary treated). The *E. coli* concentration of this 10^2 dilution is estimated to be approximately 8 *E. coli* per ml (estimated from a MPN of the raw sewage).

5 mussels were then added to each beaker, and mussels were left for either 4,6 or 24 hours before being analysed. The mussels in their beakers were kept in a temperature controlled room, on a magnetic stirrer, until removed for analysis. At Time 0, 5 control mussels (uncontaminated) were analysed, using the MPN method to determine the level of *E. coli* naturally present in the mussels before they were contaminated.

8.3.2 Enumeration of *E. coli* in mussels

The *E. coli* taken up by the mussels was enumerated using the MPN method described in Section 3.7.4.

8.4 EXPERIMENT 14

8.4.1 Preparation of Mussels for contamination

3 x 5000mL sterile beakers, containing 3500mL of UV treated dechlorinated freshwater, were each contaminated with a 10^1 dilution of raw sewage. The *E. coli* concentration of this 10^1 dilution is estimated to be approximately 80 *E. coli* per ml (estimated from a MPN of the raw sewage).

5 mussels were then added to each beaker, and mussels were left for either 4,6 or 24 hours before being analysed. The mussels in their beakers were kept in a temperature controlled room, on a magnetic stirrer, until removed for analysis. At Time 0, 6 control mussels (uncontaminated) were analysed, using the MPN method to determine the level of *E. coli* naturally present in the mussels before they were contaminated.

8.4.2 Enumeration of *E. coli* in mussels

The *E. coli* taken up by the mussels was enumerated using the MPN method described in Section 3.7.4.

8.5 EXPERIMENT 15

8.5.1 Preparation of the Mussels for contamination

3 x 5000mL sterile beakers, containing 3500mL of UV treated dechlorintated freshwater, were each contaminated with a 10^0 dilution of raw sewage. The *E. coli* concentration of this undiluted sewage is estimated to be approximately 800 *E. coli* per ml (from the MPN of the raw sewage).

5 mussels were then added to each beaker, and mussels were left for either 4, 6 or 24 hours before being analysed. The mussels in their beakers were kept in a temperature controlled room (approximately 15°C), on a magnetic stirrer, until removed for analysis. At Time 0, 5 control mussels (uncontaminated) were analysed, using the MPN method to determine the level of *E. coli* naturally present in the mussels before they were contaminated.

8.5.2 Enumeration of *E. coli* in mussels

The *E. coli* taken up by the mussels was enumerated using the MPN method described in Section 3.7.4.

8.6 Results and Discussion

Below are the results for Experiments 13-15. The raw data is tabled in Appendix C and Figure 8.1 is a line graph of the results from these experiments, the points have been connected for ease of reading.

Due to experimental error counts were not obtained from water samples therefore the initial *E. coli* numbers added to beakers was estimated using the MPN results of the raw sewage.

In these 3 experiments, mussels were exposed to 3 different dilutions of raw sewage, 10^0, 10^1, and 10^2. Results from the 3 experiments show rapid uptake of *E. coli* between 4 and 6 hours. The numbers recovered from Experiment 13 (using contamination of 8 *E. coli* per ml) and Experiment 15 (using contamination of 800 *E. coli* per ml) both continue to increase between 6 and 24 hours of exposure. In Experiment 14, little change has occurred between 4 and 6 hours. Numbers then begin to drop off after 6 hours of exposure.

These results suggest that mussels exposed to fresh water contaminated with *E. coli* concentrations of 8 per ml and higher, for only 4 to 6 hours, will take up detectable levels of *E. coli,* indicating faecal contamination has occurred.

Between 2 and 6 hours of exposure, mussels exposed to higher concentrations of sewage have taken up higher numbers of *E. coli* compared with those mussels exposed to lower concentrations of sewage, suggesting there may be some correlation between the concentration of contamination and the numbers of *E. coli* taken up by mussels.

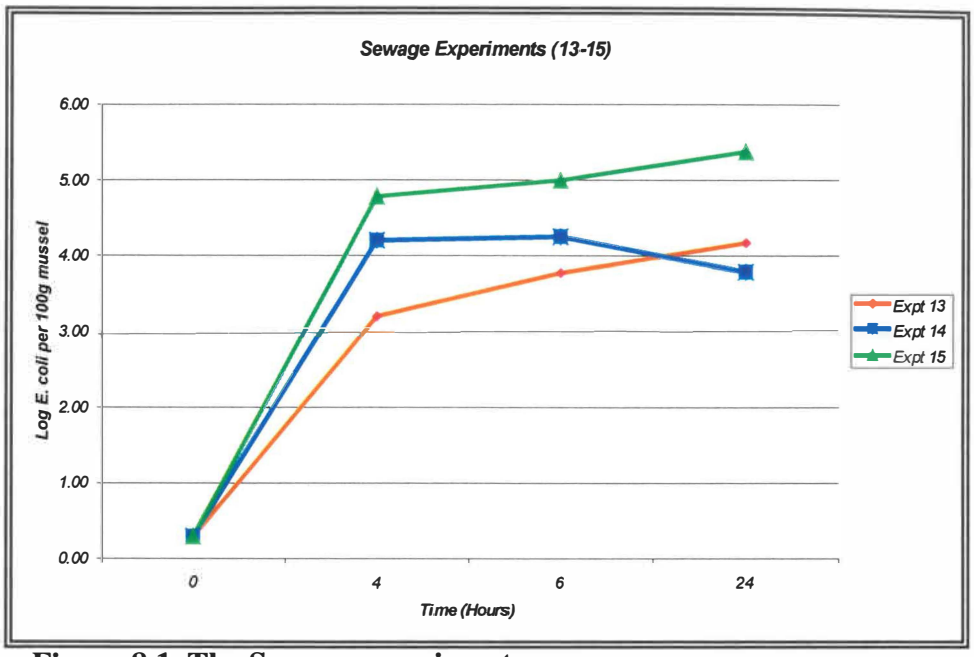

Figure 8.1. The Sewage experiments

8.7 Summary

1. *E. coli* from the different dilutions of sewage were taken up by the mussels in a similar way, there was rapid uptake within the first 4 hours of exposure.

2. These results suggest that mussels exposed to fresh water (contaminated with *E. coli* concentrations of 8 per ml and higher), for only 4 to 6 hours, will take up detectable levels of *E. coli* to determine a faecal contamination event has occurred.

3. The mussels that were exposed to higher concentrations of sewage also gave higher counts of *E. coli* during the first 6 hours. There may be a correlation between the faecal concentration in contaminated waters and the numbers of bacteria or pathogens mussels uptake.

Chapter 9

General Conclusions

Mussels (*Hyridella menziesi*) were tested for their ability to accumulate *E. coli* (representing faecal coliforms) and *Salmonella* (representing other pathogens) from their surrounding water.

These results show that mussels would be useful as biomonitors of faecal contamination because of their ability to accumulate bacteria while filtering the environment for food. By analysis of mussels, it was possible to detect the presence of *E. coli* and *Salmonella* taken up from surrounding waters. The uptake rate of these bacteria was rapid, within the first 4 hours, except in instances where the depuration process was unsuccessful leaving high background *E. coli* in the mussels prior to experimental contamination.

Once uptake had taken place and the contamination removed, results of the depuration experiment showed that there was a rapid loss of *E. coli* over the first 4 hours, although the presence of *E. coli* was still detectable. By 6 hours numbers had declined to very low levels – only some 2 % of the original numbers of *E. coli* remained.

Between 4-6 hours is suggested to be the time frame for collecting mussels from waters that have encountered a contamination event, because significant numbers of *E. coli* and *Salmonella* had been taken up. According to the results obtained from sewage experiments 13, 14 and 15, *E. coli* concentrations of 8 per ml and higher are detectable within this time.

Once mussels have been exposed to contamination for 4-6 hours the *E. coli* taken up are still detectable in the mussels up to 4 hours after the contamination has been removed. From a practical view point, this would allow several hours between a contaminating event such as a discharge and recovery of mussels for bacterial counting. This is an advantage if the discharge point is some distance from the laboratory. If grab samples, rather than mussels were the only means of monitoring sudden point discharges than the event could easily be missed.

There was also some indication in these experiments that there is a correlation between the concentration of *E. coli* present in surrounding waters and the numbers recovered from the mussels, the higher the concentration the higher the count. This was evident comparing the raw sewage experiments. Different dilutions of raw sewage indicated that within the first 6 hours of contamination the higher the concentration of sewage the higher the *E. coli* numbers recovered from the mussels. This was also indicated in Chapter 5 when comparing experiments 9 and 10. However, further work and statistical analysis is required to confirm this.

APPENDIX A

Media Ingredients

Media for E. coli MPN method.

Tryptic Soy Agar	
Ingredients	**g/l**
Tryptone	15
Soytone	5
Sodium chloride	5
Agar	15
Distilled Water	1000 ml
pH 7.03 - 7.57	

Lauryl Tryptose Broth	
Ingredients	**g/l**
Tryptose	20
Lactose	5
Dipotassium phosphate	2.75
Potassium phosphate	2.75
Sodium chloride	5
Sodium lauryl sulphate	1
Distilled Water	1000ml
pH 6.6 - 7.0	

mFC Agar	
Ingredients	**g/l**
Tryptose	10
Proteose peptone No. 3	5
Yeast extract	3
Lactose	12.5
Bile Salts no. 3	1.5
Sodium chloride	5
Aniline blue	0.1
Agar	15
Distilled water	1000 ml
ph 7.2 - 7.6	

Rosolic Acid Solution	
Ingredients	**g/l**
Rosolic acid	1
Sodium hydroxide	0.8
Distilled water	10ml

Media used for Salmonella Quantitative MPN Method.

2 % Buffered Peptone Water	
Ingredients	**g/l**
Peptone	20
Sodium chloride	5
Disodium phosphate (NaHPO4)	3.5
Potassium phosphate (KH2PO4)	1.5
Distilled Water	1000 ml
pH 7.0 - 7.4 at 25°C	

• Rappaport-Vassiliadis Soya Peptone (RVS) Broth	
Ingredients	**g/l**
Soya peptone	4.5
Sodium chloride	7.2
Potassium phosphate (KH2PO4)	1.26
Dipotassium phosphate (K2HPO4)	0.18
Magnesium chloride (anhydrous)	13.58
Malachite green	0.036
Distilled Water	1000 ml
Final pH 5.2 ± 0.2 at 25°C.	

• Xylose Lysine Deoxycholate (XLD) Agar	
Ingredients	**g/l**
Yeast extract	**3**
L-lysine	**5**
Xylose	**3.75**
Lactose	**7.5**
Saccharose (sucrose)	**7.5**
Sodium deoxycholate	**2.5**
Ferric ammonium citrate	**0.8**
Sodium thiosulphate	**6.8**
Sodium chloride	**5**
Phenol red	**0.08**
Agar	**15**
Distilled water	**1000 ml**
Final pH 7.4 0.2 at 25°C.	

APPENDIX B : MPN Probability table

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TABLE 908:II. MPN INDEX AND 95% CONFIDENCE LIMITS FOR VARIOUS COMBINATIONS OF POSITIVE RESULTS WHEN VARIOUS NUMBERS OF TUBES ARE USED PER DILUTION (10 ML, 1.0 ML, 0.1 ML)

Combination of Positives	Tubes per Dilution: 3			5		
	MPN Index /100 ml	95% Confidence Limits		MPN Index /100 ml	95% Confidence Limits	
		Lower	Upper		Lower	Upper
0-0-0	<3			<2		
0-0-1	3	<0.5	9	2	<0.5	7
0-1-0	3	<0.5	13	2	<0.5	7
0-2-0	—			4	<0.5	11
1-0-0	4	<0.5	20	2	<0.5	7
1-0-1	7	1	21	4	<0.5	11
1-1-0	7	1	23	4	<0.5	11
1-1-1	11	3	36	6	<0.5	15
1-2-0	11	3	36	6	<0.5	15
2-0-0	9	1	36	5	<0..5	13
2-0-1	14	3	37	7	1	17
2-1-0	15	3	44	7	1	17
2-1-1	20	7	89	9	2	21
2-2-0	21	4	47	9	2	21
2-2-1	28	10	150	—		
2-3-0	—			12	3	28
3-0-0	23	4	120	8	1	19
3-0-1	39	7	130	11	2	25
3-0-2	64	15	380	—		
3-1-0	43	7	210	11	2	25
3-1-1	75	14	230	14	4	34
3-1-2	120	30	380	—		
3-2-0	93	15	380	14	4	34
3-2-1	150	30	440	17	5	46
3-2-2	210	35	470	—		
3-3-0	240	36	1,300	—		
3-3-1	460	71	2,400	—		
3-3-2	1,100	150	4,800	—		
3-3-3	≥2,400			—		
4-0-0	—			13	3	31
4-0-1	—			17	5	46
4-1-0	—			17	5	46
4-1-1	—			21	7	63
4-1-2	—			26	9	78
4-2-0	—			22	7	67
4-2-1	—			26	9	78
4-3-0	—			27	9	80
4-3-1	—			33	11	93
4-4-0	—			34	12	93

TABLE 908:II. *(Continued)*

Combination of Positives	Tubes per Dilution: 3			5		
	MPN Index /100 ml	95% Confidence Limits		MPN Index /100 ml	95% Confidence Limits	
		Lower	Upper		Lower	Upper
5-0-0	—			23	7	70
5-0-1	—			31	11	89
5-0-2	—			43	15	110
5-1-0	—			33	11	93
5-1-1	—			46	16	120
5-1-2	—			63	21	150
5-2-0	—			49	17	130
5-2-1	—			70	23	170
5-2-2	—			94	28	220
5-3-0	—			79	25	190
5-3-1	—			110	31	250
5-3-2	—			140	37	340
5-3-3	—			180	44	500
5-4-0	—			130	35	300
5-4-1	—			170	43	490
5-4-2	—			220	57	700
5-4-3	—			280	90	850
5-4-4	—			350	120	1,000
5-5-0	—			240	68	750
5-5-1	—			350	120	1,000
5-5-2	—			540	180	1,400
5-5-3	—			920	300	3,200
5-5-4	—			1,600	640	5,800
5-5-5	—			≥2,400		

and 0.01 ml is planted, record 10 times the value shown in the table; if a combination of portions of 0.1, 0.01, and 0.001 ml is planted, record 100 times the value shown in the table; and so on for other combinations.

When more than three dilutions are used in a decimal series of dilutions, the results from only three of these are used in computing the MPN. To select the three dilutions to be used in determining the MPN index, taking the system of five tubes of each dilution as an example, the highest dilution that gives positive results in all five portions tested (no lower dilution giving any negative results) and the two next succeeding higher dilutions should be chosen. The results at these three volumes should then be used in computing the MPN index. In the examples given below, the significant dilution results are shown in

Faecal Coliforms in Shellfish by MPN

	Row 1					Row 2					Row 3					Row 4					Row 5				
Dilution																									
Tube No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
growth + gas in LT broth																									
growth + gas in Ec at 44.5°C																									
FC positive																									

Calculation:

MPN value (see Table) & 95% CI_____

Use the formula to calculate the most probable number of faecal coliforms per 100 mL of shellfish slurry (as provided)

*V = the volume inoculated & **DF** is the dilution factor in the first of the 3 rows used to obtain the MPN score*

$$MPN \times \frac{10}{V} \times DF =$$

Calculate the faecal coliform concentration in **100 g of the shellfish flesh** (use the information provided)

100 g of shellfish flesh contained _____ faecal coliforms

APPENDIX B : MPN Sheet

APPENDIX C : Raw Data

C.1 Results from Chapter 4 -

Preliminary Experiments (1-7)

Experiment No.→	1	2	3	4	5	6	7
A	87.6	76.4	93.9	70.1	109.1	97.2	105.0
B	3.2×10^5	<400	1.7×10^3	<4	80	<4	<4
C	7.9×10^4	2.3×10^6	3.9×10^4	1.8×10^6	4.6×10^7	1.1×10^7	3.5×10^4
D	$>3.1 \times 10^5$	5.8×10^5	1.4×10^3	1.7×10^4	10×10^6	4.4×10^4	2.2×10^4

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels (estimated from MPN of suspension)
D : *E. coli* (100/g) in mussels after 2 hours exposure to *E. coli*

C.2 Results from Chapter 5 -

Determination of the Optimum Time required to Immerse mussels in contaminated sample (8-10)

Experiment 8

Contamination → Time	0.25	0.5	1.0	1.5	2.0
A	99.8	106.8	100.4	112.9	83.3
B	1.6×10^3	1.6×10^3	1.6×10^3	1.6×10^3	1.6×10^3
C	2.9×10^5	1.8×10^4	1.4×10^5	1.8×10^5	4.8×10^4
D	1.1×10^4	1.0×10^4	1.0×10^4	4.8×10^4	4.8×10^3

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (from Control mussels)
C : Total *E. coli* in water immediately prior to adding mussels
D : *E. coli* (100/g) in mussels after exposure to *E. coli*

Contamination → Time	4.0	6.0	8.0	24
A	82.0	86.6	99.6	106.7
B	1.6×10^3	1.6×10^3	1.6×10^3	1.6×10^3
C	$.5 \times 10^4$	3.2×10^5	TNTC	1.8×10^5
D	6×10^4	1.0×10^4	2.0×10^3	1.7×10^6

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (from Control mussels)
C : Total *E. coli* in water immediately prior to adding mussels
D : *E. coli* (100/g) in mussels after exposure to *E. coli*

Experiment 9

Contamination Time→	**1**	**2**	**4**	**6**	**8**	**24**
A	112.4	117.8	141.1	140.8	137.3	125.6
B	8.02	8.02	8.02	8.02	8.02	8.02
C	4.6×10^5	6.3×10^5	4.2×10^5	4.9×10^5	8.1×10^5	4.6×10^5
D	6×10^4	4.5×10^4	2.3×10^5	3.3×10^6	2.1×10^5	$2.2\ 10^5$

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (from Control mussels)
C : Total *E. coli* in water immediately prior to adding mussels
D : *E. coli* (100/g) in mussels after exposure to *E. coli*

Experiment 10

Contamination Time→	**4**	**6**	**24**	**48**	**72**
A	62.2	63.9	69.5	58.1	52.7
B	8.02	8.02	8.02	8.02	8.02
C	1.9×10^5	1.9×10^5	2.0×10^5	1.3×10^5	1.3×10^5
D	$2.5\ 10^3$	1.4×10^3	6.0×10^6	7.5×10^5	2.5×10^5

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels
D : *E. coli* (100/g) in mussels after exposure to *E. coli*

C.3 Results from Chapter 6 -

The Retention of *E. coli* by Mussels (11)

Contamination Time➔	4	6	24	48	72
A	74.3	7.6	72.2	70.2	67.6
B	2.6×10^4	2.6×10^4	2.6×10^4	2.6×10^4	2.6×10^4
C	1.5×10^4	7.3×10^2	6.1×10^2	6.6×10^2	1.6×10^2

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels after contamination (from Control2 mussels)
C : *E. coli* (100/g) in mussels after depuration

C.4 Results from Chapter 7 -

The uptake of *E. coli* versus the uptake of *Salmonella* by Mussels (12)

E. coli

Contamination Time➔	2	4	6	24
A	83.3	85.0	86.4	110.1
B	26	26	26	26
C	2.3×10^5	1.6×10^5	1.7×10^5	2.6×10^5
D	4.8×10^4	9.8×10^4	1.6×10^5	1.1×10^6

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels
D : *E. coli* (100/g) in mussels after exposure to *E. coli*

Salmonella

Contamination Time→	2	4	6	24
A	86.3	83.9	93.3	97.83
B	<4	<4	<4	<4
C	1.8×10^5	1.8×10^5	1.8×10^5	1.8×10^5
D	7.0×10^3	4.5×10^3	1.6×10^4	4.8×10^5

A : Weight of mussel tissue (g)
B : *Salmonella* (100/g) in mussels before contamination (Controls)
C : Total *Salmonella* in water estimated from MPN of suspension
D : *Salmonella* (100/g) in mussels after exposure to *Salmonella*

C.5 Results from Chapter 8 -

The Immersion of Mussels in Raw Sewage (13-15)

Experiment 13

Contamination Time→	4	6	24
A	100.2	118.8	105.1
B	<4	<4	<4
C	2.8×10^4	2.8×10^4	2.8×10^4
D	1.6×10^3	6.0×10^3	1.5×10^4

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels (estimated from MPN of original sewage sample)
D : *E. coli* (100/g) in mussels after exposure to Sewage

Experiment 14

Contamination Time→	**4**	**6**	**24**
A	116.8	97.8	110.9
B	<4	<4	<4
C	2.8×10^5	2.8×10^5	2.8×10^5
D	1.6×10^4	1.8×10^4	6.23×10^3

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels (estimated from MPN of original Sewage)
D : *E. coli* (100/g) in mussels after exposure to sewage

Experiment 15

Contamination Time→	**4**	**6**	**24**
A	103.2	96.5	103.0
B	<4	<4	<4
C	2.8×10^6	2.8×10^6	2.8×10^6
D	6.1×10^4	1.0×10^5	2.4×10^5

A : Weight of mussel tissue (g)
B : *E. coli* (100/g) in mussels before contamination (Controls)
C : Total *E. coli* in water immediately prior to adding mussels (estimated from MPN MPN of original sewage)
D : *E. coli* (100/g) in mussels after exposure to sewage

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