ENUMERATION & IDENTIFICATION OF *Clostridium perfringens* IN RAW AND FINAL WATERS USING TWO TYPES OF MEDIA.

Presented in part-fulfillment for the
Degree of Master of Science in Environmental Protection

by

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The data in this study has been generated solely for the purpose of a student dissertation

Abstract

Enumeration & identification of *Cl. perfringens* in Raw and Final Waters using two types of media.

Brendan Bury

Cl. perfringens is a recognised indicator of faecal water pollution. There are several media that can be employed for the membrane filtration enumeration of this microorganism and so the main aim of this project was to investigate the ability of the new commercial Oxoid media, mCP, for recovering *Cl. perfringens* from drinking water, saline and river waters in comparison with an alternative, TSC Agar. In the Directive 98/83 mCP is the listed reference method for the analysis of *Cl. perfringens* and therefore all other similar methods must prove at least equivalent.

This project also tried to determine if there was any correlation between *Cl. perfringens* and coliforms in the environmental samples.

It was found the *Cl. perfringens* recovery efficiencies with TSC were significantly greater ($P<0.001$) than the corresponding values of mCP. TSC agar was found to be a better medium for large volumes of samples and was equivalent if not far better than mCP Medium.

There was only a poor correlation found between coliforms and *Cl. perfringens* in environmental samples.

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Abbreviations:

TSC	Tryptose Sulphite Cycloserine Agar
EY – TSC	Egg Yolk Free Tryptose Sulphite Cycloserine Agar
mCP	Membrane *Clostridium perfringens* Medium
KS	Kolmogorov-Smirnov Distance
m-Endo	Difco Brand name for Coliform enumeration Media
m-FC	Difco Brand name for Faecal Coliform enumeration Media
cfu	Colony Forming Unit
EU	European Union
Cl. perfringens	*Clostridium perfringens*
SD	Standard Deviation
SEM	Standard Error from Mean
r	Correlation

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1.0 Introduction:

Dublin City Council is the local authority with responsibility for local administration in the Dublin City area. The Central Laboratory is one of six divisions within the Councils Engineering Department and provides a range of analytical and advisory services to Dublin City Council and the Councils of Fingal, South Dublin and Dun Laoighre/Rathdown. The analytical services include microbiological analysis of environmental water samples for the compliance with appropriate National and EU legislation. Drinking waters from the distribution system and designated bathing waters are sampled and analysed in accordance with the requirements of the relevant items of legislation. Results are reported to the Environmental Protection Agency and the Department of the Environment, who compile monthly reports for the EU. Coliforms and *E. coli* have been traditionally used as indicators of faecal pollution and remain very important in monitoring raw and final waters. In addition to these indicators, *Cl. perfringens* analysis of surface waters used for human consumption is now required under EU legislation. Under the Council Directive 98/83/EU of 3 November 1998 On the Quality of Water for Human Consumption Annex III: Parameters for analysis using membrane filtration: Parameter *Cl.perfringens* 0cfu/100mls

The membrane *Cl. perfringens* medium (mCP) is the recommended method listed in the Directive for the analysis of *Cl. perfringens*. This includes incubation on mCP medium at 44°C/21hrs and confirmation with Crossley Milk Medium. Alternative methods can be used once Article 7(5) is met i.e. that an alternative method is "at least as reliable or equivalent" to the method stated in the Directive. It is proposed to use the media listed in the Directive along with another conventional media Tryptose Sulphite Cycloserine Agar (TSC Agar) when analyzing raw waters such as rivers, beaches, marine and estuarine samples and final drinking waters in the Dublin area.

Until recently there was no commercially produced dehydrated mCP medium available. Most labs used an alternative under Article 7(5) of the Directive (TSC, OPSP). Also, to prepare the mCP medium from the listed ingredients in the Directive would be difficult and time consuming. It would be expensive and some studies have suggested poor recoveries for mCP (M. Araujo, et al, 2001).

1.0 Introduction (contd):

Oxoid lab Media Ltd., have released their new commercial brand of mCP (Autumn 2001). It is proposed to compare this new product with a well-established media such as TSC Agar for performance and recovery of *Cl. perfringens* from raw and final waters. Another variable in this project is that there has been very little data collected on *Cl. perfringens* in water supplies both raw and final in the Dublin region. The methodology involves firstly, establishing limits of detection of both mCP and TSC using a dilution which will give 1 colony forming unit /1ml. Secondly, recoverability over a number of dilutions for both methods. Thirdly, the enumeration and isolation of *Cl. perfringens,* in raw and final water. If there is *Cl. perfringens* in environmental samples, then it is possible to determine if there is a correlation and significant difference (P value) between the performances of each media. The project will also examine if there is a correlation between *Cl. perfringens* and coliforms.

2.0 Liturature Review

2.1 Sources used in Literature Review

The sources used included the internet, UCD, DCU and DIT (Kevin St. and Cathal Brugha St.) and the British library. Supply companies like Oxoid and Technopath were also a source of useful information. A lot was also achieved by contacting people from local authority Sanitary Services Dept..

2.2 Human pathogen: *Clostridium perfringens*:

Clostridium perfringens is: a Gram +ve square ended anaerobic (ICMSF, 1996) (microaerophillic #) rod. *Cl. perfringens* is found in high concentrations in human and animal faeces aswell as sewage (USGS, 2000). 50 - 60% of humans carry *Cl. perfringens* and normally excrete 10^3 spores/g of faeces. It is commonly found in the environment such as soils, dust, flies and vegetables (ICMSF, 1996). *Cl. perfringens* is grouped into five types A - E, with A & C pathogenic to humans. Enterotoxins of type A (α toxin mw 34,000) are responsible for diarrhoeal disease. The toxin is produced as the vegetative cells sporolate in the intestine. The duration of the illness is generally short lived and mild (ICMSF, 1996).

Food vectors are usually cooked meats and poultry stored after cooking at ambient temperatures with long cooling periods. Consumption of large numbers of vegetative cells is believed to be a prerequisite for disease. A significant increase in numbers of spores in faeces is required for clinical involvement, usually > 10^6/g (ICMSF, 1996).

Therefore *Cl. perfringens* (type A & C) is a recognised pathogen found in the faeces of humans and can prove useful as a faecal indicator in pollution incidents.

Survive in up to 5% O_2 (Levett, 1990)

2.3 Taxonomy: *Cl. perfringens*

Cl. perfringens belong to the Phyla Eubacteria (16s rRNA Phylogeny). and is a member of the Kingdom Schizomycetes, Class II (sporeforming), Order II (Clostridiales), Family II (Clostridiaceae), Genus III(*Clostridium*)* (Deurden *et al*, 1991). It has low G + C (<50mol%). Clostridia are generally between 22 -55mol% with most clustered around 28% (Minton *et al*, 1989). Most Clostridia species are motile and are peritrichously flagellated. *Cl. perfringens* is different. It is non-motile which aids identification and differentiation.

Prazmowski (1880) classified *Clostridium* with 4 main characteristics (Minton *et al*, 1989):

1. Formation of an endospore
2. Anaerobic energy metabolism
3. Inability to carryout a dissimilatory reduction of Sulphate
4. Gram +ve wall which may react Gram -ve

Two reasons can be considered for the oxygen sensitivity of the Clostridia. Firstly a lack or shortage of defence mechanisms against toxic byproducts of O_2 metabolism such as hydroxides, superoxide anions and hydrogen peroxide. Secondly, interference with intermediary and biosynthetic metabolism (Minton *et al*, 1989).

Cl. perfringens differs from other Clostridia because the rods are large1 x 3-9μm (Adams *et al*, 1999) (Bergeys, 1974), encapsulated. It is also non-motile unlike *C. bifermentens* or *C. sordelli* . On TSC Agar *Cl. perfringens*, colonies are round large black 2-4mm in diameter. On m-CP, colonies are initially straw yellow which turn red/pink when exposed to ammonium hydroxide.

*The genus Clostridium was created by Prazmowski in 1880

2.4 Biochemical reactions used for confirmation of *Cl. perfringens*:

Cl. perfringens reduces *Nitrate, hydrolyzes lecithin using the enzyme lecithinase and produces acid/gas from the fermentation of lactose (unlike *C. sordelli*) (ICMSF, 1996), sucrose and raffinose. *Cl perfringens* is, also, acid phosphatase positive. Most *Cl. perfringens* are unable to ferment cellobiose (glucose β-D-glucoside) and mannitol. It was found by Cato et al (1986), between 11% and 39% of strains of *Cl. perfringens* are capable of fermenting cellobiose. It also reduces sulphite and liquefies gelatin. It can also produce acetone, butanol, ethanol, butyric acid, acetic acid, propionic acid and lactic acid (Bergeys, 1974).

Table 2.4.1

Confirmatory Tests:

[1,2]*Clostridium perfringens: Confirmatory tests*			
Test	***Result***	***Test***	***Result***
Spores	Central	***AMC***	+/-
Sporangia	Not Swollen	***Nitrate****	+
Motility	+	***Lm-Milk***	Stormy Fermentation
Glucose	+	***Gelatin***	+
Lactose	+	***Lecithinase***	+
Starch	+	***Sulphite***	+
Salicin	+/-	***Sulpher***	+

[1] Levett, 1990. [2] Mossel et al, 1991.

[2]ICMSF, 1996

***Note**: Other Clostridia have the same ability to reduce Nitrate to Nitrite (*C.celatum*, *C. paraperfringens*, *C. sardiniense*)but their inability to liquefy Gelatin within 44hrs and produce acid from raffinose within 3 days distinguishes them from *Cl. perfringens* (Labbe *et al*, 1992).

2.5 Temperature & Environmental Parameters For *Cl. perfringens*:

Table 2.5.1

[2]Limits of Growth *Cl. perfringens*

Parameters	*Min*	*Optimum*	*Max*
Temperature C	12	43 - 47	50
pH	5.5 - 5.8	7.2	8.0 - 9.0
NaCl tolerance %	----	5	----
Aw	0.97	0.95 - 0.96	0.93

[2]ICMSF, 1996

C. perfringens is mesophillic

Other authors have found that the temperature range for growth can be wider 6.5°C - 47°C or narrower 20°C - 50°C (Jay, 1996). Freezing or refrigeration of samples can prove lethal to spores and cells with reduced recoverability on selective media. It was found that vegetative cells are very sensitive to freezing, 6% survive at -23°C for 14 days. Spores on the other hand can survive at -18°C for 180 days. Only 4% of cells survive -17.7°C for 180 days in Chicken gravy (Jay, 1996). It was found though that holding samples at 5°C is more lethal to cells than at the lower temperature of - 18°C. *Cl. perfringens* can tolerate NaCL concentrations of between 5%-18%.

The resistance of spores in water samples to irradiation range from 1.2 - 3.4 Kgy. Spores are more tolerant of Chlorine Dioxide (20 - 80mg/l at pH 4.5, 6.5, 8.5) than *B. cereus* or *B stearothermophilus.* (ICMSF,1996)

2.6 Nutrient Requirements For *Cl. perfringens*:

Cl. perfringens requires proteinaceous food products to grow (poultry & meat) and a large number of growth factors. *Cl. perfringens* requires 13 amino acids 6 vitamins along with Biotin, panthothanate, pyridoxylan adenine. All 13 amino acids must be present for growth.

2.7 Samples - Saline, River and Drinking Water:

In this study samples were not pre-treated by heating to 75°C, as is common practice to eliminate vegetative cells.(Anon 1994). The proposed standard in the revised Directive (EU 1998) is for *Cl. perfringens* "including spores", and the method cited does not include heat treatment of samples prior to filtration. It is therefore, inferred that the standard is intentionally including vegetative cells if present. Previous studies (Sartory 1993) demonstrated that heat treatment at 70°C significantly reduced counts of sulphite reducing clostridia on the TSC incubated at 37°C. Omission of heat treatment allows optimum recovery.(Sartory *et al*,1998)

2.7.1 Marine and Estuarine Samples From Dublin Bay:

The proposed saline samples will be taken from Dublin Bay. The Bay lies immediately to the east of Dublin (53°21' N 6° 15' W) between Howth Head, to the North, and Dalky Island, to the South. In extent, it is about 10km from North to South. The main fresh water input is via the river Liffey with much smaller inputs from the River Tolka and Dodder to the North and south, respectively, of the Liffey (Dublin Bay Technical Report No. 6, 1992).

The old licensed disposal ground* was located c. 2 Km east of Howth Head and received sewage sludge from c. 565,000 people, following preliminary and primary sedimentation treatment at Ringsend. The treated effluent was from the Ringsend treatment plant and was discharged continuously to the mouth of the river Liffey via the ESB cooling water outfall at Poolbeg. All solid effluent is now converted to dry fertilizer (Biofert) and spread on land by farmers.

* Dumping has stopped since 1999

2.7.1 Marine and Estuarine Samples From Dublin Bay (contd):

The report found that the distribution patterns as detected for both *E. Coli* and *Clostridium* pointed to two 'Hot spots' in terms of bacterial contamination within the area investigated. One was the sewage outfall at the Nose of Howth and the other location showing high loading of bateria was centred close inshore at Dollymount Middle (fig 2.7.1 - 2.7.2 pg 9 & 10).

The report found a positve correlation between *E. Coli* and *Clostridium* ($P=<0.001$)

The recent initiated Dublin Bay Project, which started 2001, has as its main objective to investigate bacteriological contamination of Dublin Bay. Until this study no real test program had been set up for analysis of saline waters for *Cl. perfringens*..(Dublin Bay Technical Report No. 6)

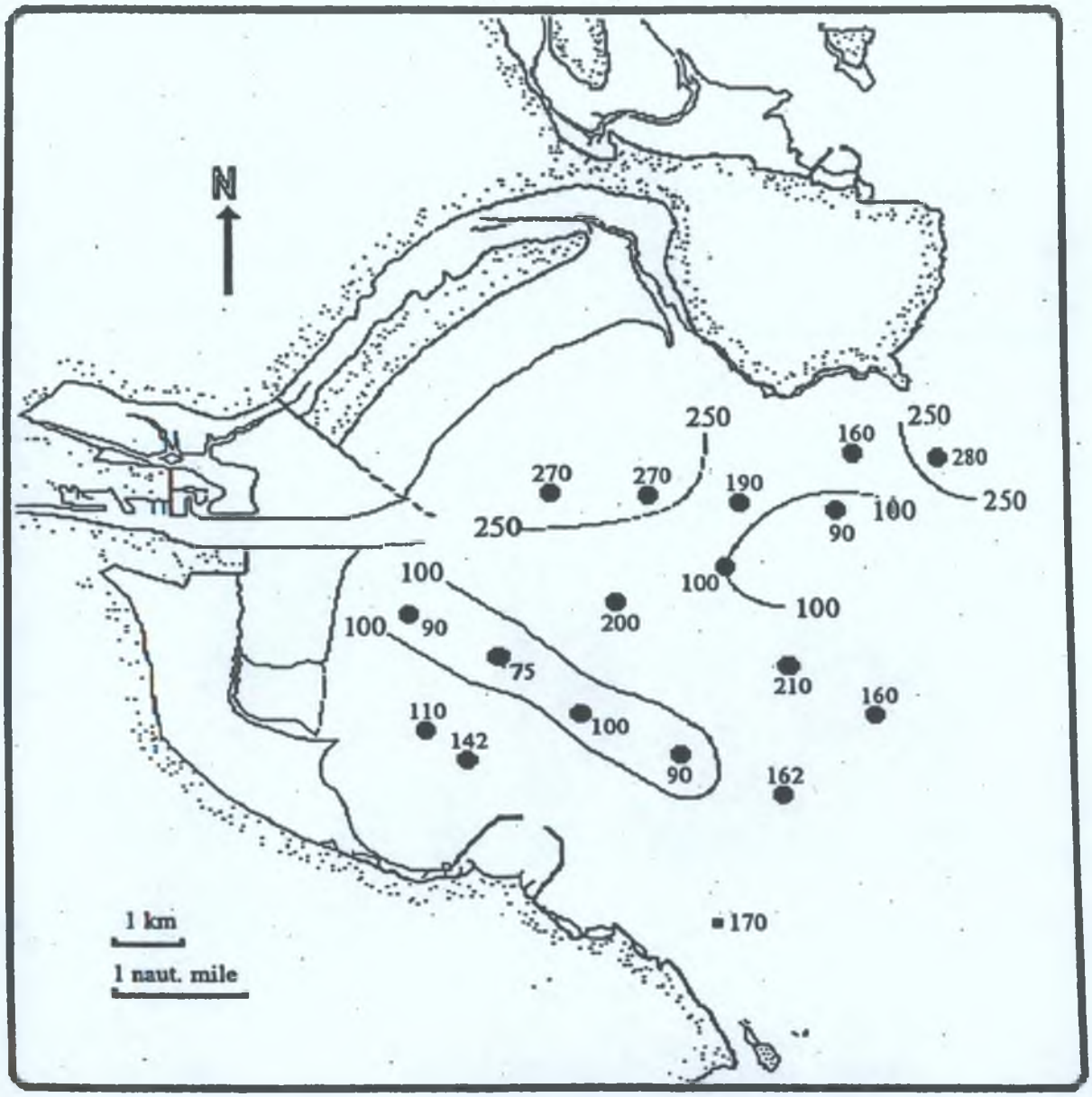
***Cl. perfringens*- Indicator of Marine and Estuarine Pollution:**

The indicator concept was first elaborated for drinking water where contamination was assessed on the basis of an operational response using multiple tube enumeration. This was later applied to Reritan Bay in the early 1900's following incidents of Thyphoid associated with the consumption of raw clams (Hackman *et al*, 1994).

Indicators provide information and can be used as an investigatory tool concerning source or age of pollution in non-impacted watersheds (Hackney *et al*, 1994).

In Ireland *Cl. perfringens* has not been widely used as an indicator organism of pollution. Most monitoring bodies such as The Environmental Protection Agency (EPA) and Local Authorities use total/ faecal coliform and faecal streptococcus as the main indicators of human pathogens and faecal contamination of water systems. This can be seen from the published yearly reports from the Irish EPA on Water Quality In Ireland*. Central Lab. Dublin Corporation (now Dublin City Council) are currently evaluating and validating methods and materials for analysis of *Cl. perfringens* , through this project, in conjunction with the parameters and method listed in 1998 EU Directive (98/83/EC).

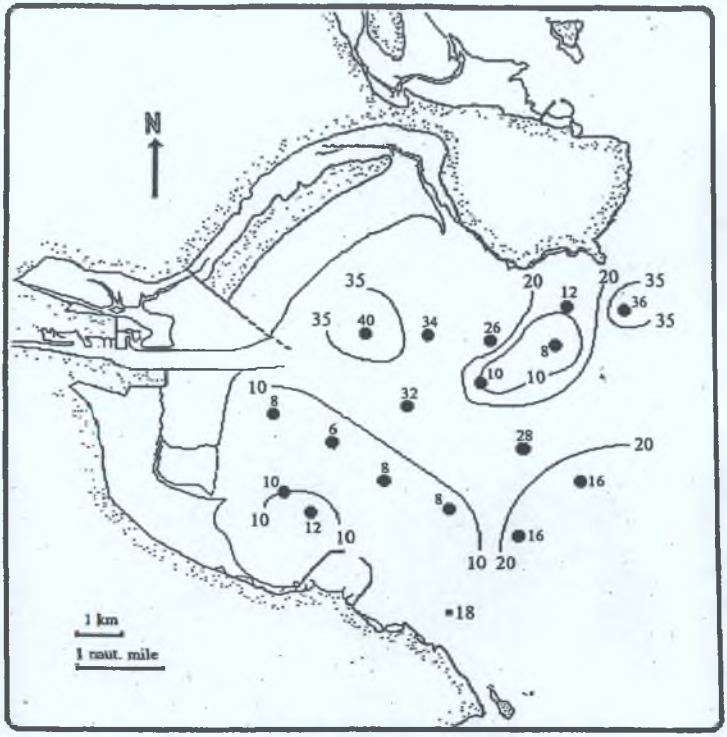
***note**: The EPA analyse water samples for *C. Perfringens* but do not print results in their reports at present.



Dublin Bay Water Quality Management Plan
— Studies on the benthos.

Distribution pattern for Clostridium (number g^{-1}).

Fig. 2.7.1



Dublin Bay Water Quality Management Plan
— Studies on the benthos.

Distribution pattern for E. coli (number g^{-1}).

Fig. 2.7.2

Cl. perfringens- **Indicator of Marine and Estuarine Pollution (contd.):**

The nearest Local Authority to Dublin, which would have comparable data is County Westmeath who carryout a yearly Spring time survey in April. The Environmental Health Officers take samples from Lough Leane, Lough Owel, Athlone, Mullingar, Moate (spring), Ballinahown (spring) and a group water well, which are analysed by the Sanitary Services within the County. They have found negligible amounts of *Cl. perfringens* in surface waters.

Some researchers have identified critical deficiencies with Coliforms in its use as an indicator of faecal contaminations or potential health risks in aquatic systems other studies have challenged the fundamental assumption that *E. Coli* is the most efficient predictor of enteric disease risk in marine waters. Pertinent factors to be considered for marine and estuarine samples are (Hackney *et al*, 1994).

Persistence

Non - Faecal Origin

Recoverability

Persistence:

There is an immediate drop off in the concentration of coliforms in sewage discharge due to dilution by physical mixing.
Light, temperatures, antagonism, inhibition, sedimentation all have an affect on Coliforms.

Also complex interactions occur of physical, biological and chemical processes. The organism has to adapt to adverse environmental conditions. In Dublin Bay Salinity measurements are 34 ppt and temperatures range from 6°C - 12°C in winter and summer respectively. Estuarine samples at the mouth of the river Liffey can vary considerably from 14 - 34 ppt salinity, which can have a drastic effect on survivability of an organism that can't osmoregulate sufficiently to cope with these fluctuations.

Non-faecal Origin:

Coliforms from a non-faecal origin can grow within the environment because of nutrients present in the water system. There can be an inability to differentiate these from a vertebrate source. Microbial grazers also consume indicator bacteria present.

Recoverability:

Significant number of cells starve in seawater and become unrecoverable and yet are still viable cells i.e. they don't grow on selective media when analyzed. It was reported up to 20% of viable cells would not grow on media, which causes an underestimate of colony forming units. *E. coli* (enterotoxegenic) was found to be nonculturable on a BHI synthetic seawater medium after 13hrs. The exceptions being, genetically engineered *E. coli* and strains with osmoregulatory differences. Coliforms and faecal streptococci, standard indicators of feacal pollution, are not expected to remain culturable under these conditions (Hill *et al*, 1993).

Cl. perfringens can be a useful as a water quality indicator because it forms spores that are resistant to disinfection and environmental stresses than other indicator bacteria. It can be useful as an index of sewage plume dispersion or transport of particulate bound material. *Cl. perfringens*, which is consistently present in sewage, has previously been proposed as an alternative indicator of faecal contamination under conditions where survival properties of the water quality indicators are critical(Hill *et al*, 1993). *Cl. perfringens* has proven useful in previous studies tracing sewage wastes in the marine environment. It is generally indicative of remote, intermittent and point source pollution.

In relation to Ringsend dried sewage biofert and Dublin Bay and estuary samples, *Cl. perfringens* is present in sewage sludge and contaminated sediments in numbers several magnitudes greater than naturally occurs in soil or sediments. *C. perfringens* counts of approximately 10^6 spores g-1 in sewage sludge and 10^1 to 10^2 spores ml-1 in effluents from sewage treatment plants have been reported. Therefore contamination of an estuary or bay with faecal matter would be readily detectable (Hill *et al*, 1993).

2.7.2 Drinking Water:

Using *Cl. perfringens* as an indicator is not without it own problems. Detection of vegetative cells in the environment is indicative of fresh untreated faecal matter. If spores are present it is difficult to index current pollution conditions because the spores are so persistent. *Cl. perfringens* is also widely distributed in soils and sediments naturally and can be transported into rivers, seas and lakes by runoff and movement of suspended solids.

In contrast, authors such as Garbow (1996) found *Clostridium perfringens* highly specific for faecal pollution. According to Payment and Franco (1993) it is the most reliable indicator for viruses and protozoa in treated drinking water. Sorensen (1989) found it a reliable indicator of faecal pathogens.

A study of three water treatment plants in Montreal, Canada, found that *C. perfringens* was the most suitable indicator for the inactivation and removal of viruses in drinking water treatment. It also appeared that there was a correlation with cysts and oocyats inactivation and removal and *C. perfringens*. Several other studies demonstrated that coliforms were inadequate to indicate the presence of pathogens especially viruses and parasites and that *C. perfringens* was a better indicator of water quality and treatment.

The three water plants in Montreal demonstrated that a high removal of both indicator microorganisms and pathogens was already attained after rapid sand filtration, between 11 - 112cfu *C. perfringens* remained after filtration. Overall removal of *C. perfringens* was >5-6$\log_{10}$ and <1 *C. perfringens* cfu in the finished water for all three plants.In the filtered water there was found to be significant correlations between *C. perfringens* and human enteric viruses ($r=0.42$, $p<0.02$). Also *C. perfringens* and somatic coliphages were significantly correlated with *Cryptosporidium* oocyst data ($r= -0.3$, $p<0.05$).This was found to be important because the removal of *C. perfringens* would also mean the removal of these other organisms. *Cl. perfringens* has a resistance to disinfection and this has been reported for chlorine dioxide and chlorine.

This ability makes it more suitable as an indicator for overall evaluation of water treatment than other organisms.

2.7.2 Drinking Water (contd):

The presence of *C. perfringens* in filtered water and not in finished water samples was found to be indicative of a less efficient treatment (i.e. Inferior flocculation or filtration) (Payment *et al*, 1993).

Another study, also, found that clostridia were present in larger numbers than viruses in river water and may have similar resistance to drinking water treatment. This may be useful for estimating the level of treatment obtained. The study found that *C. perfringens* was present at a level of 449 cfu/l from source water and 0cfu/l from finished water. Disinfectant (chlorine & chlorine dioxide was present in treated water as total chlorine (average: 0.55mg/l) or free chlorine (average: 0.37mg/l). The geometric mean were: for raw water 11,349cfu/100L, settled 83.8cfu/1000L, filtered 1.2cfu/1000L and finished water 0.0cfu/1000L. It can be seen from these figures that most of the closridia are removed after filtering (Payment, 1990).

Venczel et al (1996) proposed *C. perfringens* as a surogate or indicator organism for Cryptosporidium that could be routinely and reliably monitored to determine *Cryptosporidium* responses to disinfectants. It also demonstrated that mixed oxidant disinfectants had a synergistic affect on log reductions of both *Cryptosporidium* and *C. perfringens*.Inactivation of *C. perfringens* after 24hrs of contact, a 5mg/L dose of free chlorine produced a $1.7\log_{10}$ unit reduction of spores. Mixed oxidents with the same parameters produced $3.7\log_{10}$ unit reduction (Venczel *et al*, 1997).

Conversly , The presence of *C. perfringens* or *Cryptosporidium* does not guarantee that the other organism is present or absent.There might be no *Cryptosporidium* and thousands of *Cl. perfringens* cfu/100ml of sample.

2.7.3 Rivers & Streams:

It was found that *C. perfringens* could be a useful as a point source indicator in non-point polluted streams. It was discovered that *C. perfringens* could be detected, in decreasing concentrations, for more than 10 km downstream from municipal wastewater treatment plant effluents (Sorensen *et al*, 1989). Coliform and faecal streptococci concentrations varied widely along the streams in apparent response to non-point sources.

2.7.3 Rivers & Streams (contd):

C. perfringens appeared to be a sensitive indicator for microorganisms entering streams with municipal wastewater. It was found that animal faeces (horses, cows and sheep) faeces contained 100/g or 2-5 logs lower concentrations of *C. perfringens* than reported for human faeces. Also low concentrations of *C. perfringens* in the grazing area soil samples along the streams suggest that runoff from grazing land would contain low numbers of the organism.

Therefore it was found to be unlikely that appreciable concentrations of *Cl. perfringens* spores are contributed to streams from these non-point sources. The study showed that total coliform counts above the point of discharge and at the confluence immediately below a sewage treatment plant could both have relatively the same high numbers of coliforms present. It was also discovered that expected coliform die off with distance did not occur because of runoff from nonpoint sources. There was only a slow decrease of coliforms over a 15 km stretch of stream.

In contrast *Cl. perfringens* spores increased dramatically at the confluence with the sewage treatment plant and decreased rapidly over distance with very little interference from non point *Cl. perfringens* sources.

A study on Dublin streams and rivers for *Cl. perfringens* in comparison to other indicators has never been carried out. Total coliforms and faecal coliforms (*E. Coli*) have been the main indicators of pollution (Sorensen *et al*, 1989).

2.8 Culture Media for *Cl. perfringens*:

Different regulatory patterns in different anaerobic Clostridia can cause problems in choosing the right media. A surlpus of one amino acid might affect the biosynthesis capacity for other amino acids resulting in deregulation and growth cessation. Different species require an amino acid mix of different compositions. Trypticase-peptone, which is rich in compounds, provides the best all round media for *Clostridium*. The media must also contain reducing agents to sustain anaerobic conditions such as cysteine, sulphide, dithionate titanium or thioglycollate (Minton *et al*, 1989).

2.8 Culture Media for *Cl. perfringens* (contd)):

The media used in this project were egg-yolk free Tryptose Sulphite Cycloserine Agar (EY free-TSC Agar), membrane filtration *Cl. perfringens* Agar for presumptive identification of *Cl. perfringens* and Crossley milk medium for confirmation.

2.8.1 EggYolk free-Tryptose Sulphite Cycloserine Agar (EY-TSC Agar):

This media was proposed by Harmon et al (1971) and was a modification on the previously used Shahidi Ferguson Perfringens Agar (SFP). 400 μg of D- cycloserine per ml was substituted for Kanamycin and polymixin. It was found that 8 out of 10 strains of *Cl. perfringens* demonstrated better recoveries at 400 μg than 600 μg or 800 μg of D \cycloserine. Tolerancce of *Cl. perfringens* for D- cycloserine, its production of lecithinase, and its ability to reduce sulphite were the basis for the development of this media. The results showed that TSC allowed virtually complete recovery of most of the *Cl. perfringens* strains while inhibiting practically all faculatative anaerobes tested. SFP Agar allowed a slightly higher rate of recovery of *Cl. perfringens* but was found far less selective. The overall average recovery with the 10 strains for three trials on TSC was 90% and on SFP agar was 95% (Harmon *et al*, 1971).

The media involves incubation of plates at 37°C for 24 hrs in a anaerobic jar with a modified atmosphere of CO_2 and H_2 which provides the proper conditions for anaerobiosis. Satory et al (1986) suggested better recoveries by increasing the incubation temperature to 45°C/24hrs.

This Agar was documented as the most useful of the media for quantitative recovery of *Cl. perfringens* with adequate suppression of the growth of practically all facultative anaerobes. D - Cycloserine was used to inhibit growth of other species. Sodium metabisulphate and ferric ammonium citrate are used as an indicator of sulphite reduction by *Cl. perfringens*. These reacts with iron to form a black iron sulphide precipitate which produces black colonies (Oxoid monograph 2000). The egg-yolk (containing lecithin) present in the media can cause an opaque halo to be formed around distinctive black colonies of some presumptive *Cl. perfringens* , when hydrolysed by β-toxin (lecithinase) action. The modified egg-yolk free version was used because other anaerobes may produce a similar opaque halo reaction as *Cl. perfringens*.

2.8.1 EY free-TSC Agar (contd):

The halos may overlap causing a masking effect resulting in false positives. Studies have found that EY free-TSC Agar may give as good or better results than TSC Agar containing egg-yolk. The EY-TSC has the advantage of smaller colony formation which simplifies the counting of plates (Labbe *et al,* 1992).It also simplifies preparation and expense of the media with only one supplement required for addition at 50°C to the molten EY-TSC agar.

In a study it was found that *Serratia marcescens* and *Streptococcus lactis* were the only faculative anaerobes to grow on TSC agar. TSC also permits the growth of other sulphite reducing *Clostridium* species tested with the exception of *Cl. Sordellii,* which was completely inhibited and *C. bifermentans,* which was partially inhibited on TSC Agar (Oxoid Monograph & Harmon *et al,* 1971).

Sulphite reducing bacteria that may grow include *C. sporogenes, C. botulinum,C. paraperfringens* and *C. absonum*. (Labbe *et al,* 1992). The spores of the organism *C. sporogenes* and *C. botulinum* require at least 48 hr of incubation at 35°C in TSC Agar to obtain appreciable growth. It seems unlikely, therefore, that they would be detected within 24 hrs.

Mead (1985) concluded TSC Agar with or without egg-yolk was the best available medium for the enumeration of *Cl. perfringens*. Other authors found that out of 54 samples 10 samples displayed typical black colonies on TSC but none of those picked were confirmed as *Cl. perfringens*. (Neut *et al,* 1985).

2.8.2 Membrane *Cl. perfringens* Medium (m-CP Medium):

The m-CP Medium is recommended for the isolation and identification of *Cl. perfringens* in the European Council Directive 98/83/EC on the quality of water intended for human consumption. The problem, until recently, was that m-CP agar was not available commercially as a dehydrated media with supplements. All of the basic ingredients had to be sourced separately, mixed and rehydrated. This proved expensive and time consuming for most monitoring laboratories, so most labs resorted to tried and trusted dehydrated media such as TSC and OPSP agars for enumeration of *Cl. perfringens*.

2.8.2 m-CP Medium (contd.):

This was allowed once it could be proven, and data was available to demonstrate that, the alternative method was equivalent to the media m-CP listed in the Directive.Government monitoring laboratories in Ireland that use TSC instead of m-CP include the Environmental Protection Agency (EPA), Public Analyst and Cherry Orchard Water testing Laboratories. In Scotland, West of Scotland Water use OPSP Agar even though m-CP Agar is is specified in EU legislation and is also in the Water Supply (Water Quality) (Scotland) Regulations 2000 Schedule 4 Table A1.

The media was listed in the Difco manual of 1998 but was never produced by that company. However, in November 2001 Oxoid launched for the first time m-CP as a new product (Oxoid Culture Media Note 2001).

Bisson and Cabelli (1978) developed this media specifically for membrane filtration. Background growth was inhibited by the use of two antibiotics D - cycloserine and polymixin B sulphate and incubation at 45°C (see optimum temperature range in table 2.5.1, page 6) for 24 hr. This temperature was modified by work carried out by Armon and Payment (1988) to 44°C. It was found that incubation for 48 hr was problematic; colonies grew large and overlapped each other. In m-CP media germination of the spores occurs within a reasonable period of time, since the number of *Cl. perfringens* recovered from field samples did not increase with an increase in incubation time beyond 24 hr. Recoveries from seawater were found to be 90% (Bisson and Cabelli, 1978).

Differencial characteristics of mCP include:

I. The fermentation of sucrose differentiates *Cl. perfringens* from the greatest number of other species in the genus. Bromocresol blue is the indicator.

II.. Production of acid phosphatase also sets it apart from other *Clostridium*. Blaevic and Ederer (1975) described a test for phosphatase activity using phenolphthalein diphosphate as the substrate. The phosphate is cleaved from the substrate molecule by the action of acid phosphatase, and the typical colonies of *Cl. perfringens* turn a dark pink (phos+) after exposure to ammonium hydroxide fumes.

Differential characteristics of mCP include (contd):

III. Absence of β-D-glucosidase activity. Cellobiose (glucose β -D-glucoside) is not fermented by any strains of *Cl. perfringens* . Indoxyl β -D-glucoside was added to the media and colonies, which hydrolysed the glucoside, turn blue from the formation of indigo. Blue colonies found on plates after incubation are not *Cl. perfringens*. Armon and Payment (1988) modified the media by reducing the amount of Indoxyl β -D-glucoside required in the media, which has the benefit of reducing the cost of m-CP.
Cl. perfringens also requires iron and magnesium as growth factors.

Egg-yolk was initially added during trials for the media to look for lecithinase activity but it was found unsatisfactory because zones of percipitation (opaque halo) coalesced making enumeration difficult. The acid phosphatase test was substituted instead.
Due to the number of differential characteristics of the media it was found that *Cl. perfringens* could be separated from all but one other species of *Clostridium*, *C. Pasteurianum*. This organism has not proved a problem because it has not produced a false positive reaction (Bisson *et al*, 1979).

Colony appearance of *Cl. perfringens* on mCP:
The colonies are 1 to 3mm in diameter, convex with entire edges, somewhat opaque, slightly butryous in consistency, and a pale, yellow colour. Upon exposure to ammonium hydroxide colonies turn a pink to red colour (not purple). Bisson and Cabelli examined 500 colonies, 93% of presumptive colonies were verified as such with only 2% of presumptive negative colonies identified as *Cl. perfringens*. The combination of anaerobic incubation and the inhibitors included in the media reduced the density of heterotrophic organisms other than *Cl. perfringens* by at least three orders of magnitude. There was still, though, some background colonies observed.
The upper limit to the counting range for an unheated water sample was 85 to 90 *Cl. perfringens* per filter (Bisson *et al*, 1979)

2.8.3 Comparison of EY-TSC Agar with m-CP Agar:

Oxoid employed an accredited UKAS laboratory to carry out analysis on 366 routine water samples to compare the two media (TSC & m-CP). The accredited lab found that the sensitivity and specificity for the presence of *Cl. perfringens* in water samples was increased in m-CP Medium compared to TSC agar. The Oxoid lab also found that TSC colonies were difficult to determine because of diffusion or spread of the colonies over the entire plate. Conversely m-CP colonies were individual and easily counted.

The results from that study are tabulated below:

Table 2.8.1 Oxoid Study: Comparison of TSC and mCP Media (Summary)

Parameters	*TSC Agar*	*m-CP Medium*
True positives	64	125
False positives	133	28
% Sensitivity	47.8	93.3
% Specificity	42.7	87.9

The accredited lab concluded that m-CP medium reduces the amount of time before a result is obtained. There was found to be a reduction in false presumptive positive results, with an increase in selectivity and improved specificity.

Sartory et al (1986) discovered that TSC is considerably simpler and cheaper to prepare. In a study using domestic sewage effluents and surface waters, recovered greater numbers of **C.** *perfringens* than mCP, with similar confirmation rates (>90%). Sartory et al (1998) evaluated these two media for the recovery of *Cl. perfringens* in environmental and part-treated drinking water. It was found that m-CP was more selective and specific for the organism than TSC, but was markedly less efficient for the enumeration of both vegetative cells and spores. For samples of river water and part-treated drinking water, TSC recovered significantly greater numbers of *Cl. perfringens* than mCP Agar. The recovery of spores was 82.5% and 60% on TSC and m-CP respectively. For vegetative cells 95% and 0.7% on TSC and mCP respectively. This showed that there was a significant difference in the performance of both media (Sartory et al 1998).

2.8.3 Comparison of TSC Agar with m-CP Agar (contd.):

It was found by Sartory et al (1998) and also Burger et al (1984) that m-CP was a very poor medium for the recovery of vegetative cells. This is in contrast to Bisson and Cabelli (1979) who reported that mCP appeared to be efficient at recovering vegetative *Cl. perfringens* cells. Sartory et al (1998) demonstrated that TSC was more efficient at recovering environmentally stressed cells and spores that have passed through water treatment stages than mCP.

The confirmation rate of colonies on mCP was also found to be poor, 47.4% was reported. This was due to poor colour differentiation following exposure of colonies to ammonium fumes. This led to subjective identification of some colonies. There was also a high percentage of false negative results (16.9%) (Sartory et al 1998) compared with Bisson and Cabelli who reported 2.0% false negatives. This was probably due to atypical green or yellow colonies failing to turn pink, but were later confirmed as *Cl. perfringens* .

Another issue involved the differential characteristic of *Cl. perfringens,* which is not able to cleave Indoxyl β -D-glucoside causing the formation of straw yellow colonies after incubation. This does not prove true in all cases. It was mentioned earlier in section 2.4 that certain species of *Cl. perfringens* can cleave cellobiose and so the colonies could turn a green colour and therefore not be counted.

Araujo et al (2001) found that recovery efficiencies of five culture media, the recovery efficiency of TSC was significantly greater ($P=<0.05$) than the corresponding values of m-CP. It was reported that the average sensitivity of the methods were 66.7% and 70.6%, for recovery efficiencies 53.5% and 80.8% for m-CP and TSC respectively.

It was stated that there was no substantial reason for using mCP as a reference method all over the EU.

2.9 Membrane Filtration:

Advantages:

The speed of obtaining results, savings of labour, media, glass and cost of materials compared to MPN tube method. Samples can be filtered on site unlike MPN methods. Recommended method for water analysis.

Disadvantages:

Suspended solids such as iron, manganese, alum-floc or algae can block filter pores and prevent filtration, or can cause the development of spreading bacterial colonies that mask other colonies and prevent accurate counting.

Membrane filtration is not really suited to highly turbid waters with low concentrations of *Clostridium* densities.

Toxic materials such as metals, phenols, acids, caustics, chloroamines, and other disinfections by products may also adversely affect recovery of *Clostridium* vegetative cells on the membrane filter. Some lots of membrane filters produce low recoveries or poor differentiation of target and non-target colonies due to toxicity, chemical composition, or structural defects.(EPA manual)

2.10 Confirmatory Tests:

Crossely Milk Medium (Oxoid):

This media is made up of skimmed milk powder, peptone and bromocresol purple indicator. It was originally described by Crossley for the routine examination of canned food samples for anaerobic bacteria, This media is capable of giving rapid growth without the use of special anaerobic apparatus, yet the bacteria may be provisionally identified by their reactions upon the medium. There are seven different reactions which can occur with this media depending on the bacillus present in a sample. For Cl. perfringens the reaction is acid (bright yellow colour), with formation of firm 'stormy' clot and gas* (Oxoid manual 1998)

A loop of incubated Crossely milk medium with suspect *Cl. perfringens* was examined with Gram stain under a microscope.

*note: *C. tertium* also produces the same reaction.

2.11 The History of Relavent Water Legislation:

Directive (75/440/EEC)

Concerning the quality required of surface water intended for the abstraction of drinking water in the member states. The purpose of this directive is to ensure that surface water abstracted for drinking purposes is of a certain quality based on physical, chemical and microbiological parameters. There are some forty six parameters listed. The directive classifies waters into three categories:

A1: Water requires only simple physical treatment (filtration) and disinfection.

A2 : Requires physical treatment, chemical treatment and disinfection.

A3: Requires intensive physical, chemical treatment and disinfection.

The onus is on the sanitary authorities to monitor surface water.

Directive (79/869/EEC as amended by 81/855/EEC)

Concerning the methods of measurement and frequency of sampling and analysis of surface water intended for the abstraction of drinking water in member states. This directive is a supplement for 75/440/EEC and states the recommended methods for measurement of parameters, frequency of sampling in relation to each category (A1, A2, A3) and population size.

EC (Quality of surface water intended for the Abstraction of Drinking Water) Regulations, 1989:

The above two directives (75/440EEC & 79/869/EEC) were combined and transposed into this Irish legislation.

EC (Quality of Water intended for Human Consumption) Regulations, 1988:

This legislation brought into effect directive 80/778/EEC as amended by 81/858/EEC in Ireland. This piece of legislation sets out quality standards for waters intended for human consumption.

The Quality standards are divided into six tables:

A: Organoleptic parameters, e.g. Colour, taste.

B: Physio-chemical Parameters e.g. PH, Chlorides.

C: Substances which are undesirable in excessive amounts e.g. Phenol.

D: Toxic substances e.g. Cyanides, mercury.

E: Microbiological parameters e.g. coliforms.

Softened water parameters e.g. Alkalinity.

There are 48 maximum admissible concentrations (MAC) and 2 minimum required

EC (Quality of Water intended for Human Consumption) Regulations, 1988:

Concentrations are given in part I of the schedule. Part II of the schedule covers frequency of monitoring and part III reference methods.

Bathing Waters:

EC (Quality of Bathing Water) Regulations, 1988 and EC (Quality of Bathing Waters)(Amendment) Regulations, 1989.

These two pieces of Irish legislation implemented directive (76/160/EEC) concerning the quality of bathing waters. The legislation includes sampling plans, methods of analysis, inspection to comply with standards and an annex with a lists of parameters (microbiological, chemical and physical).

Local authorities have the responsibility to conduct regular monitoring:

First schedule: Designated bathing areas

Second Schedule: listed parameters to be used

Third Schedule: parameters under certain circumstances

Fourth Schedule: Methods of analysis and inspection to be used.

Directive 98/83/EC

Relating to the quality of water intended for human consumption. This directive updated directive 80/778/EEC (which is to be repealed after entry into force of the new directive) and reclassified parameters under three headings:

Annex I

Part A: Microbiological parameters

Part B: Chemical parameters

Part C Indicator parameters.

The relevant parts of this legislation which relate to this project includes:

Annex 1, Part A: Microbiological parameters for *E. Coli* (0 /100mls)

Annex 1, Part C Indicator parameters for *C. perfringens* (0 /100mls)

In Annex III, Specifications for the analysis of parameters, under the heading 1. "Parameters for which methods of analysis are specified" it specifies in detail the method to be used for the analysis of *C. perfringens*. The legislation states that membrane filtration using m-CP agar is the approved method in member states. It also lists the ingredients of m-CP agar in note 1. The reason for listing all components was due to the fact that there was no commercially available dehydrated media at the time. Laboratories would have to locate and purchase each individual component to formulate the media.

There was however, a clause, which allows member states to use an alternative method. This was found under the provision of Article 7(5b). This states, " other methods may be used, providing it can be demonstrated that the results obtained are at least as reliable as those produced by the methods specified. Member state shall provide the commission with all relevant information concerning such methods and their equivalence." This was the basis for this project, to discover if TSC agar was comparable, equivalent and at least as reliable as the new Oxoid m-CP agar.

EC(Quality of Water Intended for Human Consumption)(Amendment)) Regulations, 1999:

A number of provisions of directive 98/83/EC were implemented by this Irish legislation. Consumers must be informed of unacceptable risk to public health and action plans must be implemented to improve quality of water as soon as practicable of both public and private water supplies.

2.12 Method Validation and Statistical Analysis:

Principle:

Validation of a method is established by a systemic laboratory study that the performance characteristic of the method meet the specifications related to the intended use of analytical results. The performance characteristics determined for each method are:

(a) Limit of detection

(b) Recoverability

Limit of Detection:

This is determined by the analysis of 100 samples at a dilution with a maximum of 1 cfu/plate. The results will be statistically analysed for standard deviation, mean, standard error and confidence interval.

The degrees of freedom must also be recorded.

Recoverability:

This is determined by the analysis of 300 samples, 100 sample at 1:40 dilution,1:50 dilution and at 1:70 under repeatable conditions. The results will be statistically analysed for standard deviation, mean, standard error and confidence interval.

Statistical Analysis:

Statistical analysis was performed on all data obtained from analysis of water samples using Instat Version 3, Graph Pad (Instat Biostatistics, San Diego, California, USA). Significant differences were determined at the 0.1% level of significance ($P<0.001$).

Linearity:

This was determined by the analysis of paired samples for both methods, with the colony forming units per 100ml of sample spanning the range of the method. The results were used to calculate a regression line using the least squares method. Linearity is stated in terms of a correlation coefficient (r). The coefficient r is a measure of the relationship between two or more variables. Coefficient r measures only the strength of a straigth line dependence between variables.

Linearity (contd):

The coefficient of determination (r^2) on the other hand represents the proportion of common variation in the two variables (i.e. The strength or magnitude of the relationship) where:

$$r^2 = \frac{\Sigma\{(xi-x)(yi-y)\}}{\{[\Sigma(xi-x)2][\Sigma(yi-y)2]\}}$$

+1 represents perfect positive correlation
-1 represents perfect negative correlation
The closer to either -1 or +1 the greater the correlation between methods

(i) Line of Best Fit:

The r^2 value is used to determine if results could be predicted from a plotted excel graph containing the line of best fit. It determines if there is a correlation between the two sets of results. Corresponding values for each method can be extrapolated from the line of best fit. The corresponding values must be consistently higher, lower or equal to each other to produce an r^2 value close to 1.

(ii)Line of Equality:

The r^2 value is used in assessing if there is agreement or comparison between the two methods. The line is forced through the zero intercept of the x and y axis on a plotted excel graph. Corresponding extrapolated values from both methods need to be very similar or equal to produce an r^2 value close to 1 for good agreement.

Standard Deviation (SD):

This can be defined as the measure of spread or dispersion of the data. Normal distributed data range (95%) will lie in the range:

Mean - 2 * SD to mean + 2 * SD

Standard Error (SEM):

The mean value is only an estimate of the true population mean.

The standard error measures the precision of that estimate. It measures how much the mean varies from the actual true population mean.

$$SEM = \frac{s}{\sqrt{n}}$$

95% of the normally distributed data of the true population mean lies in the interval:

Confidence interval = Mean - 2 * SEM to mean + 2 * SEM

SEM vs SD:

SD describes the dispersion of the data. SEM describes the uncertainty (due to sampling error) in the mean of the data.

P value:

P-values measure the compatibility between the data and the Ho (null hypothesis i.e. That there is no difference between populations). Large P value indicates compatibility and small P indicates the opposite.

P-value measures how easily the observed deviation could be explained as chance variation rather than the alternative explanation provided by Ha (i.e. The alternative hypothesis that there is a significant difference in populations).

P=<0.05 Significant

P=<0.01 Very Significant

P=<0.001 Extremely Significant

The P-value of the data is the probability of getting a result as extreme as, or more extreme than the result that was actually observed.

A parametric student t test (paired/two tailed and unpaired/two tailed) is used for normally distributed data. A nonparametric Wilcoxon sign ranking test is used for data which is not normally distributed. Both tests can be used to achieve a P value to determine statistical significance levels (Samuels, 1989).

2.13 Cost Comparison of both Media:

TSC-EY Agar (Oxoid):

Perfringens Agar Base CM587B	€207.50/500g
Perfringens (TSC) Supplement SR88	€130.00/10 vials

500mls of media produces 60 plates (overlayed)

Agar/500ml	€09.43
Supplement/500ml	€13.00
Total/500ml(60plate)	**€22.43**

m-CP Agar(Oxoid):

m-CP *C. perfringens* base AgarCM992	€127.93/500g
Supplements:	
m-CP supplement SR188	€114.73/10 vials
Ammonium	€13.97/L
Phenolphthalein diphosphate	€64.40/1g
Ferric Chloride Hexahydrate	€29.00/100g
Indoxyl β-D-glucoside	€353.80/0.5g
Sartorius Minisarts (0.45µm) filters	€500.00/500filters
Cost of mCP Per 500ml	
m-CP *C. perfringens* base Agar	€9.08
m-CP supplement SR188	€11.47
Ammonium	-------*
Phenolphthalein diphosphate	€0.32
Ferric Chloride Hexahydrate	€0.05
Indoxyl β -D-glucoside	€21.23
Sartorius Minisarts (0.45µm) filters	€1.00
Total/500ml(100 plates) =	**€43.15**

TSC-EY costs €0.37/agar plate

m-CP costs €0.43/agar plate

note*: Ammonium is reusable and so the cost is negligeable.

3.0 Methods and Materials

3.1 Membrane Filter Method For *Cl. Perfringens* using membrane – *Clostridium perfringens* medium (m-CP).

3.1.1 Scope and Application:

This procedure enumerates *Clostridium perfringens* spores from surface and drinking water. Since *Cl. perfringens* is present in large numbers in human and animal wastes and its spores are resistant to wastewater treatment practices, extremes in temperatures and environmental stresses, it is an indicator of present faecal contamination as well as a conservative tracer of past faecal contamination. Some investigators have proposed *Cl. perfringens* as an indicator of the presence and the density of pathogenic viruses and possibly other microorganisms.

3.1.2 Summary of Principle:

m-CP is recommended for the isolation and identification of *Cl. perfringens* in the European Council Directive 98/83/EC on the quality of water intended for human consumption. In m-CP medium lack of β-D Glucosidase means that *Cl. perfringens* does not cleave the chromogen, indoxyl- β-D Glucoside, in the medium. As the organisms ferment sucrose in the medium, reducing the pH, bromocresol purple changes from purple to yellow. This results in characteristic opaque yellow colonies.
Most other *Clostridium* species will appear as either purple colonies due to the lack of sucrose fermentation, or blue/green colonies where the organism is still cleaving Indoxyl-β-D Glucoside and also fermenting sucrose. Presumptive positive *Cl. perfringens* colonies can be further tested for acid phosphatase activity by exposure to ammonium hydroxide vapour for 20 – 30 seconds. *Cl. perfringens* colonies turn pink or red as phenolphthalein diphosphate is cleaved by acid phosphatase. No colour change will be seen with colonies of organisms that do not posses acid phosphatase. D-cycloserine, polymixin B and incubation at 44°C inhibit the growth of background flora such as Gram negative organisms and Staphylococci. (USEPA/600/R-95/178, (1996))

3.1.3 Health and Safety:

Mouth pipetting is not permitted.

Observance of normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents and materials and while operating autoclaves and other equipment and instrumentation.

3.1.4 Instruments, Equipment and Materials:

- BBL Gaspak 100 System (12 plates capacity), 4360626
- BBL CO_2 & H_2 Envelopes, 4370304
- Crossely Milk Medium CM213B (Oxoid).
- m-CP Clostridium perfringens Agar CM992 (Oxoid).
- m-CP Clostridium perfringens Supplement SR188 (Oxoid).
- Phenolphtalein Diphosphate P0126 (Sigma Aldrich). Store at -18°C.
- Ferric Chloride Hexahydrate F1513 (Sigma Aldrich). Store at 2.0 – 8.0°C.
- Indoxyl-β-D-Glucoside I6893 (Sigma Aldrich). Store at -18°C.
- BBL GasPak anaerobic indicators 4370504
- Sartorius Minisart Filters (0.45µm absolute) Sterile individually wrapped 16555K.

3.1.4 Instruments, Equipment and Materials (contd.):

- Filter membranes 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL)

- Petri dishes 50 x 11mm Advantec mfs.

- Membrane Filtration units, Triple manifold with funnels (Gelman Sciences cat. 15402), wrapped in aluminium foil and sterilized at 121°C/15mins.

- Water bath, Grant W22 Model, (Davidson & Hardy).

- Weighing scales, Mettler Toledo PB303-S Range: 0.02 – 310g.

- Incubator, Sanyo model: MIR 153 (Davidson & Hardy) 44.0°C.

- Incubator, Memmert ICP 600 37°C.

- Finntip pipettes 9402050 1 – 5mls.

- Finnpipettor, 4500 Labsystems, 1- 5mls.

- Membrane Forceps (Gelman Sciences).

- Rotary Vacuum pump, Speedivac 2, Edwards.

- Kestrel Autoclave, LTE Scientific. (Davidson & Hardy).
- Fridge, Powerpoint, 2 - 8°C.

- Freezer, Electrolux, -18°C.

- Disposable culture tubes, Dispens-A- Pak, Borex, 16 x 100mm.

3.1.4 Instruments, Equipment and Materials (contd.):

- 300ml Glass bottles with screw cap able to withstand autoclaving.
- Hand tally (Upgreen Counter).
- Loops, sterile.
- Paper clips.

3.1.5 Reagents, Standards and Media:

Surgical Spirits - 95% pure, for flame-sterilization of forceps.

Virkon - 1% solution in dioniosed water.

Liquid Ammonium sp. Gr. 0.88 – 0.89 BDH Analar, 100126T.

Rinse Solution. Ringer Tablets (Oxoid). 1 tablet/ L of deionised water, sterilized.

Ferric Chloride Solution – Weigh out 4.5g of $FeCl_3.6H_2O$ and dissolve in 100ml of deionised water. Filter sterilize using minisart filters. Store in refrigerator.

Phenolphthalein diphosphate Solution – Weigh out 0.5g of phenolphthalein diphosphate and dissolve in 100ml of deionised water. Filter sterilize and store in the refrigerator.

Indoxyl β-D Glucoside Solution – Weigh out 0.03g of Indoxyl β-D Glucoside and dissolve in 4mls of deionised water. Filter sterilize directly into the m-CP media held at 50°C in water bath.

m-CP Agar

Composition/L

Tryptose	30.0	g
Yeast Extract	20.0	g
Sucrose	5.0	g
L-cysteine Hydrochloride	1.0	g
$MgSO_4.7H_2O$	0.1	g
Bromocresol Purple	0.04	g
Agar	15.0	g

Preparation of m-CP Agar:

1. Suspend 35.55g in 500mls of distilled water in a1L Duran bottle. Heat to boiling and mix well.

2. Sterilize by autoclaving at 121°C for 15 minutes.

3. Cool to 50°C and aseptically add the contents of one vial of m –CP Selective Supplement (SR0188E), reconstituted with 2mls of deionised sterile water.

4. Use sterile 10ml syringes fitted with Sartorius minisart filters (0.45µm absolute) to aseptically add the following sterile solutions dissolved in deionised water:

   10mls of 0.5% Phenolphthalein diphosphate
   1ml of 4.5% Ferric Chloride Hexahydrate
   4mls of water containing 30mg of Indoxyl β-D Glucoside. Mix well.

5. Dispense 4 – 4.5 mls into each petri dish using sterile 5ml pipette. Store plates inverted in a plastic bag in a refrigerator for no more than a month.

Modified Crossley Milk Medium:

Composition/L

Skim Milk Powder	100.0	g
Peptone	10.0	g
Bromocresol Purple	0.1	g

Preparation:

1. Cream 110g of the powder with a little deionised water in a 1L beaker. Gradually, dilute to 1L with continuous mixing (no heat required).

2. Dispense 10ml quantities into disposable culture tubes in a, grid referenced, tube rack. Cover each tube with aluminium foil or cap.

3. Autoclave the rack of tubes at 121°C for 5 minutes.

4. Using a Bunsen burner heat up the bottom of a small paper clip until it turns bright orange and then add one to each of the tubes just before inoculation with a colony.

Gram Stain Reagents:

Difco BBL Commercial Kit Containing:

Ammonium oxalate-crystal violet (BBL 212525)
Lugol's solution, Gram modification (BBL 212529)
Counter stain, Safranin (BBL 212531)
Acetone alcohol, Gram decolourizer (BBL 212527).

3.1.6 Sample Collection and Holding Times:

Collection – The drinking water, river saline samples are collected in sterile glass sample containers with metal caps containing rubber inserts to prevent leakage, using aseptic technique, The Dublin bay samples are collected from surface waters, not sediment.

Holding Time – Samples are analysed within 8hrs after collection. *Cl.perfringens* spores can survive for extended periods at 1-4°C. However, since a correlation is planned with other indicators, the holding time for *Cl. perfringens* must be limited to that of the other indicators.

3.1.7 Quality Control:

1. Adherence to sampling procedures and holding time limits is critical to the production of valid data.

2. Check and record temperatures in incubators, autoclaves, refrigerators and fridges daily to insure operation within stated limits.

3 Check and calibrate incubators, autoclaves, refrigerators and fridges, under service contract, twice yearly with an array of thermocouples linked to a specialized software package.

4. Inclusion in an external QA Scheme with the EPA and the Public Health Laboratory Service (PHLS)

Positive Controls:

1. One white SVM *Cl. perfringens* capsule is removed from the –18°C freezer and added aseptically to 10 mls of sterile ringers solution in a test tube.

2. The test tube, containing the capsule suspension, is then held at 38°C for 10 mins and then vortexed for 15 seconds.

3 Step 2 is then repeated another 3 times. The whole process should take 40 mins.

4 Take 1ml of the capsule suspension and add to 39mls of sterile Ringers solution to make a 1:40 dilution. A 1:50 & 1:70 dilution is also prepared for recoverability and limits of detection.

5. Filter 1ml of the 1:40 dilution through a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL). Use the filter membrane to inoculate m-CP agar plates with pure cultures of *Cl. perfringens*. Repeat for other dilutions 1:50 & 1:70.

Negative Controls:

1. Using a sterile swab soaked in sterile deionised water, swab the lab bench surface. Place swab into 50mls of sterile deionised water. Filter the 50mls through a a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL).

2 Place filter on m-CP plate and incubate along with other samples.

3 Expose a m-CP plate to the lab atmosphere for 1min. Use to determine level of *Cl. Perfringens* in Lab atmosphere.

Negative Controls (contd):

4 Filter 100mls of sterile diluent through a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL). Place the filter on an m-CP plate.

5 *E. coli* (EPA/600/R-95/178) SVM capsules are used as a negative control. Preparation and filtration of the capsule suspension is the same as the positive control

Carry these plates through the entire analytical procedure, as positive and negative controls along with an unused negative m-CP plate.

3.1.8 Procedure for Analyses of Water samples:

1. Prepare m-CP Agar according to instructions on page 34

2. Mark the bottom of the petri dish with sample number and volumes.

3. Grasp a sterile membrane filter by its edge using a sterile forceps and place on the filter base, grid side up. Attach the funnel to the base of the filter unit; the membrane is now held between the funnel and the base.

4. For greatest accuracy, it is necessary to filter a sample volume that will yield a countable plate. The desired range per membrane filter plate is 20-80 *Cl .perfringens* colonies.
   Volumes chosen for this project:

Rivers:	5ml and 10ml volumes
Saline:	1ml and 5ml volumes
Drinking water:	100mls.

3.1.8 Procedure for Analyses of Water samples (contd):

5. Shake the sample bottles vigorously 20 times and measure the desired volume of sample into the graduated funnel either directly as with drinking water or with sterile pipettes for rivers and saline samples.

6. After adding the sample to filter funnel, turn on vacuum and filter the sample. Rinse the sides of the funnel walls with 20-30mls of sterile quarter strength Ringers solution. Turn off vacuum and remove the funnel from the filter base.

7. Flame forceps, cool and aseptically remove the membrane filter from the filter base. Place the filter, grid side up , on the m-CP Agar using a rolling motion to prevent air bubbles. Reseat the filter if bubbles occur.

8. Remove the lids from the m-CP Agar plates. Invert lids and nest them under the corresponding plate bottom for identification. Stack the plates in layers in the anaerobic jar. Add 10mls of deionised water/tap water to 1 BBL Gaspak CO_2 & H_2 envelope and place envelope into the anaerobic jar. Place one anaerobic indicator slip into the anaerobic jar. Screw-clamp the jar lid in place to ensure the atmosphere within the jar is isolated from the atmosphere outside.

9 Incubate the anaerobic jar at 44°C for 24hrs, maintaining anaerobic conditions through the use of a commercial anaerobic system. If visible condensation does not occur within 60mins after the BBL GasPak is activated, then, remove the BBL GasPak by opening the jar and terminating the reaction. Inspect the chamber seal for alignment. Insert a new GasPak and seal the chamber.

10. After 24 hrs, remove one agar plate at a time from the jar and re-close the chamber. Examine the m-CP plate for straw-colour colonies. If such colonies are present, invert and expose the open agar plate 10-30 sec, inside a fume hood, to the fumes from an open container of concentrated ammonium hydroxide.

3.1.8 Procedure for Analyses of Water samples (contd):

11. If *Cl. perfringens* colonies are present, the phosphate in the phenolphthalein diphosphate will be cleaved from the substrate by acid phosphatase and typical colonies of *Cl. perfringens* will turn a dark pink or magenta after exposure to fumes of ammonium hydroxide.

12. Count pink or magenta colonies as presumptive *Cl.perfringens*.

13. Repeat steps 10 – 12 with the other culture plates.

Table 3.1.1. The typical appearance of colonies on mCP Medium

Organism	Typical Colony Colour
Clostridium perfringens	**Opaque Yellow** Sucrose positive/Glucosidase negative Then pink/red after exposure to NH_4OH
Other *Clostridium* spp.	**Blue/Green** Sucrose positive/Glucosidase positive (e.g. *Cl. barasii, Cl. paraputrificum, Cl. tertium*)
	Purple Sucrose negative/Glucosidase positive or negative (e.g. *Cl. biferentans, Cl.difficile, Cl. sporogenes*
	Opaque Yellow Sucrose positive/ Glucosidase negative Remain yellow after exposure to NH_4OH

3.1.9 Confirmation Tests

Modified Crossley Milk Medium

1. Pick 10 typical isolated *Cl. perfringens* colonies from the m-CP plate and inoculate ten tubes of sterile Crossley milk medium, containing an iron paper clip, and incubate at 35°C for 48hrs.

2. Examine hourly for stormy fermentation with rapid coagulation and fractured rising curd with a colour change to bright yellow.

Gram Stain

1. Flame loop and take a loopful from a typical tube and carry out a Gram stain to confirm that the colonies present are gram positive, non-motile short bacilli.

3.1.10 Reporting Results:

Pink or magenta colonies counted on m-CP medium are adjusted to a count/100ml and reported as: Presumptive *Cl. perfringens* colony forming units (CFU)/100mls.

If confirmation tests are performed, original counts on m-CP are adjusted based on the percent of colonies picked and confirmed. Report as confirmed *Cl. perfringens* CFU/100mls of water sample.

3.2 Membrane Filter Method For *Cl. perfringens* using Egg Yolk Free Tryptose Sulphite Cycloserine (EY-TSC) Agar.

3.2.1 Scope and Application:

This procedure enumerates *Clostridium perfringens* spores from surface and drinking water. Since *Cl.perfringens* is present in large numbers in human and animal wastes and its spores are resistant to wastewater treatment practices, extremes in temperatures and environmental stresses, it is an indicator of present faecal contamination as well as a conservative tracer of past faecal contamination. Some investigators have proposed *Cl. perfringens* as an indicator of the presence and the density of pathogenic viruses and possibly other microorganisms.

3.2.2 Summary of Principle:

Egg Yolk Free TSC Agar (AOAC method (**46.092**)) has the advantage over TSC with Egg yolk because smaller colonies are formed which simplifies the counting of plates with high numbers of colonies.
Sodium metabisulphite and ferric ammonium citrate are used as an indicator of sulphite reduction by *CL. perfringens*, which produces black colonies. It was found in one study that D-cycloserine inhibits all facultative anaerobes except *Serratia marcescens*, *Streptococcus lactis*. TSC allows the growth of other sulphite reducing *Clostridium* species with the exception of *Cl. sordellii* and *Cl. bifermentans*.
Incubation at 44°C in combination with D-cycloserine inhibits the growth of background flora such as Gram-negative organisms and Staphylococci. (BS EN 26461-2 : (1993) Part 2.)

3.2.3 Health and Safety:

Mouth pipetting is not permitted.

Observance of normal good laboratory practices and safety procedures required in a microbiology laboratory while preparing, using and disposing of cultures, reagents and materials and while operating autoclaves and other equipment and instrumentation.

3.2.4 Instruments, Equipment and Materials:

- BBL Gaspak 100 System (12 plates capacity), 4360626
- BBL CO2 & H2 Envelopes, 4370304
- Perfringens Agar Base (T.S.C.) CM587B (Oxoid)
- Perfringens (T.S.C.) Supplement SR88 (Oxoid).
- Crossely Milk Medium, CM213B (Oxoid).
- Filter membranes 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL)
- Petri dishes 50 x 11mm Advantec mfs.
- Membrane Filtration units, Triple manifold with funnels (Gelman Sciences cat. 15402), wrapped in aluminium foil and sterilized at 121°C/15mins.

3.2.4 Instruments, Equipment and Materials (contd.):

- Waterbath, Grant W22 Model, (Davidson & Hardy).
- Weighing scales, Mettler Toledo PB303-S Range: 0.02 – 310g.
- Incubator, Sanyo model: MIR 153 (Davidson & Hardy) 44.0°C.
- Incubator, Memmert ICP 600 37°C.
- Finntip pipettes 9402050 1 – 5mls.
- Finnpipettor, 4500 Labsystems, 1- 5mls.
- Membrane Forceps, (Gelman Sciences).
- Rotary Vacuum pump, Speedivac 2, Edwards.
- Kestrel Autoclave, LTE Scientific. (Davidson & Hardy).
- Fridge, Powerpoint, 2 - 8°C.
- Freezer, Electrolux, -18°C.
- Disposable culture tubes, Dispens-A- Pak, Borex, 16 x 100mm.
- 300ml Glass bottles with screw cap able to withstand autoclaving.
- Hand tally, (Upgreen Counter).
- BBL GasPak anaerobic indicators 4370504

3.2.4 Instruments, Equipment and Materials (contd.):

- Loops, sterile.
- Paper clips.

3.2.5 Reagents, Standards and Media:

Surgical Spirits - 95% pure, for flame-sterilization of forceps.

Virkon - 1% solution in dioniosed water.

Rinse Solution. Ringer Tablets (Oxoid). 1 tablet/ L of deionised water, sterilized.

TSC Agar

Composition/L

Tryptose	15.0	g
Soya peptone	5.0	g
Yeast Extract	5.0	g
Sodium metabisulphate	1.0	g
Ferric ammonium citrate	15.0	g
Agar	19.0	g

Preparation of EY-TSC Agar:

1. Suspend 23g in 500mls of deionised water in a1L Duran bottle. Heat to boiling and mix well.
2. Sterilize by autoclaving at 121°C for 10 minutes.

Preparation of EY-TSC Agar (contd.):

3. Cool to 50°C and aseptically add the contents of one vial of TSC Selective Supplement (SR88), reconstitute with 2mls of deionised sterile water. Mix well. Ensure enough media is prepared and set aside to overlay plates later on.

4. Dispense 4 – 4.5 mls into each petri dish using sterile 5ml pipette. Store plates inverted in a plastic bag in a refrigerator for no more than a month.

Modified Crossley Milk Medium:

Composition/L

Skim Milk Powder	100.0	g
Peptone	10.0	g
Bromocresol Purple	0.1	g

Preparation:

1. Cream 110g of the powder with a little deionised water in a 1L beaker and gradually dilute to 1L with continuous mixing (no heat required).

2. Dispense 10ml quantities into disposable culture tubes in a grid referenced, tube rack. Cover each tube with aluminium foil or cap. Autoclave the rack of tubes at 121°C for 5 minutes.

3. Using a Bunsen burner heat up the bottom of a small paper clip until it turns bright orange and then add one to each of the tubes just before inoculation with a presumptive colony of *Cl. perfringens*.

Gram Stain Reagents:

Difco BBL Commercial Kit Containing:

Ammonium oxalate-crystal violet (BBL 212525)

Lugol's solution, Gram modification (BBL 212529)

Counter stain, Safranin (BBL 212531)

Acetone alcohol, Gram decolourizer (BBL 212527).

3.2.6 Sample Collection and Holding Times:

Collection – Water samples are collected in sterile glass sample containers with metal caps containing rubber inserts to prevent leakage.

Holding Time – Samples are analysed within 8hrs after collection. *Cl. perfringens* spores can survive for extended periods at 1-4°C. However, since a correlation is planned with other indicators, the holding time for *Cl. perfringens* must be limited to that of the other indicators.

3.2.7 Quality Control:

1. Adherence to sampling procedures and holding time limits is critical to the production of valid data.

2. Check and record temperatures in incubators, autoclaves, refrigerators and fridges daily to insure operation within stated limits.

3. Check and calibrate incubators, autoclaves, refrigerators and fridges, under service contract, twice yearly with an array of thermocouples linked to a specialized software package.

4. Inclusion in an external QA Scheme with the EPA and the Public Health Laboratory Service (PHLS)

Positive Controls:

1. One white SVM *Cl. perfringens* capsule is removed from the –18°C freezer and added aseptically to 10 mls of sterile ringers solution in a test tube.

2. The test tube, containing the capsule suspension, is then held at 38°C for 10 mins and then vortexed for 15 seconds.

3 Step 2 is then repeated another 3 times. The whole process should take 40 mins.

4 Take 1ml of the capsule suspension and add to 39mls of sterile Ringers solution to make a 1:40 dilution. A 1:50 & 1:70 dilution is also prepared for recoverability and limits of detection, respectively.

5 Filter 1ml of 1:40,1:50 or 1:70 dilutions through a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL). Use the filter membrane to inoculate TSC agar plates with pure cultures of *Cl. perfringens* .

6 Overlay the plates with 3-4mls of molten TSC Agar held at 50°C containing supplement SR88.

Negative Controls:

1. Using a sterile swab soaked in sterile deionised water, swab the lab bench surface. Place swab into 50mls of sterile deionised water. Filter the 50mls through a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL).

2. Place filter on TSC plate and incubate along with other samples.

Negative Controls (contd):

3. Expose a TSC plate to the lab atmosphere for 1min. Use to determine level of *Cl.perfringens* in Lab atmosphere.

4 Filter 100mls of sterile diluent through a filter membrane 0.45µm ± 0.02 µm, 47mm diameter (sterile) mixed cellulose esters (Gelman Labs PALL). Place a filter on a TSC plate.

5 *E. coli* (EPA/600/R-95/178) SVM capsules are used as a negative control. Preparation and filtration of the capsule suspension is the same as the positive control

6. Overlay plates with molten TSC Agar held at 50°C in the water bath and containing SR88 supplement.

Carry these plates through the entire analytical procedure, as positive and negative controls along with an unused negative TSC plate.

3.2.8 Procedure for Analyses of Water samples:

1. Prepare EY-TSC Agar according to instructions on page 45.

2. Mark the bottom of the petri dish with sample number and volumes.

3. Grasp a sterile membrane filter by its edge using a sterile forceps and place on the filter base, grid side up. Attach the funnel to the base of the filter unit; the membrane is now held between the funnel and the base.

4. For greatest accuracy, it is necessary to filter a sample volume that will yield a countable plate. The desired range per membrane filter plate is 20-80 *Cl. perfringens* colonies.

3.2.8 Procedure for Analyses of Water samples (contd):

5. Volumes chosen for this project:

Rivers:	5ml and 10ml volumes
Saline:	1ml and 5ml volumes
Drinking water:	100mls.

6. Shake the sample bottles vigorously 20 times and measure the desired volume of sample into the graduated funnel either directly as with drinking water or with sterile pipettes for rivers and saline samples.

7. After adding the sample to filter funnel, turn vacuum and filter the sample. Rinse the sides of the funnel walls with 20-30mls of sterile quarter strength Ringers solution. Turn off vacuum and remove the funnel from the filter base.

8. Flame forceps, cool and aseptically remove the membrane filter from the filter base. Place the filter, grid side up , on the TSC Agar using a rolling motion to prevent air bubbles. Reseat the filter if bubbles occur.

9. Remove lids and add 3-4mls of remaining molten TSC Agar held at 50°C containing SR88 supplement.

10. Allow 15mins for Agar to set. Leave the lids off to prevent condensation build up on the inside of lids.

11. Invert lids and nest them under the corresponding plate bottom for identification. Stack the plates in layers in the anaerobic jar. Add 10mls of deionised water/tap water to 1 BBL Gaspak CO_2 & H_2 envelope and place envelope into the anaerobic jar. Place one anaerobic indicator slip into the anaerobic jar. Screw-clamp the jar lid in place ensuring the atmosphere within the jar is isolated from the atmosphere externally.

3.2.8 Procedure for Analyses of Water samples (contd):

12. Incubate the anaerobic jar at 44°C for 24hrs, maintaining anaerobic conditions through the use of a commercial anaerobic system. If visible condensation does not occur within 60mins after the BBL GasPak is activated, remove the BBL GasPak by opening the jar and terminating the reaction. Inspect the chamber seal for alignment. Insert a new GasPak and seal the chamber.

13. After 24 hrs, remove one agar plate at a time from the jar and re-close the chamber. Examine the TSC plate for black coloured colonies.

14. Repeat step 13 with the other culture plates.

3.2.9 Confirmation Tests

Modified Crossley Milk Medium

1. Pick 10 typical isolated *Cl. perfringens* colonies and 10 atypical colonies (small colonies) from the EY-TSC plate and inoculate ten tubes of sterile Crossley milk medium, containing an iron paper clip, and incubate at 35°C for 48hrs.

2. Examine hourly for stormy fermentation with rapid coagulation and fractured rising curd with a colour change to bright yellow.

Gram Stain

1. Flame loop and take a loopful from a typical tube and carry out a Gram stain to confirm that the colonies present are gram positive, non-motile short bacilli.

3.2.10 Reporting Results:

Black colonies counted on EY-TSC medium are adjusted to a count/100ml and reported as: Presumptive *Cl.perfringens* colony forming units (CFU)/100mls.

If confirmation tests are performed, original counts on EY-TSC are adjusted based on the percent of colonies picked and confirmed. Report as confirmed *Cl. perfringens* CFU/100mls of water sample.

3.3 Membrane Filter Method For Coliforms and *E. coli* using mEndo & mFC Media respectively.

Summary:

The same drinking water, river and saline samples tested for *Cl. perfringens* are also analysed simultaneously for coliforms and *E. coli* within 6 hrs of sampling on selective media, mEndo & mFC, respectively. On mEndo coliform colonies have a metallic sheen, on mFC *E. coli* appears as dark blue colonies. Another technician conducted the faecal coliform analysis within the same lab.

For saline samples a 2 ml & 4 ml volume is filtered and transferred to mEndo plates 37°C/24hrs. For *E. coli*, a 10ml quantity of a saline is filtered and incubated on mFC broth at 44°C/24hrs.

River samples are only tested for *E. coli* by filtering a 1ml & 10 ml quantity and incubating on mFC broth with the plates held at 44°C/24hrs.

Dublin Bay samples are analysed by filtering 0.1ml & 1 ml quantities and incubating duplicates of both dilutions on mEndo & mFC broths.

Drinking water is tested using the Colilert method. The method uses 100mls of sample, which is incubated with Colilert reagents at 34°C/24hrs. The Colilert method is a Most Probable Numbers (MPN) method

All principles and SOPs for these methods are found in appendix 1.

4.0 Results:

4.1 Limits of Detection of *Cl. perfringens* at 1: 70 Dilution

The raw data for the limits of detection are in appendix 2.

5 reference capsules were analyzed at a 1:70 dilution prepared for a target abundance of 1 colony forming unit/ml. 10 paired replicates of each capsule were plated on mCP and TSC media. 100 determinations were carried out. See tables 4.1.1 – 4.1.3

The Data was not converted to log values.

Table 4.1.1 Results of Colony Count at 1:70 Dilution

	Capsule 1		Capsule 2		Capsule 3		Capsule 4		Capsule 5	
Replicates	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP
1	4	3	2	2	5	0	5	5	3	1
2	2	1	2	1	4	2	5	5	3	0
3	3	1	2	2	4	2	5	5	3	2
4	5	3	3	1	3	1	6	6	2	0
5	2	3	5	1	2	1	4	4	5	1
6	3	1	5	1	3	1	4	4	2	1
7	3	0	5	3	2	1	3	3	3	1
8	4	1	4	3	2	2	2	2	3	3
9	4	2	2	2	2	2	4	4	2	1
10	5	0	3	2	2	0	5	5	6	0
Total:	**35**	**15**	**33**	**18**	**29**	**12**	**43**	**43**	**32**	**10**
Mean:	**3.5**	**1.5**	**3.3**	**1.8**	**2.9**	**1.2**	**4.3**	**4.3**	**3.2**	**1**
St.Dev.	**1.08**	**1.18**	**1.34**	**0.79**	**1.10**	**0.79**	**1.16**	**1.16**	**1.32**	**0.94**

Table 4.1.2 Summary of mean values of Colony Count at 1: 70 Dilution (see Table 4.1.1)

Capsule:	EY-TSC Mean	m-CP Mean
1	3.5	1.5
2	3.3	1.8
3	2.9	1.2
4	4.3	1.5
5	3.2	1.0

4.1 Limits Of Detection (contd):

Table 4.1.3 Summary of Statistical Data for 1:70 Dilution

	EY-TSC	m-CP	Difference
Mean	3.44	1.40	2.04
Std. Dev.	0.53	0.31	0.50
Std. Error	0.24	0.14	0.22
Min	2.90	1.00	1.50
Max	4.30	1.80	2.80
Lower CI 95%	2.79	1.02	1.42
Upper CI 95%	4.10	1.78	2.66

4.1.1 Statistical t Test Paired & Unpaired (two tailed) of 1:70 Dilutions on mCP & EY-TSC:

Assumption Tests:

Comparison of Standard Deviations of 1: 70 data on mCP & EY-TSC Media

F = 2.93

The P Value was 0.16.

The test suggested that the difference between the two standard deviations was not significant.

Gaussian Distributions:

The Kolmogorov and Smirnov (KS) method was used to test the assumption that the data was sampled from populations that follow Gaussian distributions. This test indicated whether the data were normal or not. See table 4.1.4.

Table 4.1.4 KS Distance and Gaussian Distribution For 1:70 Dilution.

Media	KS	P Value	Data Normal
TSC	0.25	>0.10	YES
m-CP	0.23	>0.10	YES

Statistical P – Value for 1:70 Recoveries of *Cl. perfringens*

Unpaired t Test (two tailed)

The two-tailed P value was <0.001, considered very significant

t = 7.47 with 8 degrees of freedom.

Paired t Test (two tailed)

This test produced the same data as above except the P- Value was slightly different.

The two-tailed P value was 0.001, considered very significant.

t = 9.07 with 4 degrees of freedom

4.2 Recoverability Of *Cl. perfringens* at 1:40 Dilution:

The raw data for the recoverability of *Cl. perfringens* are in appendix 2.

Reference capsules were analyzed at a 1:40 dilution, prepared for a target abundance of 10 cfu/ml (1:40) colony forming unit/ml. 10 paired replicates of each capsule were plated on mCP and EY-TSC media at 1:40 and 1:50. 100 determinations were carried out. (see table 4.2.1 – 4.2.3). The data was not converted to log values

Table 4.2.1 Results of Colony Count at 1:40 Dilution

	Capsule 1		Capsule 2		Capsule 3		Capsule 4		Capsule 5	
Replicates	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP
1	10	8	7	5	7	7	11	8	14	7
2	13	7	8	6	9	7	7	10	8	5
3	8	7	8	5	9	10	8	9	9	6
4	14	6	7	5	10	9	4	7	10	6
5	11	5	8	7	9	8	8	6	11	4
6	14	6	7	4	7	6	6	5	8	3
7	10	6	10	4	13	7	10	11	10	7
8	5	6	4	7	15	3	15	8	14	5
9	9	8	9	8	12	11	14	7	10	2
10	7	8	13	8	13	10	14	5	9	8
Total	101	67	81	59	104	78	97	76	103	53
Mean	10.10	6.7	8.10	5.9	10.40	7.8	9.70	7.6	10.30	5.3
St. Dev.	3.00	1.06	2.33	1.52	2.72	2.35	3.74	2.01	2.16	1.89

Table 4.2.2 Summary of mean values of Colony Count at1: 40 Dilution (See Table 4.2.1)

Capsule:	EY-TSC Mean	m-CP Mean
1	10.1	6.7
2	8.1	5.9
3	10.4	7.8
4	9.7	7.6
5	10.3	5.3

The average mean of the 5 capsules for both methods:

Table 4.2.3 Summary of Statistical Data for 1:40 Dilution

	EY-TSC	m-CP	Difference
Mean	9.7	6.7	3.06
Std. Dev.	0.94	1.07	1.20
Std. Error	0.42	0.48	0.53
Min	8.10	5.30	2.10
Max	10.40	7.80	5.00
Lower CI 95%	8.54	5.32	1.57
Upper CI 95%	10.89	7.99	4.55

4.2.1 Statistical Paired & Unpaired t Test (two tailed) of 1:40 Dilution:

Assumption Tests:

Comparison of Standard Deviations for Dilution 1:40 (mCP & TSC):

F = 1.293

The P value was 0.4048.

This test suggested that the difference between the two Standard Deviations (m-CP & EY-TSC) was not significant.

Gaussian Distribution Test For 1:40 Dilution Data (mCP & EY-TSC):

The Kolmogorov and Smirnov (KS) method was used to test the assumption that the data was sampled from populations that follow Gaussian distributions. This test indicated whether the data were normal or not

Table 4.2.4 KS Distance and Gaussian Distribution For 1:40 Dilution Data.

Media	KS	P Value	Data Normal
TSC	0.29	>0.10	YES
m-CP	0.21	>0.10	YES

Statistical P – Value for 1:40 Recovery of *Cl. perfringens*

Paired t Test (two tailed).

The two-tailed P-Value was 0.005, considered very significant.

t = 5.71 with 4 degrees of freedom

Unpaired t Test

The two-tailed P-Value was 0.001, considered very significant.

t = 4.79 with 8 degrees of freedom.

4.3 Recoverability Of *Cl. perfringens* l at 1:50 Dilution

The raw data for the recoverability of *Cl. perfringens* are in appendix 2.

Reference capsules were analyzed at 1:50 dilution, prepared for a target abundance of 5 cfu/ml (1:50) colony forming unit/ml. 10 paired replicates of each capsule were plated on mCP and EY-TSC media at 1:50. 100 determinations were carried out. (see table 4.3.1 – 4.3.3). The data was not converted to log values

Table 4.3.1 Results of Colony Count of *Cl. perfringens* at 1:50 Dilution

	Capsule 1		Capsule 2		Capsule 3		Capsule 4		Capsule 5	
Replicates	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP	TSC	m-CP
1	11	0	3	2	6	0	9	4	3	0
2	7	5	2	6	14	0	5	3	5	0
3	4	6	3	9	6	9	6	8	6	2
4	8	5	5	9	11	3	8	7	5	3
5	9	3	10	3	9	3	13	8	5	2
6	5	8	4	4	4	10	5	0	7	4
7	10	2	6	5	8	6	7	11	9	4
8	5	4	9	8	4	7	9	8	6	3
9	9	0	6	3	10	6	10	8	8	1
10	6	2	11	0	11	3	10	3	10	0
Total:	**74**	**35**	**59**	**49**	**83**	**47**	**82**	**60**	**64**	**19**
Mean:	**7.40**	**3.5**	**5.90**	**4.9**	**8.30**	**4.7**	**8.20**	**6**	**6.40**	**1.9**
St.Dev.	**2.37**	**2.59**	**3.14**	**3.07**	**3.30**	**3.47**	**2.53**	**3.33**	**2.12**	**1.60**

Table 4.3.2 Summary of mean values of Colony Count of *Cl. perfringens* at1: 50 Dilution (See Table 4.3.1)

Capsule:	EY-TSC Mean	m-CP Mean
1	7.4	3.5
2	5.9	4.9
3	8.3	4.7
4	8.2	6.0
5	6.4	1.9

The average mean of the 5 capsules for both methods:

Table 4.3.3 Summary of Statistical Data for Recovery of Cl. perfringens at 1:50 Dilution

	EY-TSC	m-CP	Difference
Mean	7.24	4.20	3.04
Std. Dev.	1.07	1.56	1.42
Std. Error	0.48	0.70	0.63
Min	5.90	1.90	1.42
Max	8.30	6.00	4.50
Lower CI 95%	5.91	2.26	1.28
Upper CI 95%	8.57	6.14	4.80

4.3.1 Statistical Paired & Unpaired t Test (two tailed) of 1:50 Dilution Data:

Assumption Tests:

Comparison of Standard Deviations for Dilution 1:50 Data (mCP & EY-TSC):

F = 2.14

The P Value was 0.24.

The test suggested that the difference between the two standard deviations was not significant.

Gaussian Distribution Test For 1:40 Dilution Data (mCP & EY-TSC):

The Kolmogorov and Smirnov method was used to test the assumption that the data was sampled from populations that follow Gaussian distributions. This test indicated whether the data were normal or not

Table 4.3.4 KS Distance and Gaussian Distribution For 1:50 Dilution

Media	KS	P Value	Data Normal
TSC	0.22	>0.10	YES
m-CP	0.23	>0.10	YES

Statistical P – Value for 1:50 Recovery of *Cl. perfringens*

Unpaired t Test (two tailed)

The two-tailed P value was 0.007, considered very significant

t = 3.59 with 8 degrees of freedom.

Paired t Test (two tailed)

This test produced the same data as above except the P- Value was slightly different.

The two-tailed P value was 0.009, considered very significant.

t = 4.79 with 4 degrees of freedom

4.4 Saline Sample Results:

92 samples were analyzed for *Cl. perfringens*, Total and Faecal coliforms (*E. coli*). A statistical summary of the log values for EY-TSC & mCP are found on tables 4.4.1-4.4.2. Summary of false positives for EY-TSC & mCP Media saline samples found on table 4.4.3. Raw data for Saline samples can be found in Appendix 2. All data was converted to log values. The graph plot for Line of Best Fit and Line Of Equality (table 4.4.2) are found on fig. 4.4.1 – 4.4.6; pages 64-69

Table 4.4.1 Summary of Statistical Data for Saline Samples

	EY-TSC	m-CP	Difference
Mean	2.08	1.68	0.41
Std. Dev.	0.68	0.71	0.53
Std. Error	0.07	0.07	0.06
Min	0.48	0.48	-1.21
Max	3.14	3.05	2.45
Median	2.26	1.82	0.39
Lower CI 95%	1.94	1.53	0.30
Upper CI 95%	2.23	1.82	0.52

All data in table 4.4.1 was converted to log values.

Table 4.4.2 Correlation between EY-TSC & mCP Saline Samples

Correlation	Line of Best fit (r^2)	Line of Equality (r^2)
TSC/m-CP	0.50	0.15
TSC/mFC	0.42	0.08
m-CP/mFC	0.17	0.12

Table 4 4.3 False Positives For TSC & mCP Saline Samples

Presumptive Cl. perfringens colonies that proved negative out of 92 samples		
Dilution	***EY-TSC Agar**	***m-CP Medium**
1 ml	28	6
5ml	159	43

*Values in Table 4.4.3 are not log values

4.4.1 Statistical Paired t Test (two tailed) of EY-TSC & mCP Saline Samples:

Assumption Tests

Effective Pairing of mCP & EY-TSC Data Results

Correlation coefficient (r) = 0.70

The one tailed P value was <0.001, considered extremely significant.

The pairing was effective.

Gaussian Distribution Test of mCP & EY-TSC Data Results:

The Kolmogorov-Smirnov (KS) method was used to test that the differences sampled from a Gaussian distribution.

The KS distance was 0.12

The P value was >0.10. The data passed the normality test with P>0.05.

Statistical P Value for Saline samples:

The two-tailed P value was <0.001, considered extremely significant.

t = 7.29 with 91 degrees of freedom.

4.4.2 Saline Wilcoxon Matched-Pairs Signed-Ranks Test (Non-parametric) For Saline Samples

Assumption Test:

Effective Pairing of mCP & EY-TSC Data Results:

Nonparametric Spearman correlation coefficient (r) = 0.79.

The one tailed P value was <0.0001, considered extremely significant.

The matching or pairing was effective.

4.4.2 Saline Wilcoxon Matched-Pairs Signed-Ranks Test (Non-parametric) For Saline Samples (contd).

Calculation Details:

Sum of all Signed Ranks	(W)	=	3030.0
Sum of positive ranks	(T+)	=	3517.5
Sum of negative ranks	(T-)	=	-487.5

Number of Pairs = 89

3 pairs were excluded from the calculations because both values were equal

P Value:

The two-tailed P value was <0.001, considered extremely significant.

(The P value was an estimate based on a normal approximation).

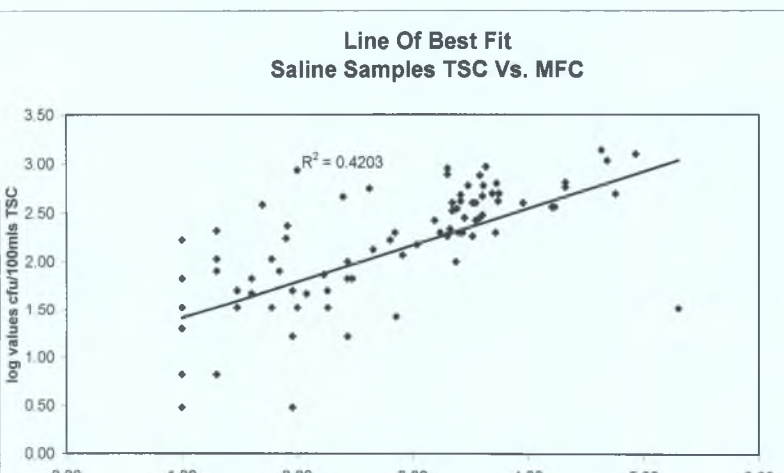

Line Of Best Fit
Saline Samples TSC Vs. MFC
log values cfu/100mls TSC
R² = 0.4203
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Fig. 4.4.2 log values cfu/100mls MFC
65

Fig 4.4.2
65

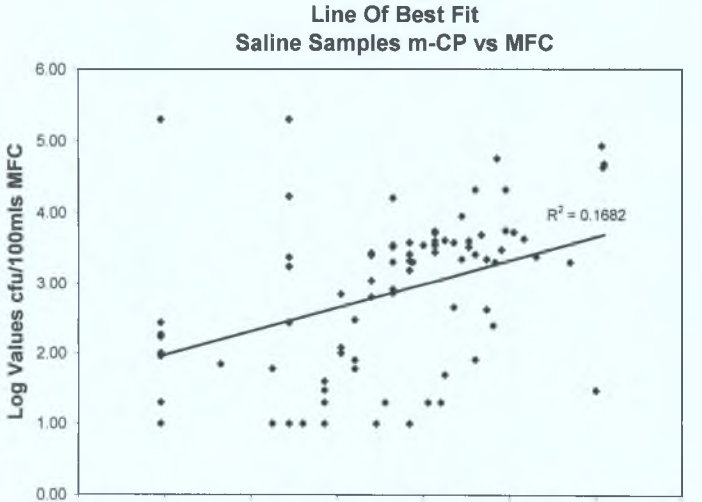


Fig.4.4.3 Log Values cfu/100mls m-CP

Fig.4.4.3
66

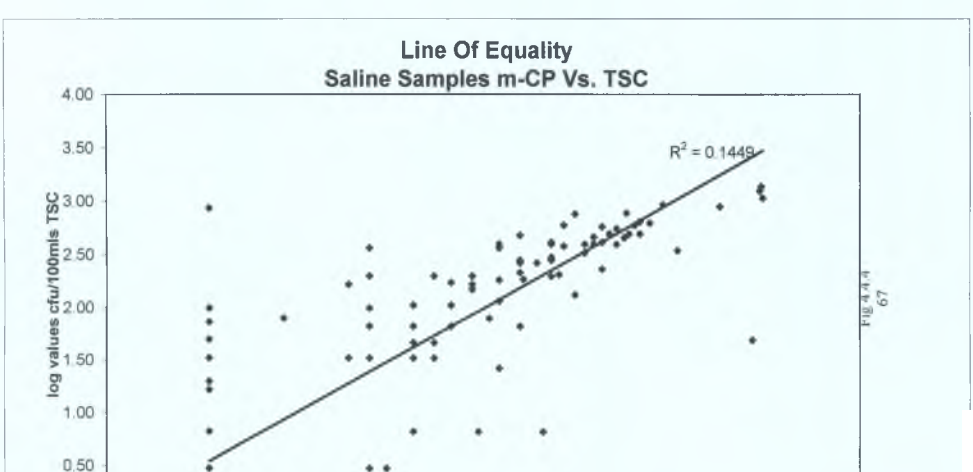


Fig. 4.4.4 log values cfu/100mls m-CP

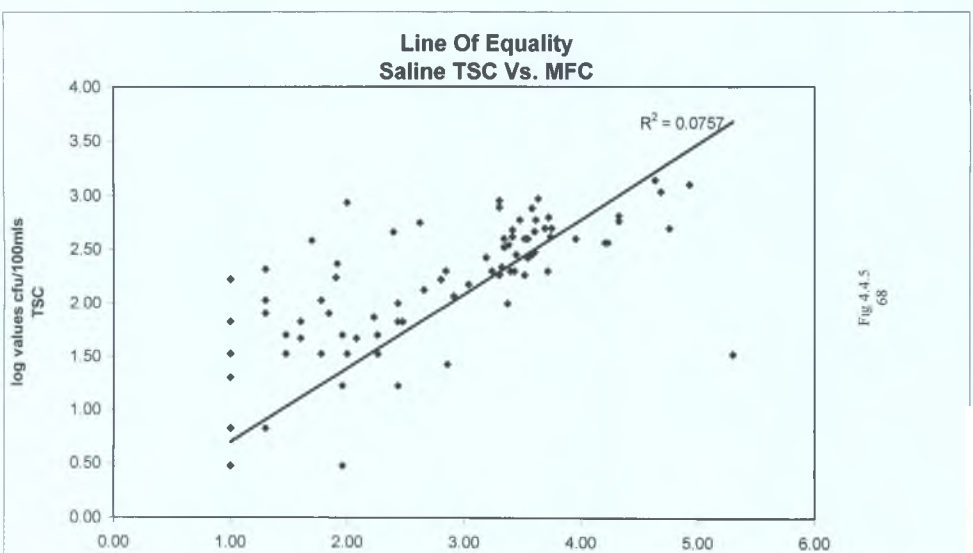


Fig 4.4.5 log values CFU/100mls MFC
68

Fig 4.4.5
68

4.5 River Samples:

132 samples were analyzed for *Cl. perfringens*, Total and Faecal coliforms (*E. coli*). A statistical summary of the log values for EY-TSC & mCP are found on tables 4.5.1-4.5.2. Summary of false positives for EY-TSC & mCP Media saline samples found on table 4.5.3. The graph plot for Line of Best Fit and Line Of Equality (table 4.5.2) are found on fig. 4.5.1 – 4.5.6. Raw data for Saline samples can be found in Appendix 2. All data was converted to log values

Table 4.5.1 Summary of Statistical Data for TSC & mCP River Samples

	EY-TSC	m-CP	Difference
Mean	2.17	1.74	0.43
Std. Dev.	0.48	0.59	0.40
Std. Error	0.04	0.05	0.03
Min	0.82	0.82	-0.48
Max	3.30	3.30	1.61
Median	2.26	1.78	0.32
Lower CI 95%	2.09	1.64	0.37
Upper CI 95%	2.26	1.84	0.50

Table 4.5.2Correlation between the media For Saline Samples

Correlation	Line of Best fit (r^2)	Line of Equality (r^2)
EY-TSC/m-CP	0.54	0.02
EY-TSC/mFC	0.28	0.16
m-CP/mFC	0.29	0.30

Table 4 5.3 False Positives For Saline Samples

Presumptive Cl. perfringens colonies that proved negative out of 132 samples		
Dilution	**EY-TSC Agar**	**m-CP Medium**
5 ml	232	70
10 ml	534	90

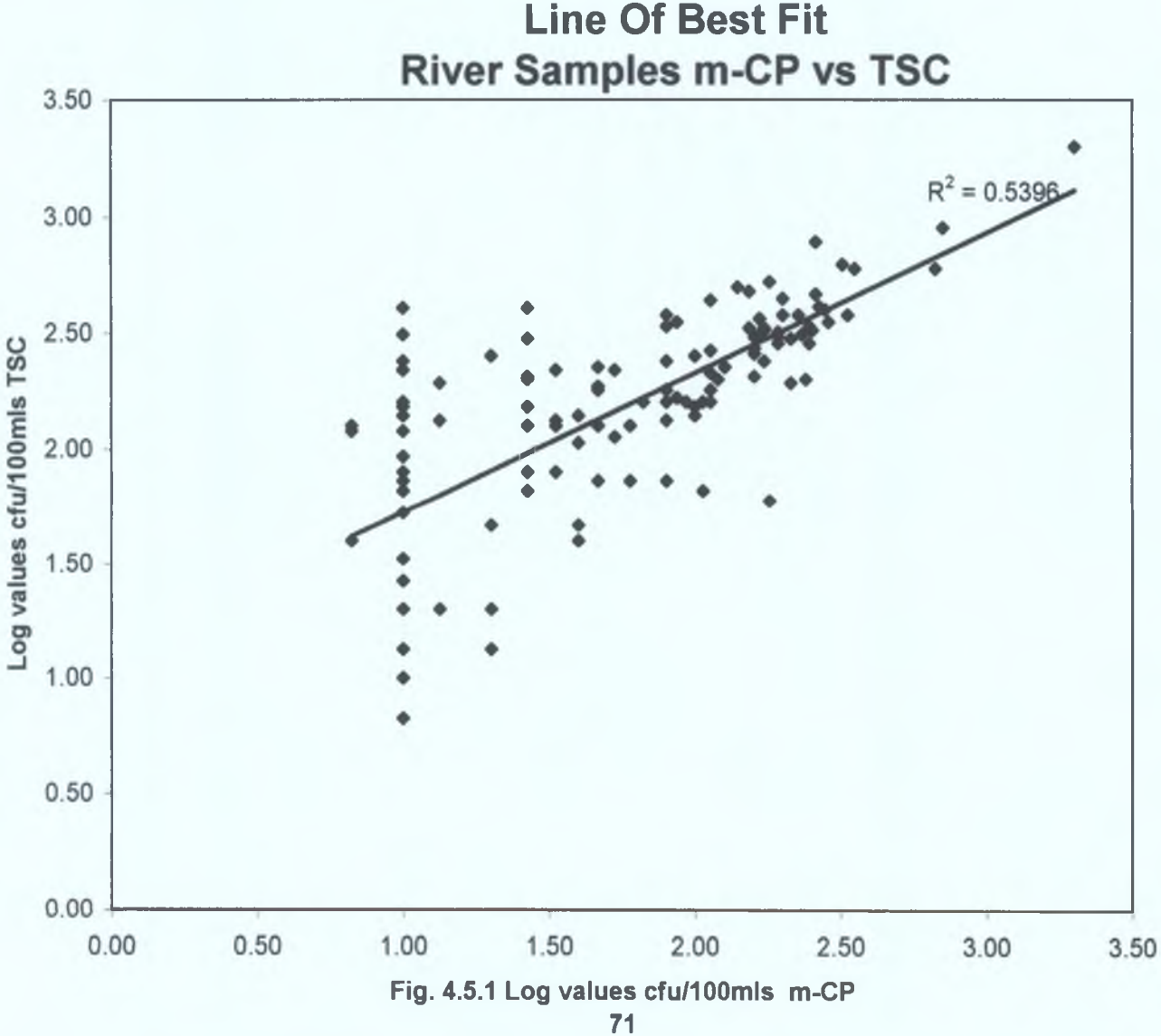


Fig. 4.5.1 Log values cfu/100mls m-CP
71

Fig. 4.5.1
71

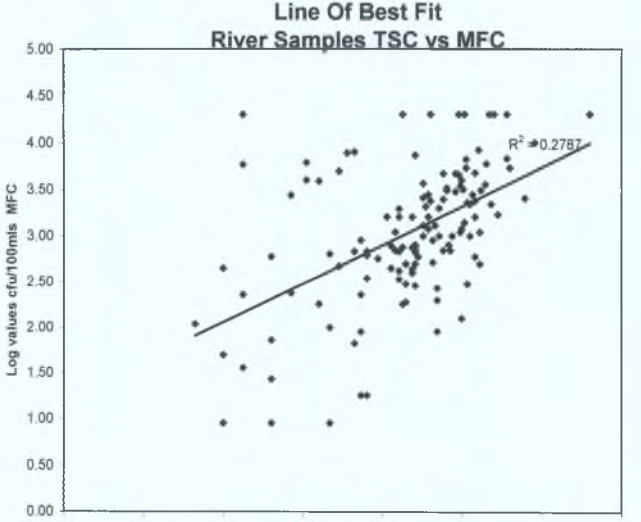


Fig. 4.5.2 Log values cfu/100mls TSC
72

Fig. 4.5.2
72

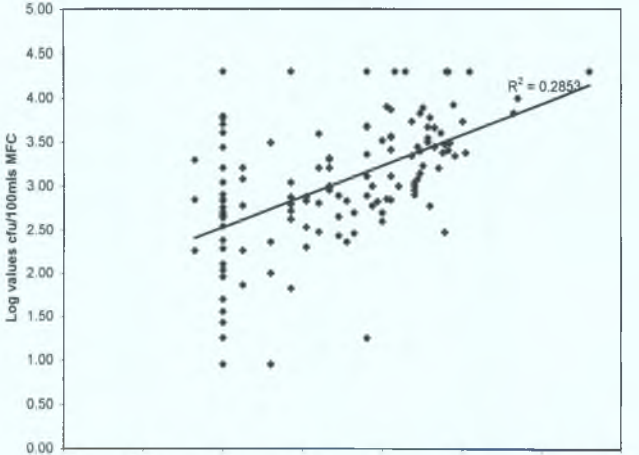


Fig. 4.5.3 Log values cfu/100mls m-CP

73

Fig. 4.5.3
73

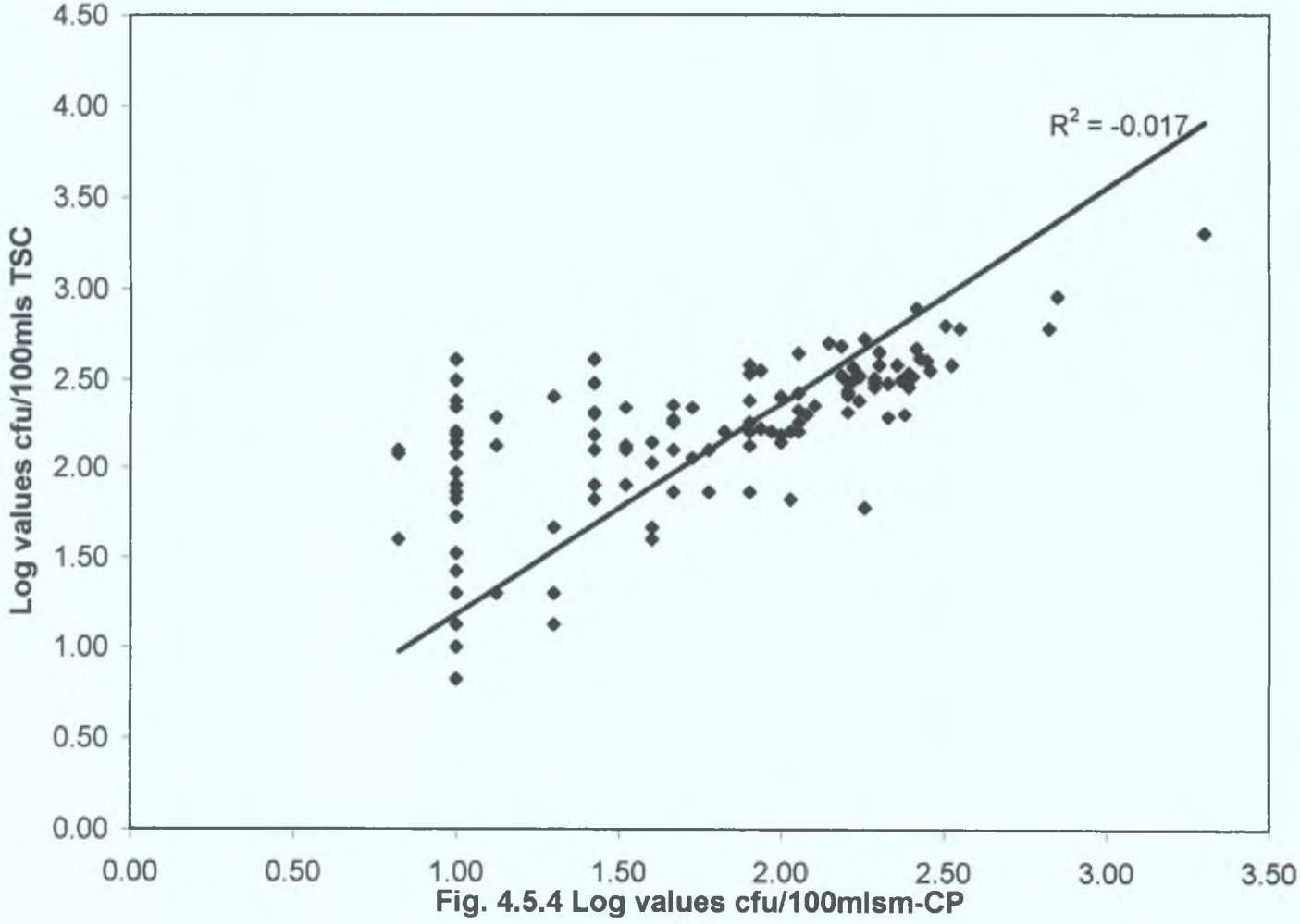


Fig. 4.5.4 Log values cfu/100mlsm-CP

74

Fig. 4.5.4
74

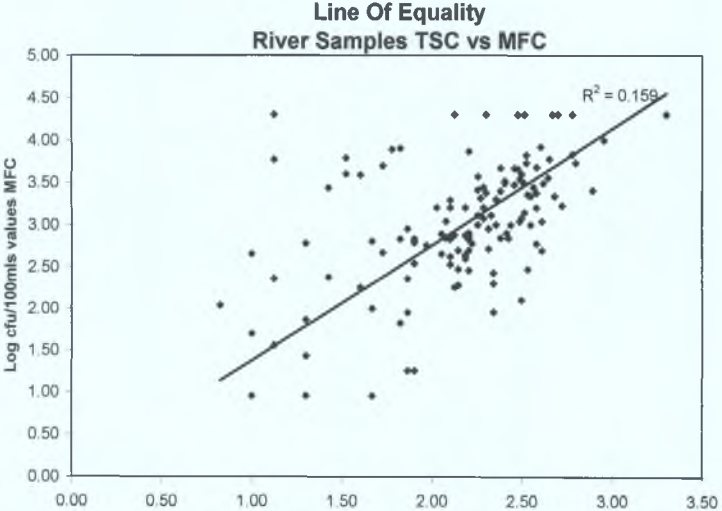


Fig. 4.5.5 Log values cfu/100mls TSC

Fig. 4.5.5
75

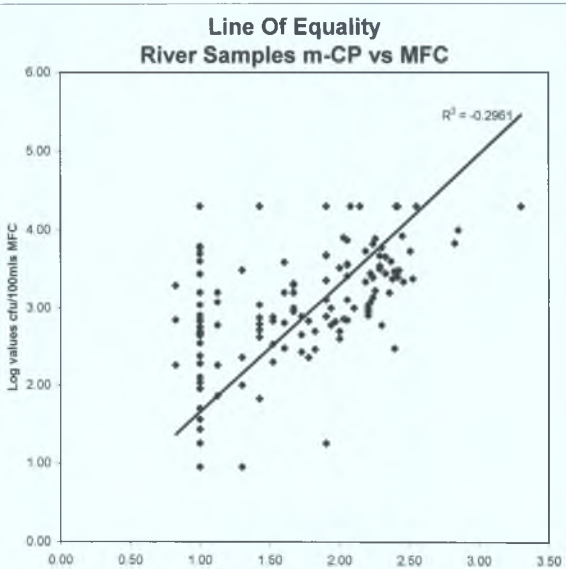


Fig. 4.5.6 Log values cfu/100mls m-CP

76

Fig. 4.5.6

76

4.5.1 Statistical Results for TSC & mCP River Samples:

All results were converted to log values. The Kolmogorav-Smirnov distance (KS) was 0.14 and the P value was 0.015. The data failed the normality test for Gaussian distribution with the P value <0.05. A non- parametric test was preferred instead of a paired t test because the data was not normal. A t test was still carried out as a comparison with the Wilcoxon Ranks Test

Number of sample pairs analyzed was 132. 8 pairs were excluded from the calculations because both values were equal.

4.5.2 Wilcoxon Matched-Pairs Signed-Ranks Test (Non-parametric) of mCP & EY-TSC River Samples

Assumption Test:

Effective Pairing of EY-TSC & mCP Samples:

Nonparametric Spearman correlation coefficient (r) = 0.75.

The one tailed P value was <0.0001, considered extremely significant.

The matching or pairing was effective.

Calculation Details:

Sum of all Signed Ranks	(W)	=	7395.0
Sum of positive ranks	(T+)	=	7572.5
Sum of negative ranks	(T-)	=	-177.5

Number of Pairs = 124

Wilcoxon Matched Pairs Signed Ranks P Value:

The two-tailed P value was <0.001, considered extremely significant.

(The P value was an estimate based on a normal approximation).

4.5.3 Paired t Test (two-tailed) of EY-TSC & mCP River Data:

The data for this test failed a normality test

P value:

The two-tailed P value was <0.001, considered extremely significant.

t = 12.40 with 131 degrees of freedom.

4.6 Drinking Mains Survey Results:

114 aseptically collected random samples were analyzed for *Cl. perfringens* using EY-TSC Agar and m-CP Medium. The samples were also simultaneously tested for coliforms and *E. Coli* on m-Endo and mFC broths respectively. All samples were paired and results were compared to determine if there was any correlation between methods.

Table 4.6.1 Summary of Raw Data For Drinking Water Samples:

Confirmed Colony Forming Units (CFU) /100mls				
	Cl. perfringens		*Coliforms*	*E.coli*
Sample	***EY-TSC***	***m-CP***	****m-Endo***	****m-FC***
1	**10**	**4**	<1	**1**
2	**1**	0	<1	<1
3	**1**	0	<1	<1
4	**3**	0	**49**	<1
5	**tntc**	**tntc**	**1733**	**345**
6	**1**	0	<1	<1
7	**1**	0	<1	<1
8	**1**	0	<1	<1
9	**1**	0	<1	<1
10	0	**1**	<1	<1
11	0	**1**	<1	<1

*m-Endo & m-FC methods were carried out using Colilert (a variation/modification of the Most Probable Numbers (MPN) Method). That is why <1 values are quoted instead of 0 values.

5.0 Discussion:

5.1 Comparison of Limits of Detection For *Cl. perfringens* at Dilution 1:70

The term 'limit of detection' is not strictly accurate in this context; rather the assay seeks to determine the relative efficiency of the methods in the recovery of a target abundance of 1 cfu per ml. This would be the lowest *Cl. perfringens* abundance that would be reported in practice.

The use of pure cultures, diluted appropriately ensures that there were no interferences from other Clostridia. 100 individual determinations were recorded, 50 paired samples each for EY-TSC and mCP. Comparing the mean values for Capsules 1 - 5 (table 4.1.2;pg 53) there is a noticeable difference in values. EY-TSC has a higher recovery rate for *Cl. perfringens* than mCP Medium. The mCP at dilution 1:70 was right at its detection limit where as EY-TSC had the potential for further dilution.

In table 4.1.3 the difference in the two means was 2 *Cl. perfringens* colonies. The standard error from the true population mean was low for both methods 0.24 and 0.14. (table 4.1.3;pg 54) The difference between min and the max number of colonies recovered on table shows that EY-TSC recovered nearly 2 - 3 colonies on average more than mCP at dilution1: 70. The mean of paired differences was 2.04 and the 95% confidence interval of the difference was 1.42 to 2.66. (The confidence interval must contain only positive values or negative values and must not contain a zero value).

Statistical P Value For 1:70 Dilution Data:

Paired t Test (two tailed):

An assumption test was carried out to demonstrate that the differences are sampled from a Gaussian distribution. The Kolmogorov and Smirnov (KS) method was used and the KS distance was used and the P value was >0.10. The data passed the normality test with P>0.05 (Table 4.1.4;pg 54).

The two-tailed P value was <0.001, considered very significant. The t value was 9.07 with 4 degrees of freedom.

Statistical P Value For 1:70 Dilution Data (contd):

Unpaired t test (two tailed):

Assumption tests were carried out to see if the data had equal standard deviations and came from a Gaussian distribution. It was found that the difference between the standard deviations was not significant. The F value=2.93 and the P value was 0.16. The data also passed the normality test using the Kolmogorov - Smirnov method. The two-tailed P value was <0.001, considered very significant. The t value was = 7.47 with 8 degrees of freedom.

There was a definite difference between both methods with TSC able to recover significantly more colonies than mCP at the dilution 1/70. The EY-TSC Agar had significantly higher results whether the test was statistically paired or unpaired. The EY-TSC Agar method could be potentially diluted further and hence have a better and lower limit of detection than mCP Medium. This has legal implication also. The legislation (Dir 98/83/EU) states that mCP is the reference method and yet in this project it has been found inadequate compared to EY-TSC in limits of detection at 1: 70 dilution of reference material. The mCP Medium is not equivalent to TSC Agar at 1: 70, EY-TSC out-performed mCP significantly in recovering *Cl. perfringens.*

5.2 Recoverability of *Cl. perfringens* at 1: 40 and 1: 50 Dilution on TSC & mCP Media:

There were 200 individual determinations carried out. 100 at 1:40 and 100 at 1:50 dilution for *Cl. perfringens* on EY-TSC and mCP Media. The mean of the five capsules in tables 4.2.2 & 4.3.2 (pages 56 & 58) for both methods clearly bears out the differences in the mean values. TSC, again, consistent with the 1: 70 dilution results, had higher recoveries for *Cl. perfringens* than mCP for both dilutions 1:40 and 1:50.

Statistical P Value For 1:40 and 1:50 Data:

Paired t Test (two tailed):

The data was assumption tested first for normality. Data for both dilutions passed the normality test for Gaussian distribution using the Kolmogorov-Smirnov method (see tables 4.2.4-4.3.4; pages 57 & 59). KS distance and P values were the same for both dilutions.

Statistical P Value For 1:40 and 1:50 Data:

Paired t Test (two tailed) (contd):

The actual P values were:

1:40 dilution P= 0.005, t= 5.71 with 4 degrees of freedom.

1:50 dilution P= 0.009, t = 4.79 with 4 degrees of freedom.

(See pages 57 & 60)

Both P values highlight that there was a very significant difference between both methods. EY-TSC had significantly higher mean recoveries than mCP.

The paired t-Test (two-tailed) was the preferred statistical analysis because the samples were paired and it was known whether or not mCP or EY-TSC was better for the recovery of *Cl. perfringens.*

Unpaired t Test (two tailed):

The data, again, passed the normality test and the standard deviations were not significantly different for EY-TSC and mCP at both dilutions.

The actual P values were:

1:40 dilution P= 0.001, t= 4.79 with 8 degrees of freedom.

1:50 dilution P= 0.007, t = 3.59 with 8 degrees of freedom.

(See pages 57 & 60)

Again, it didn't matter if the samples were paired or not, there was still a very significant difference between both methods.

5.3 Comparison of EY-TSC and mCP Media:

Combining all the evidence from all three dilutions (1:40, 1:50 & 1:70) it can be clearly observed that EY-TSC is by far a better medium for the recovery of *Cl. perfringens.* The results confirm the findings of various authors. Using Wilcoxon sign ranking test Sartory et al (1998) and Araujo et al (2001) found that there was a significant difference between EY-TSC and mCP (P=<0.001, Sartory 1998);(P=<0.05, Araujo 2001) with EY-TSC producing higher recoveries for *Cl. perfringens*. The mCP medium was found to be inhibitory and less efficient for the enumeration of both vegetative cells and spores (Burger et al, 1984)

5.3 Comparison of EY-TSC and mCP Media (contd):

Oxoid released the first commercially available mCP Medium in the autumn of 2001 (first proposed by Bisson & Cabelli (1979)). The data published by Oxoid stated that mCP had a greater sensitivity for the detection of *Cl. perfringens* than EY-TSC Agar (TSC = 47.8%; mCP = 93.3%). The Oxoid study also found that the true positive results of 366 samples were 64 and 125 for EY-TSC and mCP respectively. In contrast this study discovered the opposite was true. As mentioned above along with other studies, EY-TSC recovered significantly higher numbers of the organism than mCP. Sartory et al (1998) found recoveries of vegetative cells was 95% and 0.7% on TSC and mCP respectively. This study did agree on the false positive results obtained by Oxoid. Oxoid found the false positives to be 133 for TSC and 28 for mCP. This study discovered for river samples a false positive result of 232 (EY-TSC) and 70 (mCP) (table 4.5.3;pg 70); for saline samples 159 (EY-TSC) and 43 (mCP) on the 5ml dilution plates (see table 4.4.3;pg 61). The mCP is by far superior to TSC in this respect but it means very little in the light of all the evidence. There is no point in having a very selective media (mCP) if it recovers significantly less *Cl. perfringens* than its rival EY-TSC. The major drawback with EY-TSC is the opposite, it is not selective enough, it allows other Clostridia to grow and it can be painstaking to confirm a suitable proportion of colonies on every plate.

There was only a negligible difference in cost between both media. The mCP medium was harder to prepare and more time consuming than EY-TSC Agar making mCP less adapt for large volumes of samples. (Sartory et al, 1998).

5.4 Saline Samples (Dublin Bay)

Discussion:

All colony forming units counts /100mls were converted to log values to normalize the data. The log mean value for EY-TSC was 2.08 compared to 1.68 for m-CP, Median values were similar. This indicated that on average EY-TSC recovered more *Cl. perfringens* than m-CP. The standard error from the true population mean was only 0.07 for both media. The mean of paired differences was 0.41 and the 95% confidence interval of the difference was 0.30 to 0.52. (The confidence interval must contain only positive values or negative values and must not contain a zero value) (table 4.4.1; pg 61). The coefficient of determination (r^2) and line of best fit between EY-TSC and m-CP was low (r^2=0.50). This revealed that there was a poor correlation between both methods. For two methods testing for one organism the correlation should have been much higher (nearer r^2=0.99). Prediction values from intercept coordinates with the line of best fit would be inaccurate & unreliable (table 4.4.2; pg 61).

The r^2 value for the line of equality (r^2=0.15) was lower than the line of best fit (r^2=0.50). The line of equality determined if there was any agreement/comparison between the two methods. Forcing the intercept of the line through 0 is a better indication of how the two sets of values compare with each other. This result (r^2=0.15) indicates that there was little comparison or agreement between either method, the correlation was weak.The correlations between EY-TSC/m-CP and mFC were also poor. The mCP media had the worst correlation (coefficient of determination r^2=0.17) with mFC medium (E. coli) (table 4.4.2; pg 61. See also fig. 4.4.1-4.4.6; pg64-69).

This is contrary to the conclusions of the Dublin Bay Technical Report No. 6 which found a positive correlation between *E. coli* and *Clostridium*. (P=<0.001) EY-TSC Agar had more presumptive colonies, which were negative on confirmation than mCP. This demonstrated that mCP is more selective for *Cl. perfringens* than TSC. But mCP recovered less than EY-TSC. Recovery of *Cl. perfringens* outweighs the selectivity. Sartory et al (1998) found that of 76 isolates from mCP only 36 (47.4%) confirmed as *Cl. perfringens*, while 137 (86.7%) of 158 isolates from TSC confirmed positive. The mCP Medium was found to be inhibitory of *Cl. perfringens* spores and vegetative cells.

Though there was an observed difference in the recoveries from each method, with EY-TSC recovering more of the organism, was there a statistical significant difference between the two media EY-TSC and mCP?

Statistical P – Value For Saline Waters:

The data was normally distributed ($p>0.05$) and the pairing was effective ($p<0.001$). The parametric test carried out was a Two Tailed Paired t Test, which resulted in a very significant difference between EY-TSC and mCP ($p<0.001$). The non-parametric test carried out was a Wilcoxon Matched Pairs Signed Ranks Test, also very significant ($p<0.001$) (see section 4.4.1 & 4.4.2; pg 62). This result ($p<0.001$) confirms that there is a considerable significant difference between the two methods, in saline samples TSC Agar recovers more *Cl. perfringens* than mCP Medium. This is supported by the higher mean/median value for TSC and the poor correlation values mentioned above.

The null hypothesis (Ho) (i.e. that there is no difference between populations) is rejected in favour of the alternative hypothesis (Ha) for this set of samples (i.e. there is a difference).

5.5 *Cl. perfringens* vs Coliforms As an Indicator in Saline Waters

Comparison of the actual data (not the log values) in appendix 2 between the *Cl. perfringens* media TSC/mCP and mEndo/mFC media demonstrates a difference in the volume of colonies counted. The confirmed colony counts on mEndo/ mFC are mostly in the thousands or tens of thousands. In contrast very few counts are above a thousand on the mCP/ EY-TSC media. Also the sample volume filtered for coliforms and *E. coli* were small 0.1ml & 1ml/ plate. This would incur errors in the final calculated results due to high dilution and high number of colonies per sample plate.

Cl. perfringens are found in much lower concentration than coliforms. They are also more persistent indicators of remote pollution and because of smaller colony counts might make it a useful tool in measuring pollution dispersion within the Bay.

5.5 *Cl. perfringens* vs Coliforms As an Indicator in Saline Waters (contd)

Cl. perfringens spores appear not to be affected by either predators or die off even after a month (Davies, 1995). Coliforms 'die off' to only 10% of their original concentration after 85 days. The cause of coliform reduction is due to high salinity, toxic agents, predation and low available nutrition (Davies, 1995). Even with such a die off over time there appears to be still a huge number of Coliforms present in the marine environment in Dublin Bay. It must be remembered that dumping at sea was stopped in 1999. This might be due to bacteria adhering to sediment particles that may protect from harmful factors and actually aid growth and persistence in the marine environment. The main reason is probably due to Ringsend wastewater treatment facility not being fully online yet. It will be operational at the end of 2002.

5.6 River Samples

Discussion:

All sample results/100mls were converted to log values to normalize the data. The statistical data was similar to that of the saline samples. The log mean value for EY-TSC was 2.17 compared to 1.74 for m-CP, Median values were similar. This indicated that on average EY-TSC recovered more *Cl. perfringens* than m-CP. The standard error from the true population mean was only 0.04 & 0.05 for EY-TSC & m-CP respectively. The mean of paired differences was 0.43 and the 95% confidence interval of the difference was 0.36 to 0.50. (The confidence interval must contain only positive values or negative values and must not contain a zero value) (table 4.5.1; pg 70).

The coefficient of determination (r^2) and line of best fit between EY-TSC and m-CP was low (r^2=0.54). This revealed that there was a poor correlation between both methods. For two methods testing for one organism the correlation should have been much higher (nearer r^2=0.99). Prediction values from intercept coordinates with the line of best fit would be inaccurate (table 4.5.2; pg 70). The r^2 value for the line of equality (r^2=0.02) was lower than the line of best fit (r^2=0.54). The line of equality determined if there was any agreement/comparison between the two methods.

5.6 River Samples (contd)

Forcing the intercept of the line through 0 is a better indication of how the two sets of values compare with each other. This result (r^2=0.02) indicates that the two methods had poor comparison or agreement; the correlation was weak. The correlations between EY-TSC/m-CP and mFC were also poor. The EY-TSC media had the worst correlation and agreement (Best Fit r^2=0.28, Equality r^2=0.16) with mFC medium (E. coli). The opposite was true for saline samples; mCP was lower (table 4.5.2).

EY-TSC Agar had more presumptive colonies, 232 and 534 on 5mls and 10mls plates respectively, which were negative on confirmation. The mCP media had 70 & 90 colonies on 5ml and 10mls plates respectively (see table 4.5.3 pg 70). This demonstrated that mCP is more selective for *Cl. perfringens* than EY-TSC. But mCP recovered less than EY-TSC. As was mentioned for saline samples, recovery of *Cl. perfringens* outweighs the selectivity.

Though there was an observed difference in the recoveries from each method, with EY-TSC recovering more of the organism, was there a statistical significant difference between the two media EY-TSC and mCP?

Statistical P – Value River Samples:

The data was not normally distributed and a non-parametric test was chosen instead.

The non-parametric test carried out was a Wilcoxon Matched Pairs Signed Ranks Test.

The pairing was effective with a non-parametric Spearman correlation coefficient (r=0.75),

There was a significant difference between mFC and EY-TSC ($p<0.001$) (see pg 77)

This result ($p<0.001$) confirms that there is a considerable significant difference between the two methods. EY-TSC Agar, for river samples, recovers more *Cl. perfringens* than mCP Medium. This is supported by the higher mean/median value for EY-TSC and the poor correlation values.

The null hypothesis (Ho) (i.e that there is no difference between populations) is rejected in favour of the alternative hypothesis (Ha) for this set of samples (i.e. there is a difference).

5.7 Drinking Water Survey Samples

There was no real statistical analysis that could be done on these results because out of 114 samples only a few samples proved positive. These water samples had undergone chlorination prior to introduction to the public supply and had chlorine levels in the range 0.02 - 0.40mg/l, maintaining a bactericidal function. The r or r^2 values would be meaningless. Values would be close to 1 and would be misleading. The presence of so many zero results does not prove either way, whether or not, that there is a significant difference between the methods (see Appendix 2 Raw Data).

EY-TSC Agar:

12 samples had presumptive *Cl. perfringens* colonies. 3 samples proved to be negative on confirmation in Crossely milk medium. Another EY-TSC plate had greater than 200 colonies and was too numerous to count (tntc), (sample 5). The eight remaining EY-TSC plates had an overall total of 36 presumptive *Cl. perfringens* colonies. On confirmation only 19 colonies proved to be *Cl. perfringens*, samples 1-4 & 6-9 (see table 4.6.1; pg 78).

m-CP Medium:

4 samples had presumptive *Cl. perfringens* colonies. 3 samples proved to be positive on confirmation in Crossely milk medium for *Cl. perfringens*. The other mCP plate had greater than 200 colonies and was too numerous to count (tntc). The 3 remaining mCP plates had an overall total of 8 presumptive *Cl. perfringens* colonies. On confirmation only 6 colonies proved to be *Cl. perfringens*, samples 1,10 and 11 (see table 4.6.1; pg 78).

Discussion:

Table 4.6.1 displays the 9 confirmed positive samples found in the 114 drinking water samples obtained. It can be observed from the table that there were 9 positive results on EY-TSC compared with only 4 on m-CP Medium. On Sample one, 21 colonies on EY-TSC, initially, were presumptive for *Cl. perfringens* on confirmation 10 were positive. For m-CP 6 were presumptive with 4 proving positive. Sample four the result was 3 and 0 for EY-TSC and m-CP respectively.

5.7 Drinking Water Survey Samples (contd)

Discussion:

Sample five there were too many colonies observed for enumeration to be effective on both media. For the remaining six samples EY-TSC had 4 positive colonies on samples 6-9 and m-CP had 2 positive colonies on samples 10 and 11. Overall it can be observed from the 11 samples on table 4.6.1 that, though EY-TSC is not as selective as m-CP Medium, it can recover a lot more positive colonies from drinking water samples than m-CP. From table 4.6.1 excluding sample five, 19 positive colonies were recovered from 114 samples compared with only 6 from m-CP. Also a rough correlation can be seen between the *Cl. perfringens* media and the mFC and mEndo broths. Sample one had 10, 4 and 1 colonies for EY-TSC, m-CP and mFC, sample four had 3 and 49 colonies for EY-TSC and mEndo respectively. Sample five, there were too many colonies observed to enumerate on the perfringens plates with a corresponding, countable, high number of colonies on the mEndo/mFC plates.

As discussed in the literature review, *Cl. perfringens* is a reliable indicator of faecal pollution (Garbow, 1996, Sorensen 1989). All the drinking water samples containing Coliforms or *E. Coli* also contained *Cl. perfringens*. There was one draw back; six samples contained *Cl. perfringens* but no Coliforms. This may have been due to *Cl. perfringens* producing spores, which are persistent and are more indicative of remote or distant contamination of the water supply. The six samples containing *Cl perfringens* might have been contaminated in the past but are free of Coliforms now. Therefore presence of *Cl. perfringens* might not be a good indicator of recent pollution incidents. The spores are also chlorine tolerant.

Sample five had a very high level of all three organisms. This might have occurred if there was a problem with filter beds in the waterworks e.g. a 'breakthrough' in the sand filter. The filters are the main defense against spores and parasites (Payment, 1990). Presence of *Cl. perfringens* could be a useful tool in determining the efficiencies of both the rapid gravity and slow sand filtration systems.

6.0 Conclusions:

- EY-TSC Agar recovers significantly more *Cl. perfringens* than m-CP Medium in relation to detection of limits, recoverability and environmental samples ($P<0.001$).

- Based on this study and evidence from other work it is very questionable that mCP is used as a standard reference medium in Directive 98/83/EU. The EY-TSC proved not only equal to mCP but it also out performed it in a number of set criteria.

- EY-TSC Agar was easier to prepare and was less time consuming than mCP Medium. There was relatively little difference in cost of both media. The mCP Medium, though, would prove more expensive in the long term because of labour costs.

- *Cl. perfringens* can be a useful indicator of faecal pollution in drinking water, river and Saline Samples.

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Appendix 1

Standard Operating Procedure for Coliforms and Faecal Coliforms.

DUBLIN COPORATION

MICROBIOLOGY LABORATORY

LABORATORY METHODS MANUAL

Date	Issue	S.O.P.	Determination of:	Page
24/11/00	2	290	Presumptive Total coliforms by Membrane Filtration	1of 12

PRINCIPLE: A measured volume of sample is filtered through a cellulose ester membrane with a nominal pore size of 0.45 µm. Bacteria to be enumerated are retained on the membrane which is then placed on a differential medium selective for coliform bacteria. After a specified incubation period, coliform colonies of characteristic morphology and colour on the membrane are counted. Other non-coliform organisms are either inhibited or can be distinguished by their colonial appearance. The number of coliform colonies counted is used to estimate the number of colony forming units (CFU) per 100 ml of sample.

REFERENCES:

1. The Microbiology of Water 1994. Part 1 - Drinking Water. Report on Public Health and Medical Subjects No. 71. Methods for the examination of water and associated materials. London: HMSO.

2. Standard Methods for the Examination of Water and Wastewater, 19th Edition, 1995. (APHA - AWWA - WEF).

SAFETY: Dehydrated M-Endo broth may cause eye, skin or respiratory irritation. Basic fuchsin, present in the nutritive broth, is a potential carcinogen. Gloves should be worn to avoid contamination of the skin. Inhalation of the powder during preparation of the dehydrated broth should be avoided and dust masks (conforming to British Standard No. 6016) should be worn while handling dehydrated media. Use in properly ventilated area. Wash thoroughly after handling.

Methylated spirits used for flaming forceps must only be dispensed in small volumes sufficient for immersion of forcep tips to a depth of approximately 1 - 1.5 cm. When in use place in a position away from bunsen so that when retrieving forceps from flame, the forceps does not pass over the methylated spirit.

EQUIPMENT:

1. Filtration Apparatus - Sterile.
2. Autoclave.
3. Incubator at 37 ± 1.0 °C
4. Microscope providing 10-15 diameters magnification, and fluorescent illumination.
5. Sterile mixed cellulose ester Membranes. Pore size 0.45 µm; diameter 47 mm; white; gridded.
6. Sterile disposable 50 mm Petri Dishes containing absorbent pads.
7. Membrane Forceps - smooth blunt tips.
8. Refrigerator at 2 to 8 °C.
9. Sterile applicator swabs.

REAGENTS:

1. Ringer Solution. OXOID - Code BR52. Prepare daily sufficient quarter strength Ringer Solution by dissolving 1 tablet in 500 ml distilled or deionised water. Autoclave at 121 ± 2 °C for 15 minutes and cool to room temperature.

2. M-Endo Broth. DIFCO - Code 0749-17. Reconstitute the dehydrated base by suspending 48 g of the powder in 1 litre distilled or deionised water containing 20 mls of 95% ethanol. Mix thoroughly. Do not autoclave. Heat the medium to

boiling, but avoid overheating. The pH of the medium should be in the range 7.1 to 7.3 at 25 °C. To measure pH of the medium, (SOP?) aseptically dispense 20mls medium into a 50 ml beaker. Measure the pH of the medium in the beaker. If it is not in the range 7.1 to 7.3, adjust with a measured volume of 1N hydrochloric acid, or 1N sodium hydroxide. Add 20X ml of the required solution (1N hydrochloric acid to increase the pH reading, or 1N sodium hydroxide to decrease the pH reading) where X is the total ml of media being measured. Record the final pH value on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 890. Record any pH adjustments made to the medium in the Microbiology Laboratory Diary.

3. Prepared broth may be stored in batches in sterile 100 ml Duran bottles in the refrigerator at 2 to 8 °C for up to 96 hours. pH of each batch must be checked immediately before use and any necessary adjustments made with 1N hydrochloric acid, or 1N sodium hydroxide. Record the final pH value on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 890. Record any pH adjustments made to the medium in the Microbiology Laboratory Diary.

4. Flaming Alcohol. Dispense 10 to 20 ml of methylated spirits into a 50 ml glass beaker to be used in flaming forceps.

5. Positive Organism Control Suspension. Prepare a suspension of *Enterobacter cloacae* using one gelatin capsule of reference material *Enterobacter cloacae* 500 (WR3) as described in SOP 810.

6. Negative Organism Control Suspension. Prepare a suspension of *E.coli* using one gelatin capsule of reference material *E.coli* 500 (WR3) as described in SOP 810.

PROCEDURE:

1. Swab down the work area of the benchtop with surgical spirits prior to analysis. Perform an initial check for benchtop contamination using the method described at 10 (vi) below.

2. Saturate an absorbent pad in a sterile 50 mm petri dish with 2.1 ml m-Endo Broth. Pour off any excess medium to avoid confluent colony growth.

3. Aseptically place a sterile gridded, 0.45 µm membrane filter in the filtration apparatus, gridded side uppermost.

4. Slowly filter a known volume of sample under partial vacuum of about 65 kPa (500 mm of mercury). An ideal volume will yield growth of about 50 coliform colonies, and not more than 200 colonies of all types.

For drinking water, filter 100 ml of sample dispensed directly into the funnel using the funnel volume graduation. Samples of 20 to 100 ml volume should be measured using a sterile graduated cylinder. Samples of 1 to 20 ml volume are measured using air displacement pipettes with sterile

disposable tips. When filtering less than 20 ml of sample, add at least 20 ml sterile, quarter strength Ringer Solution to the funnel before filtration to ensure uniform dispersion of the bacterial suspension. Do not pipette less than 1 ml aliquots of the sample. If smaller volumes are required to be filtered, prepare appropriate dilutions in quarter strength Ringer Solution.

5. Close the stopcock to break the vacuum as soon as the sample has been filtered so that as little air as possible is drawn through the membrane. Aseptically remove the membrane. Place it on the absorbent pad in the petri dish, grid side uppermost. Avoid trapping air bubbles beneath the membrane to ensure complete contact between it and the pad.

6. Replace the petri dish lid, invert the dish and incubate for 18 to 24 hours at 37 ± 1.0 °C.

7. Rinse the funnel without splashing with three 20 to 30 ml aliquots of sterile, quarter strength Ringer Solution.

8. After incubation, count presumptive total coliform colonies on each membrane, using low power magnification and fluorescent illumination. Typical coliform colonies are red with a greenish-gold, metallic sheen. Record the actual number of colonies counted in the Laboratory Work Book "Total and Faecal Coliforms".

9. If no confirmatory tests are to be carried out on presumptive total coliform colonies, discard counted plates into Biohazard Bin for incineration. Otherwise retain plate for confirmatory tests (SOP 289).

10. The following controls should be run with each batch of samples for presumptive total coliform analysis:

(i) Positive Organism Control - *Enterobacter cloacae* suspension (reagent 4 above). Analyse 1 ml of this suspension as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 886.

(ii) Media Control. Saturate an absorbent pad in a sterile petri dish with 2.1 mls of m-Endo broth. Pour off any excess medium, cover the dish and incubate with the samples as in analysis procedure (steps 6 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 891.

(iii) Ringer Solution Control. To check for contamination of the quarter strength Ringer Solution, analyse 50 mls of this solution as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 892.

(iv) Evironmental Air Plate. Saturate an absorbent pad in a sterile petri dish with 2.1 mls m-Endo Broth. Pour off excess broth and place the uncovered dish on the bench while samples are being processed. When all samples have been filtered, cover the dish and incubate with the samples as in analysis procedure (steps 6 to 9) above. Record the number of colonies counted on Quality Control

Sheet for Total and – Faecal Coliforms (Sample Type 890) under procedure number 888.

(v) Before starting analysis, swab the cleaned benchtop with a sterile applicator swab by drawing the swab across approximately 1m of the bench surface in a zigzag pattern. Place the swab in a sterile flask containing 25 mls Ringer Solution and shake to suspend bacteria from the swab. Analyse the suspension as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 878.

RESULTS: Preferentially compute results from samples that produce membrane filter counts in the range 20 to 80 colonies. If all counts are below 20 colonies, use the counts for the largest volume filtered to compute the results. If all counts are above 80 use counts for the smallest volume filtered to compute the results.

Report results as :

Presumptive Total Coliforms CFU/100ml = $\frac{\text{(Number Coliform Colonies) x 100 ml}}{\text{Sample Filtered}}$

If no presumptive total coliforms are detected when less than 100 ml volume of a sample is filtered, results should be reported as CFU per actual volume filtered i.e.

0 CFU / x ml where x is the volume of sample filtered.

Where confirmation of presumptive total coliform colonies is required follow SOP 289.

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COLIFORMS

Survey: _____

Sample Date: ___/___/___ Counted By: _____

	Total Coliforms (SOP 290)				Faecal Coliforms (SOP 291)			
Site	mls Filtered	Plate Count	Nos/100ml	Mean	mls Filtered	Plate Count	Nos/100ml	Mean
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								

Figure 1: Results Sheet for Total Coliforms

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LABORATORY METHODS MANUAL

Date	Issue	S.O.P.	Determination of:	Page
24/11/00	2	291	Presumptive Faecal coliforms by Membrane Filtration	1of 12

PRINCIPLE: A measured volume of sample is filtered through a cellulose ester membrane with a nominal pore size of 0.45 µm. Bacteria to be enumerated are retained on the membrane which is then incubated on a differential medium, and at a temperature which is selective for coliform bacteria of faecal origin. After a specified incubation period, faecal coliform colonies of characteristic morphology and colour on the membrane are counted. Other non-coliform organisms are either inhibited or can be distinguished by their colonial appearance. The number of faecal coliform colonies counted is used to estimate the number of colony forming units (CFU) per 100 ml of sample.

REFERENCES:

1. The Microbiology of Water 1994. Part 1 - Drinking Water. Report on Public Health and Medical Subjects No. 71. Methods for the examination of water and associated materials. London: HMSO.

2. Standard Methods for the Examination of Water and Wastewater, 19th Edition, 1995. (APHA - AWWA - WEF).

SAFETY:

Inhalation of fine powders may be harmful, causing irritation of the upper respiratory tract, especially with bile salt products. Dust masks (conforming to British Standard No. 6016) should be worn while handling dehydrated media. Gloves may be worn to prevent mild skin rashes on prolonged contact. Work in adequately ventilated areas.

Methylated spirits used for flaming forceps must only be dispensed in small volumes sufficient for immersion of forcep tips to a depth of approximately 1 - 1.5 cm. When in use place in a position away from bunsen so that when retrieving forceps from flame, the forceps does not pass over the methylated spirit.

EQUIPMENT:

1. Filtration Apparatus - Sterile.
2. Autoclave.
3. Water Bath at 44.0 ± 0.5 °C
4. Sterile mixed cellulose ester Membranes. Pore size 0.45 µm; diameter 47 mm; white; gridded.
5. Sterile applicator swabs.
6. Sterile disposable 50 mm Petri Dishes containing absorbent pads.
7. Membrane Forceps - smooth blunt tips.
8. Refrigerator at 2 to 8 °C.

REAGENTS:

1. Ringer Solution. OXOID - Code BR52. Prepare daily sufficient quarter strength Ringer Solution by dissolving 1 tablet in 500 ml distilled or deionised water. Autoclave at 121 ± 2 °C for 15 minutes and cool to room temperature.

2. 1% Rosolic Acid Solution. Prepare 0.2 N NaOH by dissolving 0.8 g NaOH in 100 ml distilled or deoinized water. Into this dissolve 1.0g Rosolic acid (DIFCO - Code 3228-09-1). Do not autoclave. Store this 1% Rosolic Acid Solution in the dark in the fridge at 2 to 8 °C and discard after 2 weeks, or sooner if its colour changes from dark red to muddy brown.

3. m-FC Broth. DIFCO - Code 0883-17-3. Reconstitute the dehydrated base by suspending 37 g of the powder in 1 liter distilled or deionised water. Add 10 ml of a 1% solution of Rosolic Acid in 0.2 N NaOH (Reagent 2 above). Mix thoroughly. Do not autoclave. Heat the medium to boiling, but avoid overheating. Promptly remove from heat and cool. The pH of the medium should be in the range 7.2 to 7.6 at 25 °C. To measure pH of the medium, aseptically dispense 20 mls medium into a 50 ml beaker. The beaker need not be sterile. Measure the pH of the medium in the beaker. If it is not in the range 7.2 to 7.6, adjust with a measured volume of 1N hydrochloric acid, or 1N sodium hydroxide. Add 20X ml of the required solution (1N hydrochloric acid to increase the pH reading , or 1N sodium hydroxide to decrease the pH reading) where X is the total ml of media being measured. Record the final pH value on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 893. Record any pH adjustments made to the medium in the Microbiology Laboratory Diary.

Prepared broth may be stored in batches in sterile 100 ml Duran bottles in the refrigerator at 2 to 8 °C for up to 96 hours. pH of each batch must be checked immediately before use and any

necessary adjustments made with 1N hydrochloric acid, or 1N sodium hydroxide. Record the final pH value on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 893. Record any pH adjustments made to the medium in the Microbiology Laboratory Diary.

4. Flaming Alcohol. Dispense 10 to 20 ml of methylated spirits into a 50 ml glass beaker to be used in flaming forceps.

5. Positive Organism Control Suspension. Prepare a suspension of *Escherichia coli* using one gelatin capsule of reference material *Escherichia coli* 500 (WR1) as described in SOP 810.

6. Negative Organism Control Suspension. Prepare a suspension of *Enterobacter cloacae* using one gelatin capsule of reference material *Enterobacter cloacae* 500 (WR3) as described in SOP 810.

PROCEDURE:

1. Swab down the work area of the benchtop with surgical spirits prior to analysis. Perform an initial check for benchtop contamination using the method described at 10 (vi) below.

2. Saturate an absorbent pad in a sterile 50 mm petri dish with 2.1 ml m-FC Broth. Pour off any excess medium to avoid confluent colony growth.

3. Aseptically place a sterile gridded, 0.45 µm membrane filter in the filtration apparatus, gridded side uppermost.

4. Slowly filter a known volume of sample under partial vacuum of about 65 kPa (500 mm of mercury). An ideal volume will yield growth of 20 to 80 faecal coliform colonies, and not more than 200 colonies of all types. For drinking water, filter 100 ml of sample dispensed directly into the funnel using the funnel volume graduation. Samples of 20 to 100 ml volume should be measured using a sterile graduated cylinder. Samples of 1 to 20ml volume are measured using air displacement pipettes with sterile tips. When filtering less than 20ml of sample, add

at least 20 ml sterile, quarter strength Ringer Solution to the funnel before filtration to ensure uniform dispersion of the bacterial suspension. Do not pipette less than 1 ml aliquots of the sample. If smaller volumes are required to be filtered, prepare appropriate dilutions in quarter strength Ringer Solution.

5. Close the stopcock to break the vacuum as soon as the sample has been filtered so that as little air as possible is drawn through the membrane. Aseptically remove the membrane. Place it on the absorbent pad in the petri dish, grid side uppermost. Avoid trapping air bubbles beneath the membrane to ensure complete contact between it and the pad.

6. Replace the petri dish lid, invert the dish and place in a water-tight container. Fully immerse the container in the water bath and incubate for 18 to 24 hours at

44.0 ± 0.5 °C.

7. Rinse the funnel without splashing with three 20 to 30 ml aliquots of sterile, quarter strength Ringer Solution.

8. After incubation, count presumptive faecal coliform colonies on each membrane. Typical faecal coliform colonies are blue in colour. Record the actual number of colonies counted in the Laboratory WorkBook "Total and Faecal Coliforms".

9. If no confirmatory tests are to be carried out on presumptive faecal coliform colonies, discard counted plates into Biohazard Bin for incineration. Otherwise retain plate for confirmatory tests (SOP 289).

10. The following controls should be run with each batch of samples for presumptive total coliform analysis:

(i)Positive Organism Control - *Escherichia coli* suspension (reagent 4 above). Analyse 1 ml of this suspension as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 887.

(ii)Negative Organism Control - *Enterobacter cloacae* suspension (reagent 5 above). Analyse 1 ml of this suspension as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 881.

iii) Media Control. Saturate an absorbent pad in a sterile petri dish with 2.1 mls of m-FC broth. Pour off any excess medium, cover the dish and incubate with the samples as in analysis procedure (steps 6 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 894.

(iv)Ringer Solution Control. To check for contamination of the quarter strength Ringer Solution, analyse 50 mls of this solution as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms. (Sample Type 890) under procedure number 895.

(v) Evironmental Air Plate. Saturate an absorbent pad in a sterile petri dish with 2.1 mls m-FC Broth. Pour off excess broth and place the uncovered dish on the bench while samples are being processed. When all samples have been filtered samples as in analysis procedure (steps 6 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 889.

(vi) Before starting analysis, swab the cleaned benchtop with a sterile applicator swab by drawing the swab across approximately 1m of the bench surface in a zigzag pattern. Place the swab in a sterile flask containing 25 mls Ringer Solution and shake to suspend bacteria from the swab. Analyse the suspension as in sample analysis procedure (steps 2 to 9) above. Record the number of colonies counted on Quality Control Sheet for Total and Faecal Coliforms (Sample Type 890) under procedure number 879.

RESULTS: Preferentially compute results from samples that produce membrane filter counts in the range 20 to 80 colonies. If all counts are below 20 colonies, use the counts for the largest volume filtered to compute the results. If all counts are above 80 use counts for the smallest volume filtered to compute the results.

Report results as **Presumptive Faecal Coliforms CFU/100ml**

$$= \frac{\text{(Number Faecal Coliform Colonies) x 100}}{\text{ml Sample Filtered}}$$

If no presumptive faecal coliforms are detected when less than 100 ml volume of a sample is filtered, results should be reported as CFU per actual volume filtered i.e.

0 CFU / x ml where x is the volume of sample filtered.

Where confirmation of presumptive faecal coliform colonies is required follow SOP 289.

COLIFORMS

Survey: _____

Sample Date: ___/___/___ Counted By: _____

	Total Coliforms (SOP 290)				Faecal Coliforms (SOP 291)			
Site	mls Filtered	Plate Count	Nos/100ml	Mean	mls Filtered	Plate Count	Nos/100ml	Mean
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								

Figure 1: Results Sheet for Faecal Coliforms

DUBLIN COPORATION

MICROBIOLOGY LABORATORY

LABORATORY METHODS MANUAL

Date	Issue	S.O.P.	Determination of:	Page
15/11/00	2	290d	Total coliforms	1of 9

PRINCIPLE: The concept of the coliforms is of strains of Enterobacteriacae able to ferment lactose, which invariably begins with the cleavage of lactose into glucose and galactose by the enzyme β-galactosidase. Therefore, a fundamental characteristic of a coliform is the possession of a gene coding for the production of β-galactosidase. The presence of the β-galactosidase gene in these tests is demonstrated by the metabolism of the chromogenically labelled enzyme specific substrate ortho-nitrophenol galactopyranoside (ONPG). Metabolism of ONPG releases free ortho-nitrophenol which imparts a bright yellow colour to the medium.

The inoculated medium is dispensed into a series of wells of various volumes and incubated. A count of wells for positive for Total Coliform (yellow colouration) is used to estimate coliform abundance by reference to Most Probable Number (MPN) tables.

The following genera have been isolated in routine practice:

Citrobacter, Enterobacter, Escherichia, Hafnia, Klebsiella, Serratia.

REFERENCE:

1.The Microbiology of Water 1994. Part 1-Drinking water. Report on Public Health and Medical Subject No.71 . Methods for the Examination of Waters and Asociated Materials. London:HMSO.

2.Evaluation trials for two media for the simultaneous detection and enumeration of *Escherichia coli* and coliform organisms 1998. Methods for the Examination of Waters and Associated Materials. London :HMSO.

SAFETY:

Inhalation of fine powders may be harmful, causing irritation of the upper respiratory tract, especially with bile salt products. Dust masks (conforming to British Standard No. 6016) should be worn to prevent mild skin rashes on prolonged contact. Work in adequately ventilated areas.

EQUIPMENT

1.Autoclave

2.Incubator at 35.0 ± 0.5°C

3. Quanti -Tray or Quanti-Tray 2000

4. Quanti-Tray Sealer

5. Refrigerator at 2-8°C

6. Reaction Vessel - 120ml disposable polystyrene vessel. Non-autoflourescesing, sterile, with calibrated fill line.

7. UV Viewing Cabinet with 365nm UV source.

8.IDEXX P/A Compartor Cell.

REAGENTS:

1.Enzyme specific substrate ONPG. Colilert®-18 Dehydrated Reagent in unit doses for addition to sample in Reaction vessel.

2a.Control Organism Suspension. Prepare a suspension of *Escherichia coli* using one gelatin capsule of reference material *Escherichia coli* 500 (WR1) as described in SOP ____.

2b. Control Organism Suspension. Prepare a suspension of *Enterobacter cloacae* using one gelatin capsule of reference material *Enterobacter cloacae* 500(WR3) as described in SOP_____.

3. Sterile Quarter strength Ringers Solution

PROCEDURE:

1. Add unit dose of Colilert®-18 Dehydrated Reagent to reaction vessel.

2. In the case of Saline samples add 1ml of sample and 99ml sterile deionised water to the reaction vessel. In the case of fresh water samples add 100ml of sample to the reaction vessel. Cap the vessel and shake until the medium is dissolved.

3. Aseptically transfer the 100ml sample and reagent from the reaction vessel into a Quanti-Tray or Quanti-Tray/2000. Quanti-Tray will allow abundance estimates up to 20,000/100ml at the 1:100 sample dilution used here. If higher bacterial abundance is anticipated use the Quanti-Tray/2000 which allows estimates up to 241,900/100ml at the 1:100 sample dilution used here.

4. Seal the Quanti-tray in the Quanti-Tray sealer and incubate at 35± 0.5°C for 18 hours. Ensure the wells of the Quanti-Tray are facing upwards throughout the whole incubation period.

5. After incubation check the Quanti-Tray in normal lighting. Count the number of wells showing a colour change to bright yellow indicating the presence of coliforms. Colour

development should be equal to, or greater than that shown in the IDEXX comparator.

6. The following controls should be run with each batch of confirmatory tests:

(i) Positive Organism Control- Mix 100ml of *E.coli* suspension with Colilert® Reagent and treat as in analysis procedure.

(ii) Negative Control Organism- Mix 100ml of *Enterobacter cloacae suspension* with Colilert® Reagent and treat as in analysis procedure.

RESULTS Having counted the number of positive wells, refer to the appropriate MPN tables below (i.e. 51 Well Quanti-Tray MPN Table for Quanti-Tray, or IDEXX Quanti-Tray/2000 MPN Table for Quanti -Tray/2000) and read the corresponding Most Probable Number of coliforms. Since saline samples are diluted 1:100 in this method, the MPN value read in the table must be multiplied by 100 to obtain:

Coliform Abundance MPN/100ml

Note: Since *E.coli* itself is a coliform, if the *E.coli* count (SOP 291d) exceeds the Total Coliform Count, then the *E.coli* count must also be recorded as the Total Coliform Count.

COLIFORMS

Survey: _____

Sample Date: ___/___/___ Counted By: _____

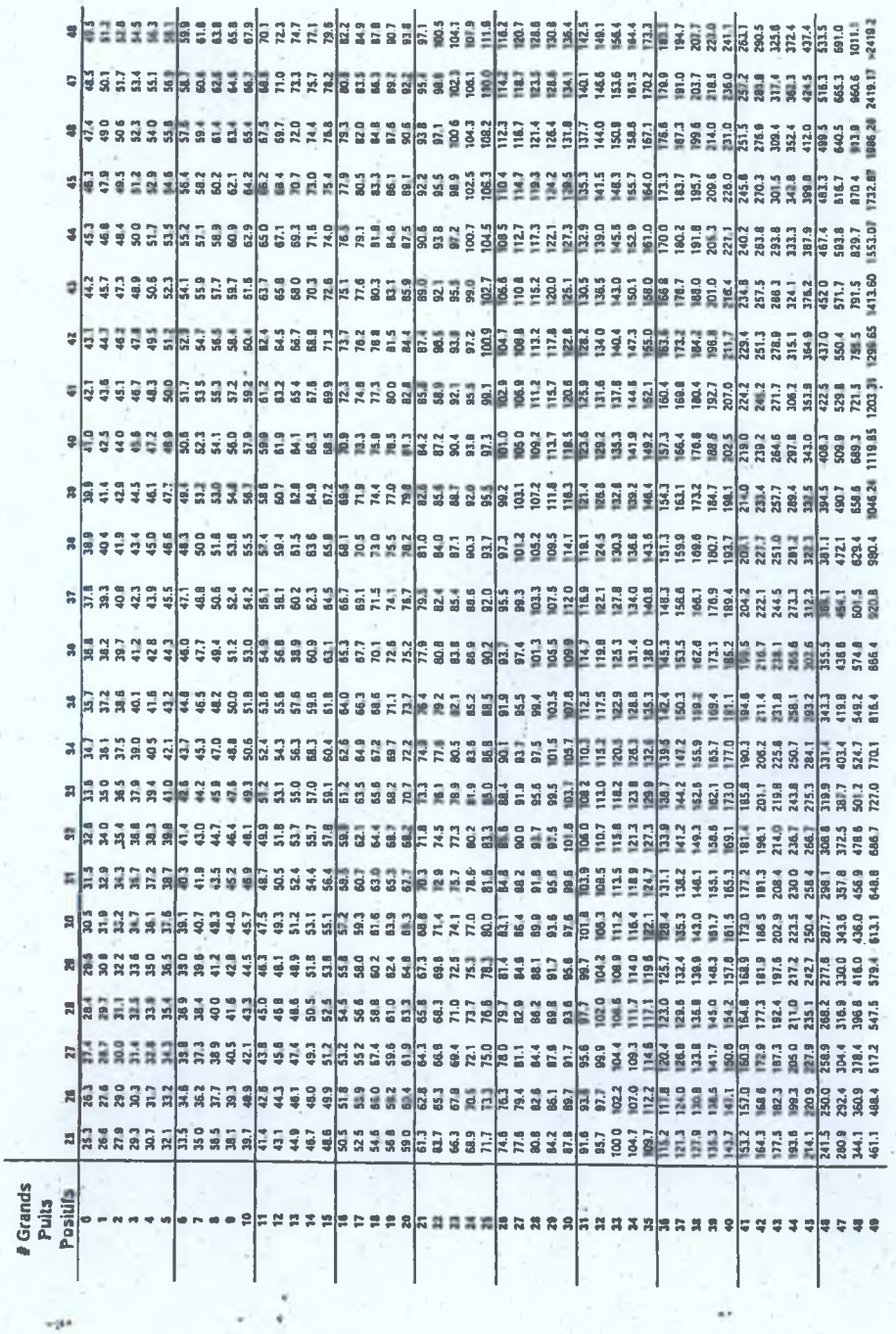
	Total Coliforms (SOP 290)				Faecal Coliforms (SOP 291)			
Site	mls Filtered	Plate Count	Nos/100ml	Mean	mls Filtered	Plate Count	Nos/100ml	Mean
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								

Figure 1: Sample sheet for Faecal and Total Coliforms

Table MPN IDEXX Quanti-Tray/2000

# Grands Puits Positifs	# Petits Puits Positifs																							
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0	<1	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.0	15.1	16.1	17.1	18.1	19.1	20.2	21.2	22.2	23.2	24.3
1	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.1	9.1	10.1	11.1	12.1	13.2	14.2	15.2	16.2	17.3	18.3	19.3	20.4	21.4	22.4	23.5	24.5	25.6
2	2.0	3.0	4.1	5.1	6.1	7.1	8.1	9.2	10.2	11.2	12.2	13.3	14.3	15.3	16.4	17.4	18.5	19.5	20.6	21.6	22.6	23.7	24.8	25.8	26.9
3	3.1	4.1	5.1	6.1	7.2	8.2	9.2	10.3	11.3	12.4	13.4	14.4	15.5	16.5	17.6	18.6	19.7	20.8	21.8	22.9	23.9	25.0	26.1	27.1	28.2
4	4.1	5.2	6.2	7.2	8.3	9.3	10.4	11.4	12.5	13.5	14.6	15.6	16.7	17.8	18.8	19.9	21.0	22.0	23.1	24.2	25.2	26.3	27.4	28.5	29.6
5	5.2	6.3	7.3	8.4	9.4	10.5	11.5	12.6	13.7	14.7	15.8	16.9	17.9	19.0	20.1	21.2	22.2	23.3	24.4	25.5	26.6	27.7	28.8	29.9	31.0
6	6.3	7.4	8.4	9.5	10.6	11.6	12.7	13.8	14.9	15.9	17.0	18.1	19.2	20.3	21.4	22.5	23.6	24.7	25.8	26.9	28.0	29.1	30.2	31.3	32.4
7	7.4	8.5	9.6	10.7	11.8	12.8	13.9	15.0	16.1	17.2	18.3	19.4	20.5	21.6	22.7	23.8	24.9	26.0	27.1	28.3	29.4	30.5	31.6	32.8	33.9
8	8.6	9.7	10.8	11.9	13.0	14.1	15.2	16.3	17.4	18.5	19.6	20.7	21.8	22.9	24.1	25.2	26.3	27.4	28.6	29.7	30.8	32.0	33.1	34.3	35.4
9	9.8	10.9	12.0	13.1	14.2	15.3	16.4	17.5	18.7	19.8	20.9	22.0	23.2	24.3	25.4	26.6	27.7	28.9	30.0	31.2	32.3	33.5	34.6	35.8	37.0
10	11.0	12.1	13.2	14.3	15.5	16.6	17.7	18.9	20.0	21.1	22.3	23.4	24.6	25.7	26.9	28.0	29.2	30.3	31.5	32.7	33.8	35.0	36.2	37.4	38.6
11	12.2	13.4	14.5	15.6	16.8	17.9	19.1	20.2	21.4	22.5	23.7	24.8	26.0	27.2	28.3	29.5	30.7	31.9	33.0	34.2	35.4	36.6	37.8	39.0	40.2
12	13.5	14.6	15.8	16.9	18.1	19.3	20.4	21.6	22.7	23.9	25.1	26.3	27.5	28.6	29.8	31.0	32.2	33.4	34.6	35.8	37.0	38.2	39.4	40.7	41.9
13	14.8	16.0	17.1	18.3	19.5	20.6	21.8	23.0	24.2	25.4	26.6	27.8	29.0	30.2	31.4	32.6	33.8	35.0	36.2	37.5	38.7	39.9	41.1	42.4	43.6
14	16.1	17.3	18.5	19.7	20.9	22.1	23.3	24.4	25.7	26.9	28.1	29.3	30.5	31.7	33.0	34.2	35.4	36.7	37.9	39.1	40.4	41.6	42.9	44.2	45.4
15	17.5	18.7	19.9	21.1	22.3	23.5	24.7	25.9	27.2	28.4	29.6	30.9	32.1	33.3	34.6	35.8	37.1	38.4	39.6	40.9	42.2	43.4	44.7	46.0	47.3
16	18.9	20.1	21.3	22.6	23.8	25.0	26.2	27.5	28.7	30.0	31.2	32.5	33.7	35.0	36.3	37.5	38.8	40.1	41.4	42.7	44.0	45.3	46.6	47.9	49.2
17	20.3	21.6	22.8	24.0	25.3	26.5	27.8	29.1	30.3	31.6	32.9	34.1	35.4	36.7	38.0	39.3	40.6	41.9	43.2	44.5	45.9	47.2	48.5	49.8	51.2
18	21.8	23.1	24.3	25.6	26.9	28.1	29.4	30.7	32.0	33.3	34.6	35.9	37.2	38.5	39.8	41.1	42.4	43.8	45.1	46.4	47.8	49.1	50.5	51.9	53.2
19	23.3	24.6	25.9	27.2	28.5	29.8	31.1	32.4	33.7	35.0	36.3	37.6	39.0	40.3	41.6	43.0	44.3	45.7	47.1	48.4	49.8	51.2	52.6	54.0	55.4
20	24.9	26.2	27.5	28.8	30.1	31.4	32.8	34.1	35.4	36.8	38.1	39.5	40.8	42.2	43.6	44.9	46.3	47.7	49.1	50.5	51.9	53.3	54.7	56.1	57.6
21	26.5	27.8	29.2	30.5	31.8	33.2	34.5	35.9	37.3	38.6	40.0	41.4	42.8	44.1	45.5	46.9	48.4	49.8	51.2	52.6	54.1	55.5	56.9	58.4	59.9
22	28.2	29.5	30.9	32.3	33.6	35.0	36.4	37.7	39.1	40.5	41.9	43.3	44.7	46.2	47.6	49.0	50.5	51.9	53.4	54.8	56.3	57.8	59.3	60.7	62.2
23	29.9	31.3	32.7	34.1	35.4	36.8	38.2	39.7	41.1	42.5	43.9	45.4	46.8	48.3	49.7	51.2	52.7	54.2	55.6	57.1	58.6	60.2	61.7	63.2	64.7
24	31.7	33.1	34.5	35.9	37.3	38.8	40.2	41.6	43.1	44.6	46.0	47.5	49.0	50.5	51.9	53.4	55.0	56.5	58.0	59.5	61.1	62.6	64.2	65.8	67.3
25	33.5	35.0	36.4	37.9	39.3	40.8	42.2	43.7	45.2	46.7	48.2	49.7	51.2	52.7	54.3	55.8	57.3	58.9	60.5	62.0	63.6	65.2	66.8	68.4	70.0
26	35.5	36.9	38.4	39.9	41.3	42.8	44.3	45.9	47.4	48.9	50.4	52.0	53.5	55.1	56.7	58.2	59.8	61.4	63.0	64.7	66.3	67.9	69.6	71.2	72.9
27	37.4	38.9	40.4	41.9	43.5	45.0	46.5	48.1	49.6	51.2	52.8	54.4	56.0	57.6	59.2	60.8	62.4	64.1	65.7	67.4	69.1	70.8	72.5	74.2	75.9
28	39.5	41.0	42.6	44.1	45.7	47.2	48.8	50.4	52.0	53.6	55.2	56.9	58.5	60.1	61.8	63.5	65.2	66.9	68.6	70.3	72.0	73.7	75.5	77.3	79.0
29	41.6	43.2	44.8	46.4	48.0	49.6	51.2	52.8	54.5	56.1	57.8	59.5	61.2	62.9	64.6	66.3	68.0	69.8	71.5	73.3	75.1	76.9	78.7	80.5	82.3
30	43.9	45.5	47.1	48.7	50.4	52.0	53.7	55.4	57.1	58.8	60.5	62.2	64.0	65.7	67.5	69.3	71.0	72.8	74.7	76.5	78.3	80.2	82.1	84.0	85.9
31	46.2	47.9	49.5	51.2	52.9	54.6	56.3	58.1	59.8	61.6	63.3	65.1	66.9	68.7	70.5	72.4	74.2	76.1	78.0	79.9	81.8	83.7	85.7	87.6	89.6
32	48.7	50.4	52.1	53.8	55.6	57.3	59.1	60.9	62.7	64.5	66.3	68.1	70.0	71.9	73.8	75.7	77.6	79.5	81.5	83.5	85.4	87.5	89.5	91.5	93.6
33	51.2	53.0	54.7	56.5	58.3	60.1	62.0	63.8	65.7	67.6	69.5	71.4	73.3	75.2	77.2	79.2	81.2	83.2	85.2	87.3	89.3	91.4	93.5	95.7	97.8
34	53.9	55.7	57.6	59.4	61.3	63.1	65.0	66.9	68.9	70.8	72.8	74.8	76.8	78.8	80.8	82.9	85.0	87.1	89.2	91.4	93.5	95.7	97.9	100.1	102.4
35	56.8	58.6	60.5	62.4	64.4	66.3	68.3	70.3	72.3	74.3	76.3	78.4	80.5	82.6	84.7	86.9	89.1	91.3	93.5	95.7	98.0	100.3	102.6	105.0	107.3
36	59.8	61.7	63.7	65.7	67.7	69.7	71.7	73.8	75.9	78.0	80.1	82.3	84.5	86.7	88.9	91.2	93.5	95.8	98.1	100.5	102.9	105.3	107.7	110.2	112.7
37	62.9	65.0	67.0	69.1	71.2	73.3	75.4	77.6	79.8	82.0	84.2	86.5	88.8	91.1	93.4	95.8	98.2	100.6	103.1	105.6	108.1	110.7	113.3	115.9	118.6
38	66.3	68.4	70.6	72.7	74.9	77.1	79.4	81.6	83.9	86.2	88.6	91.0	93.4	95.8	98.3	100.8	103.4	105.9	108.6	111.2	113.9	116.6	119.4	122.2	125.0
39	69.9	72.2	74.4	76.6	78.9	81.3	83.6	86.0	88.4	90.9	93.3	95.9	98.4	101.0	103.6	106.3	109.0	111.8	114.5	117.4	120.3	123.2	126.1	129.2	132.2
40	73.8	76.2	78.5	80.9	83.3	85.7	88.2	90.7	93.3	95.9	98.5	101.2	103.9	106.7	109.5	112.4	115.3	118.2	121.2	124.2	127.3	130.5	133.7	137.0	140.3
41	78.0	80.5	83.0	85.5	88.0	90.6	93.3	95.9	98.7	101.4	104.3	107.1	110.0	113.0	116.0	119.1	122.2	125.4	128.7	132.0	135.3	138.8	142.3	145.9	149.5
42	82.6	85.2	87.8	90.5	93.2	96.0	98.8	101.7	104.6	107.6	110.6	113.7	116.9	120.1	123.3	126.7	130.1	133.6	137.1	140.8	144.5	148.3	152.2	156.1	160.2
43	87.6	90.4	93.2	96.0	99.0	101.9	105.0	108.1	111.2	114.5	117.8	121.1	124.6	128.1	131.7	135.4	139.1	143.0	147.0	151.0	155.1	159.4	163.8	168.2	172.8
44	93.1	96.1	99.1	102.2	105.4	108.6	111.9	115.3	118.7	122.3	125.9	129.6	133.4	137.4	141.4	145.5	149.7	154.1	158.5	163.1	167.8	172.7	177.7	182.9	188.2
45	98.3	102.5	105.8	109.2	112.6	116.2	119.8	123.6	127.4	131.3	135.4	139.6	143.9	148.3	152.9	157.6	162.4	167.4	172.6	177.9	183.5	189.2	195.1	201.2	207.5
46	106.3	109.8	113.4	117.2	121.0	125.0	129.1	133.3	137.6	142.1	146.7	151.5	156.5	161.6	166.9	172.5	178.2	184.2	190.4	196.8	203.5	210.5	217.8	225.4	233.3
47	114.3	118.3	122.4	126.6	130.9	135.4	140.1	145.0	150.0	155.3	160.7	166.4	172.3	178.5	185.0	191.8	198.9	206.3	214.2	222.4	231.0	240.0	249.5	259.5	270.0
48	123.9	128.4	133.1	137.9	143.0	148.3	153.9	159.7	165.8	172.2	178.9	186.0	193.5	201.4	209.8	218.7	228.2	238.2	248.9	260.2	272.3	285.1	298.7	313.0	328.2
49	135.5	140.8	146.4	152.3	158.5	165.0	172.0	179.3	187.2	195.6	204.6	214.3	224.7	235.9	248.1	261.3	275.5	290.9	307.6	325.5	344.8	365.4	387.3	410.6	435.2

Figure 2: MPN Table IDEXX Quanti-Tray - 2000



# Grands Puits Positifs	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48
0	25.3	26.3	27.4	28.4	29.5	30.5	31.5	32.6	33.6	34.7	35.7	36.8	37.8	38.9	39.9	41.0	42.1	43.1	44.2	45.3	46.3	47.4	48.5	49.5
1	26.6	27.6	28.7	29.7	30.8	31.9	32.9	34.0	35.0	36.1	37.2	38.2	39.3	40.4	41.4	42.5	43.6	44.7	45.7	46.8	47.9	49.0	50.1	51.2
2	27.9	29.0	30.0	31.1	32.2	33.2	34.3	35.4	36.5	37.5	38.6	39.7	40.8	41.9	42.9	44.0	45.1	46.2	47.3	48.4	49.5	50.6	51.7	52.8
3	29.3	30.3	31.4	32.5	33.6	34.7	35.7	36.8	37.9	39.0	40.1	41.2	42.3	43.4	44.5	45.6	46.7	47.8	48.9	50.0	51.2	52.3	53.4	54.5
4	30.7	31.7	32.8	33.9	35.0	36.1	37.2	38.3	39.4	40.5	41.6	42.8	43.9	45.0	46.1	47.2	48.3	49.5	50.6	51.7	52.9	54.0	55.1	56.3
5	32.1	33.2	34.3	35.4	36.5	37.6	38.7	39.8	41.0	42.1	43.2	44.3	45.5	46.6	47.7	48.9	50.0	51.2	52.3	53.5	54.6	55.8	56.9	58.1
6	33.5	34.6	35.8	36.9	38.0	39.1	40.3	41.4	42.6	43.7	44.8	46.0	47.1	48.3	49.4	50.6	51.7	52.9	54.1	55.2	56.4	57.6	58.7	59.9
7	35.0	36.2	37.3	38.4	39.6	40.7	41.9	43.0	44.2	45.3	46.5	47.7	48.8	50.0	51.2	52.3	53.5	54.7	55.9	57.1	58.2	59.4	60.6	61.8
8	36.5	37.7	38.9	40.0	41.2	42.3	43.5	44.7	45.9	47.0	48.2	49.4	50.6	51.8	53.0	54.1	55.3	56.5	57.7	58.9	60.2	61.4	62.6	63.8
9	38.1	39.3	40.5	41.6	42.8	44.0	45.2	46.4	47.6	48.8	50.0	51.2	52.4	53.6	54.8	56.0	57.2	58.4	59.7	60.9	62.1	63.4	64.6	65.8
10	39.7	40.9	42.1	43.3	44.5	45.7	46.9	48.1	49.3	50.6	51.8	53.0	54.2	55.5	56.7	57.9	59.2	60.4	61.6	62.9	64.2	65.4	66.7	67.9
11	41.4	42.6	43.8	45.0	46.3	47.5	48.7	49.9	51.2	52.4	53.6	54.9	56.1	57.4	58.6	59.9	61.2	62.4	63.7	65.0	66.2	67.5	68.8	70.1
12	43.1	44.3	45.6	46.8	48.1	49.3	50.5	51.8	53.1	54.3	55.6	56.8	58.1	59.4	60.7	61.9	63.2	64.5	65.8	67.1	68.4	69.7	71.0	72.3
13	44.9	46.1	47.4	48.6	49.9	51.2	52.4	53.7	55.0	56.3	57.6	58.9	60.2	61.5	62.8	64.1	65.4	66.7	68.0	69.3	70.7	72.0	73.3	74.7
14	46.7	48.0	49.3	50.5	51.8	53.1	54.4	55.7	57.0	58.3	59.6	60.9	62.3	63.6	64.9	66.3	67.6	68.9	70.3	71.6	73.0	74.4	75.7	77.1
15	48.6	49.9	51.2	52.5	53.8	55.1	56.4	57.8	59.1	60.4	61.8	63.1	64.5	65.8	67.2	68.5	69.9	71.3	72.6	74.0	75.4	76.8	78.2	79.6
16	50.5	51.8	53.2	54.5	55.8	57.2	58.5	59.9	61.2	62.6	64.0	65.3	66.7	68.1	69.5	70.9	72.3	73.7	75.1	76.5	77.9	79.3	80.7	82.2
17	52.5	53.9	55.2	56.6	58.0	59.3	60.7	62.1	63.5	64.9	66.3	67.7	69.1	70.5	71.9	73.3	74.8	76.2	77.6	79.1	80.5	82.0	83.5	84.9
18	54.6	56.0	57.4	58.8	60.2	61.6	63.0	64.4	65.8	67.2	68.6	70.1	71.5	73.0	74.4	75.9	77.3	78.8	80.3	81.8	83.3	84.8	86.3	87.8
19	56.8	58.2	59.6	61.0	62.4	63.9	65.3	66.7	68.2	69.7	71.1	72.6	74.1	75.5	77.0	78.5	80.0	81.5	83.1	84.6	86.1	87.6	89.2	90.7
20	59.0	60.4	61.9	63.3	64.8	66.3	67.7	69.2	70.7	72.2	73.7	75.2	76.7	78.2	79.8	81.3	82.8	84.4	85.9	87.5	89.1	90.6	92.2	93.8
21	61.3	62.8	64.3	65.8	67.3	68.8	70.3	71.8	73.3	74.8	76.4	77.9	79.5	81.0	82.6	84.2	85.8	87.4	89.0	90.6	92.2	93.8	95.4	97.1
22	63.7	65.3	66.8	68.3	69.8	71.4	72.9	74.5	76.1	77.6	79.2	80.8	82.4	84.0	85.6	87.2	88.9	90.5	92.1	93.8	95.5	97.1	98.8	100.5
23	66.3	67.8	69.4	71.0	72.5	74.1	75.7	77.3	78.9	80.5	82.1	83.8	85.4	87.1	88.7	90.4	92.1	93.8	95.4	97.2	98.9	100.6	102.3	104.1
24	68.9	70.5	72.1	73.7	75.3	77.0	78.6	80.2	81.9	83.6	85.2	86.9	88.6	90.3	92.0	93.7	95.5	97.3	99.1	100.9	102.7	104.5	106.3	108.2
25	71.7	73.3	75.0	76.6	78.3	80.0	81.6	83.3	85.0	86.8	88.5	90.2	92.0	93.7	95.5	97.3	99.1	100.9	102.7	104.5	106.3	108.2	110.0	111.8
26	74.6	76.3	78.0	79.7	81.4	83.1	84.8	86.6	88.4	90.1	91.9	93.7	95.5	97.3	99.2	101.0	102.9	104.7	106.6	108.5	110.4	112.3	114.2	116.2
27	77.8	79.4	81.1	82.9	84.6	86.4	88.2	90.0	91.8	93.7	95.6	97.4	99.3	101.2	103.1	105.0	106.9	108.8	110.8	112.7	114.7	116.7	118.7	120.7
28	80.8	82.6	84.4	86.2	88.1	89.9	91.8	93.7	95.6	97.5	99.4	101.3	103.3	105.2	107.2	109.2	111.2	113.2	115.2	117.3	119.3	121.4	123.5	125.6
29	84.2	86.1	87.9	89.8	91.7	93.6	95.5	97.5	99.5	101.5	103.5	105.5	107.5	109.5	111.6	113.7	115.7	117.8	120.0	122.1	124.2	126.4	128.6	130.8
30	87.8	89.7	91.7	93.6	95.6	97.6	99.6	101.6	103.7	105.7	107.8	109.9	112.0	114.1	116.3	118.5	120.6	122.8	125.1	127.3	129.5	131.8	134.1	136.4
31	91.6	93.6	95.6	97.7	99.7	101.8	103.9	106.0	108.2	110.3	112.5	114.7	116.9	119.1	121.4	123.6	125.9	128.2	130.5	132.9	135.3	137.7	140.1	142.5
32	93.7	97.7	99.9	102.0	104.2	106.3	108.5	110.7	113.0	115.2	117.5	119.8	122.1	124.5	126.8	129.2	131.6	134.0	136.5	139.0	141.5	144.0	146.6	149.1
33	100.0	102.2	104.4	106.6	108.9	111.2	113.5	115.8	118.2	120.5	122.9	125.3	127.8	130.3	132.8	135.3	137.8	140.4	143.0	145.6	148.3	150.9	153.6	156.4
34	104.7	107.0	109.3	111.7	114.0	116.4	118.9	121.3	123.8	126.3	128.8	131.4	134.0	136.6	139.2	141.9	144.6	147.3	150.1	152.9	155.7	158.6	161.5	164.4
35	109.7	112.2	114.6	117.1	119.6	122.1	124.7	127.3	129.9	132.6	135.3	138.0	140.8	143.6	146.4	149.2	152.1	155.0	158.0	161.0	164.0	167.1	170.2	173.3
36	113.2	117.8	120.4	123.0	125.7	128.4	131.1	133.9	136.7	139.5	142.4	145.3	148.3	151.3	154.3	157.3	160.4	163.6	166.8	170.0	173.3	176.6	179.9	183.3
37	121.3	124.0	126.8	129.6	132.4	135.3	138.2	141.2	144.2	147.2	150.3	153.5	156.6	159.9	163.1	166.4	169.8	173.2	176.7	180.2	183.7	187.3	191.0	194.7
38	127.0	130.0	133.0	136.0	139.0	142.0	146.1	149.3	152.5	155.9	159.2	162.6	166.1	169.6	173.2	176.8	180.4	184.2	188.0	191.8	195.7	199.6	203.7	207.7
39	135.3	138.5	141.7	145.0	148.3	151.7	155.1	158.6	162.1	165.7	169.4	173.1	176.9	180.7	184.7	188.6	192.7	196.8	201.0	205.3	209.6	214.0	218.5	223.0
40	143.7	147.1	150.6	154.2	157.8	161.5	165.3	169.1	173.0	177.0	181.1	185.2	189.4	193.7	198.1	202.5	207.0	211.7	216.4	221.1	226.0	231.0	236.0	241.1
41	153.2	157.0	160.9	164.8	168.9	173.0	177.2	181.4	185.8	190.3	194.8	199.5	204.2	209.1	214.0	219.0	224.2	229.4	234.8	240.2	245.8	251.5	257.2	263.1
42	164.3	168.6	172.9	177.3	181.9	186.5	191.3	196.1	201.1	206.2	211.4	216.7	222.1	227.7	233.4	239.2	245.2	251.3	257.5	263.8	270.3	276.9	283.8	290.5
43	177.5	182.3	187.3	192.4	197.6	202.9	208.4	214.0	219.8	225.8	231.8	238.1	244.5	251.0	257.7	264.6	271.7	278.9	286.3	293.8	301.5	309.4	317.4	325.6
44	193.6	198.3	203.0	211.0	217.2	223.5	230.0	236.7	243.8	250.7	258.1	265.6	273.3	281.2	289.4	297.8	306.2	315.1	324.1	333.3	342.8	352.4	362.3	372.4
45	214.1	220.9	227.9	235.1	242.7	250.4	258.4	266.7	275.3	284.1	293.2	302.6	312.3	322.3	332.5	343.0	353.8	364.9	376.2	387.9	399.8	412.0	424.5	437.4
46	241.3	250.0	258.9	268.2	277.8	287.7	298.1	308.8	319.9	331.4	343.3	355.5	368.1	381.1	394.5	408.3	422.5	437.0	452.0	467.4	483.3	499.5	516.3	533.5
47	280.9	292.4	304.4	316.9	330.0	343.6	357.8	372.5	387.7	403.4	419.8	436.8	454.1	472.1	490.7	509.9	529.8	550.4	571.7	593.8	616.7	640.5	665.3	691.0
48	344.1	360.9	378.4	396.8	416.0	436.0	456.9	478.8	501.2	524.7	549.2	574.8	601.5	629.4	658.6	689.3	721.3	754.8	791.5	829.7	870.4	913.9	960.6	1011.1
49	461.1	488.4	517.2	547.5	579.4	613.1	648.8	686.7	727.0	770.1	818.4	866.4	920.8	980.4	1046.24	1119.85	1203.31	1298.65	1413.60	1553.07	1732.87	1986.28	2419.17	>2419.2

Figure 3: Quanti –Tray MPN Table

DUBLIN COPORATION

MICROBIOLOGY LABORATORY

LABORATORY METHODS MANUAL

Date	Issue	S.O.P.	Determination of:	Page
15/11/00	2	291d	Faecal coliforms	1of 8

PRINCIPLE:

E.Coli produces the β-Glucuronidase gene which is highly specific to this organism. The presence of the β-Glucuronidase gene is demonstrated by metabolism of the chromogenically labelled, enzyme specific substrate Methyl Ubeliferyl Glucuronide (MUG). Metabolism of MUG releases free Methyl Umbeliferone which can be seen as a bright blue fluorescence under UV light at 365nm.

The inoculated medium is dispensed into a series of wells of various volumes and incubated. A count of wells for positive for Total Coliform (yellow colouration) is used to estimate coliform abundance by reference to Most Probable Number (MPN) tables.

REFERENCE:

1.The Microbiology of Water 1994. Part 1- Drinking water. Report on Public Health and Medical Subject No.71. Methods for the Examination of Waters and Associated Materials. London:HMSO.

Methods for the Examination of Waters and Associated Materials. London :HMSO.

SAFETY: Inhalation of fine powders may be harmful, causing irritation of the upper respiratory tract, especially with bile salt products. Dust masks (conforming to British Standard No. 6016) should be worn to prevent mild skin rashes on prolonged contact. Work in adequately ventilated areas.

EQUIPMENT:

1. Autoclave
2. Incubator at 35.0 ± 0.5°C
3. Quanti -Tray or Quanti-Tray 2000
4. Quanti-Tray Sealer
5. Refrigerator at 2-8°C
6. Reaction Vessel- 120ml disposable polystyrene vessel. Non-autoflourescesing, sterile, with calibrated fill line.
7. UV Viewing Cabinet with 365nm UV source.
8. IDEXX P/A Compartor Cell.

REAGENTS:

1.Enzyme specific substrate ONPG. Colilert®-18 Dehydrated Regent in unit doses for addition to sample in Reaction vessel.

2a.Control Organism Suspension. Prepare a suspension of *Escherichia coli* using one gelatin capsule of reference material *Escherichia coli* 500 (WR1).

2b. Control Organism Suspension. Prepare a suspension of Enterobacter clocacae using one gelatin capsule of reference material Enterobacter clocacae 500(WR3)

3. Sterile Quarter strength Ringers Solution.

PROCEDURE:

1.Add unit dose of Colilert®-18 Dehydrated Reagent to reaction vessel.

2. In the case of Saline samples ad 1ml of sample and 99ml sterile deionised water to the reaction vessel. In the case of fresh water samples add 100ml of sample to the reaction vessel. Cap the vessel and shake until the medium is dissolved.

3.Aseptically transfer the 100ml sample and reagent from the reaction vessel into a Quanti-Tray or Quanti-Tray/2000. Quanti-Tray will allow abundance estimates up to 20,000/100ml at the 1:100 sample dilution used here. If higher bacterial abundance is anticipated use the Quanti-Tray/2000 which allows estimates up to 241,900/100ml at the 1:100 sample dilution used here.

4. Seal the Quanti-tray in the Quanti-Tray sealer and incubate at 35± 0.5°C for 18 hours. Ensure the wells of the Quanti-Tray are facing upwards throughout the whole incubation period.

5.After incubation check the Quanti-Tray in under UV light at 365nm. Count the number of wells of each type showing blue fluorescence under UV light at 365nm indicating the presence of E. coli.

6. The following controls should be run with each batch of confirmatory tests:

(iii) Positive Organism Control - Mix 100ml of E.coli suspension with Colilert® Reagent and treat as in analysis procedure.

(iv) Negative Control Organism- Mix 100ml of *Enterobacter cloacae* suspension with Colilert® Reagent and treat as in analysis procedure.

RESULTS Having counted the number of positive wells, refer to the appropriate MPN tables below (i.e. 51 Well Quanti-Tray MPN Table for Quanti-Tray, or IDEXX Quanti-Tray/2000 MPN Table for Quanti -Tray/2000) and read the corresponding Most Probable Number of coliforms. Since saline samples are diluted 1:100 in this method, the MPN value read in the table must be multiplied by 100 to obtain

E.coli Abundance MPN/100ml

Note: Since E.coli itself is a coliform, if the E.coli count (SOP 291d) exceeds the Total Coliform Count, then the E.coli count must also be recorded as the Total Coliform Count.

COLIFORMS

Survey: _____

Sample Date: ___/___/___ Counted By: _____

	Total Coliforms (SOP 290)				Faecal Coliforms (SOP 291)			
Site	mls Filtered	Plate Count	Nos/100ml	Mean	mls Filtered	Plate Count	Nos/100ml	Mean
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								

Figure 1: Sample Results Sheet for Faecal and Total Coliforms

Appendix 2

Raw Data of Saline, River and Drinking Water Samples

		TSC				
Date:	1mls	Confirmatory	5mls	Confirmatory	mean cfu/100	Log
30-Jan-02	5	5	24	19	400	2.60
Dub. Bay	5	5	22	16	350	2.54
	8	8	57	38	767	2.88
	5	5	28	20	417	2.62
	12	10	32	26	600	2.78
	4	4	31	25	483	2.68
	7	7	30	21	467	2.67
	6	6	46	33	650	2.81
	25	17	70	59	1267	3.10
	24	21	69	62	1383	3.14
	20	15	56	50	1083	3.03
	0	0	3	3	50	1.70
	0	0	1	1	17	1.22
	4	4	25	20	400	2.60
	0	0	20		400	2.60
22-02-02	2	2	33	33	583	2.77
Dub. Bay	3	3	26	21	400	2.60
	2	2	16	16	300	2.48
	3	3	9	9	200	2.30
	5	5	13	11	267	2.43
	4	4	13	13	283	2.45
	4	4	26	26	500	2.70
	2	2	12	12	233	2.37
	1	1	7	7	133	2.12
	1	1	9	9	167	2.22
	3	3	33	33	600	2.78
	7	7	49	49	933	2.97
	1	1	21	21	367	2.56
	8	8	14	14	367	2.56
	8	7	36	31	633	2.80
	4	4	20	20	400	2.60
	4	4	29	26	500	2.70
	3	3	23	22	417	2.62
	1	1	2	2	50	1.70
	0	0	2	2	33	1.52
	0	0	1	1	17	1.22
	1	1	5	5	100	2.00
Dub. Bay	2	2	28	28	500	2.70
	1	1	11	11	200	2.30
	1	1	12	12	217	2.34
	0	0	4	4	67	1.82
	1	1	10	10	183	2.26
	2	2	15	10	200	2.30
	3	3	12	8	183	2.26
	2	2	12	10	200	2.30
	3	3	13	13	267	2.43
	3	3	16	14	283	2.45
	1	1	6	6	117	2.07

Date:	1mls	TSC Confirmatory	5mls	Confirmatory	mean cfu/100	Log
	4	4	17	16	333	2.52
	0	0	7	6	100	2.00
	0	0	0	0	3	0.48
	3	3	6	6	150	2.18
	1	1	1	1	33	1.52
	0	0	2	2	33	1.52
	0	0	2	2	33	1.52
12-Feb-02	45	45	tntc	0	900	2.95
*beach	4	4	6	6	67	1.82
	43	39	0	-	780	2.89
	31	28	tntc	-	560	2.75
	0	0	0	0	3	0.48
	0	0	1	1	7	0.82
	43	43	tntc	-	860	2.93
	4	4	7	7	73	1.87
	0	0	3	3	20	1.30
	0	0	1	1	7	0.82
12-02-02	5	4	0	0	27	1.43
Beaches	1	1	0	0	7	0.82
	1	1	0	0	7	0.82
	0	0	0	0	3	0.48
	1	1	0	0	7	0.82
	1	1	0	0	7	0.82
	4	4	12	12	107	2.03
	0	0	9	7	47	1.67
	8	8	25	20	187	2.27
	3	3	28	28	207	2.32
	5	5	30	20	167	2.22
	0	0	8	5	33	1.52
27-Feb-02	0	0	12	12	80	1.90
	1	1	6	4	33	1.52
	1	1	6	6	47	1.67
	1	1	4	4	33	1.52
	0	0	6	5	33	1.52
	1	1	14	11	80	1.90
	1	1	15	15	107	2.03
	7	7	19	19	173	2.24
	14	13	58	56	460	2.66
	2	2	8	8	67	1.82
05-03-02	7	5	21	18	383	2.58
	1	1	6	3	67	1.82
	0	0	12	12	200	2.30
	0	0	3	3	50	1.70
Beaches	0	0	0	0	3	0.48
					rtscmfc	0.65
					r2	0.42

Date:	1mls	m-CP Confirmatory	5mls	Confirmatory	mean cfu/100	log
29-Jan-02	3	2	13	9	183	2.26
Dub. Bay	1	1	28	26	450	2.65
	4	4	8	5	150	2.18
	2	2	10	10	200	2.30
	1	1	18	16	283	2.45
	0	0	7	5	83	1.92
	0	0	11	11	183	2.26
	2	1	20	17	300	2.48
	12	12	60	53	1083	3.03
	12	12	54	54	1100	3.04
	7	7	64	60	1117	3.05
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	14	14	233	2.37
	0	0	4	4	67	1.82
22-02-02	0	0	13	12	200	2.30
Dub. Bay	0	0	11	10	167	2.22
	0	0	7	7	117	2.07
	0	0	3	3	50	1.70
	0	0	5	5	83	1.92
	0	0	9	7	117	2.07
	0	0	13	13	217	2.34
	0	0	12	12	200	2.30
	0	0	9	9	150	2.18
	0	0	3	3	50	1.70
	0	0	8	8	133	2.12
	5	5	18	18	383	2.58
	0	0	1	1	17	1.22
	0	0	4	4	67	1.82
	0	0	22	20	333	2.52
	0	0	7	7	117	2.07
	1	1	19	17	300	2.48
	0	0	8	7	117	2.07
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
Dub. Bay	2	2	14	14	267	2.43
	0	0	3	3	50	1.70
	0	0	5	5	83	1.92
	0	0	1	1	17	1.22
	1	1	4	3	67	1.82
	0	0	1	1	17	1.22
	0	0	4	4	67	1.82
	0	0	7	7	117	2.07
	1	1	5	5	100	2.00
	3	2	4	3	83	1.92
	0	0	4	4	67	1.82

Date:	1mls	m-CP Confirmatory	5mls	Confirmatory	mean cfu/100	log
	2	2	8	8	167	2.22
	1	1	0	0	17	1.22
	0	0	0	0	3	0.48
	0	0	3	3	50	1.70
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	1	1	17	1.22
12-Feb-02	43	40	66	66	707	2.85
*beach	1	1	2	3	27	1.43
	11	11	28	28	260	2.41
	7	7	28	28	233	2.37
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
	0	0	0	0	3	0.48
12-02-02	0	0	11	10	67	1.82
Beaches	0	0	8	8	53	1.73
	1	1	5	7	53	1.73
	0	0	3	3	20	1.30
	0	0	4	4	27	1.43
	1	1	15	15	107	2.03
	0	0	6	6	40	1.60
	0	0	4	4	27	1.43
	1	1	16	12	87	1.94
	7	7	12	12	127	2.10
	0	0	2	2	13	1.12
	3	3	1	1	27	1.43
27-Feb-02	2	2	7	7	60	1.78
	1	1	4	4	33	1.52
	0	0	5	5	33	1.52
	2	2	0	0	13	1.12
	1	1	3	3	27	1.43
	1	1	0	0	7	0.82
	0	0	4	4	27	1.43
	2	2	4	4	40	1.60
	6	6	32	32	253	2.40
	0	0	6	6	40	1.60
05-03-02	0	0	8	8	133	2.12
	0	0	5	5	83	1.92
	0	0	2	2	33	1.52
	0	0	0	0	0	3.00
	0	0	1	1	17	1.22
					mcpmfcr	0.41
					r2	0.17

		m-Endo			MFC		
Date:	0.1mls	1mls	mean cfu/100	0.1mls	1mls	mean cfu/100	log
29-Jan-02	17	42	4200	15	33	3300	3.52
	9	55	5500	5	24	2400	3.38
	6	71	7100	8	38	3800	3.58
	8	60	6000	7	26	2600	3.41
	15	74	7400	4	30	3000	3.48
	8	52	5200	4	26	2600	3.41
	8	72	7200	11	40	4000	3.60
	23	>200	23000	21	84	21000	4.32
	>200	>200	>200000	85	>200	85000	4.93
	89	>200	89000	43	>200	43000	4.63
	76	>200	76000	48	>200	48000	4.68
	1	7	727	6	2	182	2.26
	0	2	182	0	1	91	1.96
	11	43	4300	10	22	2200	3.34
	9	54	5400	4	35	3500	3.54
22-02-02	25	136	25000	21	>200	21000	4.32
Dub. Bay	23	121	23000	9	>200	9000	3.95
	16	112	16000	6	40	4000	3.60
	4	54	5400	4	27	2700	3.43
	2	46	4600	2	15	1545	3.19
	9	66	6600	3	28	2800	3.45
	11	64	6400	12	49	4900	3.69
	2	27	2700	2	7	82	1.91
	6	10	1454	0	5	455	2.66
	1	7	727	1	6	636	2.80
	9	42	4200	2	41	4100	3.61
	24	115	24000	11	43	4300	3.63
	42	>200	42000	17	>200	17000	4.23
	31	>200	31000	16	87	16000	4.20
	18	>200	18000	8	53	5300	3.72
	4	91	4000	3	35	3500	3.54
	9	>200	9000	15	56	5600	3.75
	13	72	7200	7	55	5500	3.74
	0	1	91	0	1	91	1.96
	1	2	273	0	2	183	2.26
	6	2	182	1	2	273	2.44
	0	5	455	1	2	273	2.44
Dub. Bay	>200	>200	>200,000	57	>200	57000	4.76
	28	>200	28000	10	25	2500	3.40
	21	94	21000	1	21	2100	3.32
	8	37	3700	1	2	273	2.44
	22	52	13600	3	20	2000	3.30
	17	>200	17000	4	15	1727	3.24
	6	50	5000	7	33	3300	3.52
	16	81	16000	9	32	5200	3.72
	28	135	28000	12	35	3500	3.54
	29	>200	29000	6	38	3800	3.58
	5	41	4100	3	6	818	2.91

Date:	m-Endo 0.1mls	m-Endo 1mls	m-Endo mean cfu/100	MFC 0.1mls	MFC 1mls	MFC mean cfu/100	log
	52	>200	**52000**	5	22	**2200**	3.34
	10	45	**4500**	8	18	**2363**	3.37
	1	2	**273**	1	0	**91**	1.96
	9	42	**42000**	4	8	**1091**	3.04
	>200	>200	**200000**	>200	>200	**200000**	5.30
	>200	>200	**200000**	>200	>200	**200000**	5.30
	>200	>200	**200000**	>200	>200	**200000**	5.30
11-Feb-02	>200	>200	**>10,000**	>200	**2,000**		3.30
***beach**	1	873	**67**	4	**40**		1.60
	51	119	**2550**	>200	**2,000**		3.30
	32	50	**900**	42	**420**		2.62
	0	0	**<25**	1	**10**		1.00
	1	2	**50**	2	**20**		1.30
	2	9	**184**	10	**100**		2.00
	13	2	**234**	17	**170**		2.23
	1	0	**17**	1	**10**		1.00
	1	1	**33**	2	**20**		1.30
	20	36	**950**	72	**720**		2.86
	1	0	**17**	0	**<10**		1.00
	1	0	**17**	1	**10**		1.00
	1	0	**17**	1	**10**		1.00
	0	0	**<25**	0	**<10**		1.00
	1	1	**33**	2	**20**		1.30
	5	0	**84**	6	**60**		1.78
	6	0	**100**	4	**40**		1.60
	72	75	**2738**	>200	**2,000**		3.30
	0	0	**<25**	2	**20**		1.30
	0	0	**<25**	1	**10**		1.00
	0	0	**<25**	3	**30**		1.48
27-Feb-02	0	5	**84**	2	**20**		1.30
	2	4	**217**	10	**100**		2.00
	3	7	**167**	12	**120**		2.08
	2	4	**100**	6	**60**		1.78
	1	6	**117**	1	**10**		1.00
	10	23	**575**	7	**70**		1.85
	2	1	**50**	2	**20**		1.30
	1	6	**117**	8	**80**		1.90
	4	18	**367**	25	**250**		2.40
	10	13	**384**	30	**300**		2.48
05-03-02	1	3	**67**	5	**50**		1.70
	0	0	**<25**	1	**10**		1.00
	21	0	**1050**	70	**700**		2.85
	1	6	**117**	3	**30**		1.48
	0	0	**<25**	0	**10**		1.00

RIVERS

TSC

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
30/01/02	2	2	2	2	27	1.43
	2	2	3	3	33	1.52
	2	2	3	3	33	1.52
	0	0	2	2	13	1.12
	3	3	5	5	53	1.73
	2	2	0	0	13	1.12
	3	3	17	17	133	2.12
	9	9	14	14	153	2.19
	9	9	12	12	140	2.15
	17	17	35	30	313	2.50
	6	6	17	17	153	2.19
	6	6	13	13	127	2.10
02-04-02	33	21	47	30	340	2.53
	32	20	48	33	353	2.55
	26	26	46	46	480	2.68
	14	14	24	24	253	2.40
	9	9	27	19	187	2.27
	13	8	33	26	227	2.36
	8	8	12	12	133	2.12
02-06-02	5	5	26	26	207	2.32
	5	5	19	19	160	2.20
	7	7	16	16	153	2.19
	6	6	18	18	160	2.20
	16	11	28	23	227	2.36
	14	13	38	28	273	2.44
	35	30	63	45	500	2.70
	36	27	73	43	467	2.67
02-07-02	14	14	39	34	320	2.51
	15	15	25	21	240	2.38
	18	18	29	28	307	2.49
	12	12	20	20	213	2.33
	15	11	18	16	180	2.26
	10	10	10	10	133	2.12
	20	20	33	23	287	2.46
	16	16	42	33	327	2.51
	12	12	15	15	180	2.26
	20	20	42	31	340	2.53
	16	16	38	23	260	2.41
	8	8	19	19	180	2.26
	14	14	26	26	267	2.43
	29	29	28	28	380	2.58
	15	15	32	32	313	2.50
	12	12	45	45	380	2.58
	17	17	45	28	300	2.48
	15	15	42	42	380	2.58
02-12-02	45	45	tntc	0	900	2.95
	4	4	6	6	67	1.82
	43	39	0	-	780	2.89

RIVERS

TSC

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
02-13-02	9	9	18		60	1.78
	10	8	tntc	-	160	2.20
	20	15	tntc	-	300	2.48
	23	19	tntc	-	380	2.58
	10	10	15		67	1.82
	10	10	tntc	-	200	2.30
	5	5	22	18	153	2.19
	9	9	10	10	127	2.10
	8	8	36	25	220	2.34
	3	3	18	14	113	2.05
	9	8	13	11	127	2.10
	1	1	6	6	47	1.67
	1	1	tntc	-	20	1.30
	6	6	5	5	73	1.87
	53	30	tntc	-	600	2.78
	18	18	40	35	353	2.55
	22	17	26	21	253	2.40
02-18-02	24	15	80	52	447	2.65
	11	6	19	11	113	2.05
	4	4	3	3	47	1.67
	23	16	43	33	327	2.51
	12	12	21	13	167	2.22
	5	5	9	6	73	1.87
	5	3	18	9	80	1.90
	10	10	14	14	160	2.20
	2	2	5	2	27	1.43
	tntc	-	tntc	-	2000	3.30
	9	9	25	20	193	2.29
	4	4	14	14	120	2.08
	4	4	6	6	67	1.82
	1	1	7	7	53	1.73
	2	2	0	0	13	1.12
	2	0	4	0	10	1.00
	9	6	11	8	93	1.97
	14	11	28	22	220	2.34
02-19-02	13	13	21	17	200	2.30
	6	6	18	18	160	2.20
	tntc	-	tntc	-	2000	3.30
	3	3	16	16	127	2.10
	1	1	5	5	40	1.60
	16	13	27	18	207	2.32
	13	7	19	14	140	2.15
	3	3	8	8	73	1.87
	9	8	13	13	140	2.15
	12	9	21	18	180	2.26
	1	1	5	5	40	1.60
	8	8	bo	-	160	2.20
	7	7	12	12	127	2.10

RIVERS

TSC

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
	1	0	6	2	**13**	**1.12**
	tntc	pos	tntc	pos	**2000**	**3.30**
	21	21	39	34	**367**	**2.56**
	26	26	36	36	**413**	**2.62**
	17	12	31	24	**240**	**2.38**
	10	10	20	14	**160**	**2.20**
02-20-02	26	18	25	25	**287**	**2.46**
	22	19	27	27	**307**	**2.49**
	22	12	33	18	**200**	**2.30**
	27	17	55	30	**313**	**2.50**
	bo	-	62	22	**440**	**2.64**
	34	34	60	60	**627**	**2.80**
	bo	-	71	30	**600**	**2.78**
	28	20	bo	-	**400**	**2.60**
	38	24	41	26	**333**	**2.52**
	30	16	54	34	**333**	**2.52**
	30	30	49	49	**527**	**2.72**
	1	0	4	0	**10**	**1.00**
	10	0	15	12	**80**	**1.90**
	2	2	9	9	**73**	**1.87**
	4	4	8	8	**80**	**1.90**
	6	6	6	6	**80**	**1.90**
	0	0	0	0	**10**	**1.00**
02-21-02	25	20	50	41	**407**	**2.61**
	13	12	-	-	**240**	**2.38**
	14	14	4	4	**120**	**2.08**
	14	12	17	17	**193**	**2.29**
	9	9	29	20	**193**	**2.29**
	48	32	35	29	**407**	**2.61**
02-28-02	6	6	10	10	**107**	**2.03**
	1	1	2	2	**20**	**1.30**
	2	2	5	5	**47**	**1.67**
	1	1	2	2	**20**	**1.30**
	0	0	3	3	**20**	**1.30**
	1	1	0	0	**7**	**0.82**
	16	16	17	17	**220**	**2.34**

RIVERS

m-CP

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
30/01/02	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	1	0	10	1.00
	0	0	0	0	10	1.00
	1	1	1	1	13	1.12
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	1	1	7	0.82
04-Feb-02	12	10	3	2	80	1.90
	0	0	15	13	87	1.94
	11	8	17	15	153	2.19
	1	0	4	3	20	1.30
	6	4	5	3	47	1.67
	4	3	4	4	47	1.67
	1	1	4	4	33	1.52
06-Feb-02	3	2	2	2	27	1.43
	9	6	6	6	80	1.90
	12	12	3	3	100	2.00
	5	4	6	6	67	1.82
	6	6	14	13	127	2.10
	9	8	16	16	160	2.20
	7	6	18	15	140	2.15
	17	16	25	23	260	2.41
07-Feb-02	11	10	19	19	193	2.29
	9	7	21	19	173	2.24
	11	9	19	15	160	2.20
	4	4	13	13	113	2.05
	8	8	12	9	113	2.05
	1	0	12	12	80	1.90
	10	8	30	29	247	2.39
	14	12	26	26	253	2.40
	4	4	5	3	47	1.67
	14	12	27	25	247	2.39
	15	10	14	14	160	2.20
	10	8	13	9	113	2.05
	4	3	14	14	113	2.05
	8	8	24	22	200	2.30
	10	9	18	16	167	2.22
	11	11	26	23	227	2.36
	14	14	19	18	213	2.33
	20	18	35	32	333	2.52
12-Feb-02	43	40	66	66	707	2.85
	1	1	2	3	27	1.43
	11	11	28	28	260	2.41

RIVERS

m-CP

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
13-Feb-02	17	16	13	11	180	2.26
	7	5	14	12	113	2.05
	2	2	3	2	27	1.43
	5	3	10	9	80	1.90
	5	5	11	11	107	2.03
	3	1	10	3	27	1.43
	1	1	5	3	27	1.43
	1	1	6	6	47	1.67
	3	2	5	3	33	1.52
	6	6	2	2	53	1.73
	2	2	2	2	27	1.43
	2	2	tntc	tntc	40	1.60
	2	2	11	0	13	1.12
	1	1	6	6	47	1.67
	28	28	72	72	667	2.82
	23	23	21	20	287	2.46
	6	6	10	9	100	2.00
18-Feb-02	11	10	23	20	200	2.30
	3	3	5	5	53	1.73
	1	1	2	2	20	1.30
	10	9	17	17	173	2.24
	5	5	8	8	87	1.94
	4	4	5	5	60	1.78
	1	1	3	3	27	1.43
	7	7	8	7	93	1.97
	0	0	0	0	10	1.00
	tntc	pos	tntc	pos	2000	3.30
	5	14	20	18	213	2.33
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
	0	0	0	0	10	1.00
19-Feb-02	2	2	16	16	120	2.08
	8	6	10	10	107	2.03
	tntc	pos	tntc	pos	2000	3.30
	4	3	6	6	60	1.78
	3	3	3	3	40	1.60
	6	6	20	18	160	2.20
	1	0	9	6	40	1.60
	5	5	7	7	80	1.90
	6	6	10	9	100	2.00
	2	2	10	10	80	1.90
	1	0	1	1	7	0.82
	5	4	7	6	67	1.82
	0	0	5	5	33	1.52

RIVERS

m-CP

Date:	cfu/5mls	5ml Conf.	cfu/10mls	10ml Conf.	mean cfu/100	Log
	1	1	3	2	**20**	**1.30**
	6	6	tntc	pos	**2000**	**3.30**
	0	0	25	25	**167**	**2.22**
	0	0	43	40	**267**	**2.43**
	0	0	13	12	**80**	**1.90**
	0	0	0	0	**10**	**1.00**
20-Feb-02	10	7	24	22	**193**	**2.29**
	10	9	20	20	**193**	**2.29**
	8	8	29	28	**240**	**2.38**
	10	10	25	25	**233**	**2.37**
	7	5	13	12	**113**	**2.05**
	14	13	39	35	**320**	**2.51**
	18	16	38	37	**353**	**2.55**
	11	9	35	33	**280**	**2.45**
	10	9	19	17	**173**	**2.24**
	6	4	21	19	**153**	**2.19**
	10	9	18	18	**180**	**2.26**
	0	0	0	0	**10**	**1.00**
	0	0	5	5	**33**	**1.52**
	0	0	0	0	**10**	**1.00**
	0	0	0	0	**10**	**1.00**
	0	0	0	0	**10**	**1.00**
	0	0	0	0	**10**	**1.00**
21-Feb-02	0	0	0	0	**10**	**1.00**
	0	0	0	0	**10**	**1.00**
	0	0	1	1	**7**	**0.82**
	0	0	2	2	**13**	**1.12**
	1	1	1	1	**13**	**1.12**
	1	1	3	3	**27**	**1.43**
28-Feb-02	0	0	6	6	**40**	**1.60**
	1	1	1	1	**13**	**1.12**
	2	2	1	1	**20**	**1.30**
	0	0	3	3	**20**	**1.30**
	0	0	0	0	**10**	**1.00**
	0	0	0	0	**10**	**1.00**
	3	3	5	5	**53**	**1.73**

RIVERS

Date:	MFC cfu/1ml	cfu/10mls	mean cfu/100mls	Log
30/01/02	50	56	2750	3.44
	40	96	4000	3.60
	62	97	6200	3.79
	59	101	5900	3.77
	50	97	5000	3.70
	>200	>200	20000	4.30
	6	14	182	2.26
	10	44	440	2.64
	5	16	191	2.28
	2	12	127	2.10
	25	70	1600	3.20
	35	43	1965	3.29
04-Feb-02	23	>200	2300	3.36
	10	>200	1000	3.00
	22	>200	2200	3.34
	31	142	3100	3.49
	34	76	2080	3.32
	20	135	2000	3.30
	5	76	760	2.88
06-Feb-02	9	52	520	2.72
	12	78	780	2.89
	4	93	400	2.60
	5	97	500	2.70
	10	>200	1000	3.00
	14	>200	1000	3.00
	>200	>200	20000	4.30
	>200	>200	20000	4.30
07-Feb-02	32	>200	3200	3.51
	25	>200	2500	3.40
	11	>200	1100	3.04
	13	>200	1300	3.11
	37	>200	3700	3.57
	>200	>200	20000	4.30
	30	>200	3000	3.48
	>200	>200	20000	4.30
	10	>200	1000	3.00
	3	>200	300	2.48
	8	>200	800	2.90
	26	>200	2600	3.41
	7	>200	700	2.85
	6	>200	600	2.78
	12	>200	1200	3.08
	16	>200	1600	3.20
	46	>200	4600	3.66
	24	>200	2400	3.38
12-Feb-02	>200	>200	10,000	4.00
	1	873	67	1.83
	51	119	2550	3.41

RIVERS

Date:	MFC cfu/1ml	cfu/10mls	mean cfu/100mls	Log
13-Feb-02	78	>200	7800	3.89
	74	145	7400	3.87
	>200	>200	20,000	4.30
	48	115	4800	3.68
	80	>200	8000	3.90
	>200	>200	20,000	4.30
	11	75	750	2.88
	16	90	1600	3.20
	2	20	200	2.30
	4	45	450	2.65
	18	42	420	2.62
	3	64	640	2.81
	6	102	600	2.78
	9	84	900	2.95
	68	>200	6800	3.83
	22	>200	2200	3.34
	33	>200	3300	3.52
18-Feb-02	21	59	6000	3.78
	13	78	780	2.89
	0	1	9	0.95
	14	>200	1400	3.15
	6	90	600	2.78
	3	23	230	2.36
	6	62	620	2.79
	0	67	670	2.83
	2	24	240	2.38
	>200	>200	20,000	4.30
	28	>200	2800	3.45
	11	>200	1100	3.04
	4	68	680	2.83
	1	47	470	2.67
	0	4	36	1.56
	0	45	450	2.65
	3	57	570	2.76
	2	8	91	1.96
19-Feb-02	>200	>200	20,000	4.30
	1	72	720	2.86
	>200	>200	20,000	4.30
	8	68	680	2.83
	39	>200	3900	3.59
	9	>200	900	2.95
	3	>200	300	2.48
	2	0	18	1.26
	5	>200	500	2.70
	13	>200	1300	3.11
	1	19	181	2.26
	7	29	290	2.46
	2	34	340	2.53

RIVERS

Date:	MFC cfu/1ml	cfu/10mls	mean cfu/100mls	Log
	4	23	230	2.36
	-	-	-	
	28	>200	2800	3.45
	31	>200	3100	3.49
	47	>200	4700	3.67
	5	80	800	2.90
20-Feb-02	47	>200	4700	3.67
	35	>200	3500	3.54
	24	>200	2400	3.38
	40	>200	4000	3.60
	36	>200	3600	3.56
	54	>200	5400	3.73
	>200	>200	20,000	4.30
	84	>200	8400	3.92
	67	>200	6700	3.83
	54	>200	5400	3.73
	17	>200	1700	3.23
	0	5	50	1.70
	4	68	680	2.83
	0	9	90	1.95
	4	35	350	2.54
	0	2	18	1.26
	0	1	9	0.95
21-Feb-02	5	>200	500	2.70
	7	>200	700	2.85
	7	>200	700	2.85
	16	>200	1600	3.20
	12	>200	1200	3.08
	11	>200	1100	3.04
28-Feb-02	16	>200	1600	3.20
	1	7	73	1.86
	1	10	100	2.00
	0	1	9	0.95
	0	3	27	1.43
	0	12	109	2.04
	3	27	270	2.43

DWMS

Date:	TSC CFU/100mls	CFU Confirmatory
31/01/02	0	0
	0	0
	0	0
	21	10
	0	0
	0	0
	1	0
	0	0
	0	0
	0	0
	1	0
	0	0
	0	0
	4	0
	0	0
	0	0
	0	0
	0	0
	0	0
04-02-02	0	0
	1	1
	0	0
	0	0
	1	1
	0	0
	0	0
	3	3
	tntc	200
	0	0
	0	0
	0	0
	0	0
	0	0
	1	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
06-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0

DWMS

TSC

Date:	CFU/100mls	CFU Confirmatory
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
07-02-02	0	0
	1	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	1	1
11-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
12-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0

DWMS

Date:	TSC CFU/100mls	CFU Confirmatory
	0	0
	0	0
	0	0
	1	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0

DWMS

m-CP

Date:	CFU/100mls	CFU Confirmatory
31/01/02	0	0
	0	0
	0	0
	6	4
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
04-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	tntc	200
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
06-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0

	DWMS m-CP	
Date:	CFU/100mls	CFU Confirmatory
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
07-02-02	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
11-02-02	0	0
	0	0
	0	0
	1	1
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
12-02-02	0	0
	0	0
	1	1
	0	0
	0	0
	0	0

DWMS

m-CP

Date:	CFU/100mls	CFU Confirmatory
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0
	0	0

DWMS

Date:	m-Endo CFU/100mls	m-FC CFU/100mls
31/01/02	<1	<1
	<1	<1
	<1	<1
	<1	1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
04-02-02	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	49	<1
	1733	345
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
06-02-02	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1

	DWMS	
	m-Endo	m-FC
Date:	CFU/100mls	CFU/100mls
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
07-02-02	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
11-02-02	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
12-02-02	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1

	DWMS	
	m-Endo	m-FC
Date:	CFU/100mls	CFU/100mls
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1
	<1	<1