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Indicator Organism Sources and Recreational Water Quality:

A Study on the Impact of Duck Droppings on the Microbiological Quality of Water at Hataitai Beach

A thesis presented in partial fulfillment of the requirements for the degree of
Master of Science in Microbiology
at
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Stanley Edwin Abbott
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ABSTRACT

The aim of this study was to identify the possible sources of faecal pollution of recreational water at Hataitai Beach, a small Evans Bay beach that is a popular Wellington Harbour location for swimming, canoeing, kayaking and fishing. Since 1994 the water quality at Hataitai Beach had deteriorated to the extent where the beach was closed for bathing between February 1996 and January 1997, and again in January 1998. Results of routine membrane filtration tests for indicator organisms performed on marine water samples from Hataitai Beach by the Wellington City and Regional Councils' laboratories had been unable to determine if the source of raised bacterial levels were of animal or human origin. Consequently it was not firmly established if the ducks that frequented Hataitai Beach were the main reason for the high counts or whether they contributed to a pollution problem that was thought to be exacerbated by leaking sewers and private drain faults. Following remedial work on sewer and stormwater pipes carried out by the Wellington City Council in 1998, Hataitai Beach was re-opened for swimming in December 1998 and remained open throughout the 1999-bathing season.

In this study 400 marine water samples (500 ml volumes) were collected from various sites at Hataitai Beach from July 1998 to April 2000. The Enterolert™ system (IDEXX Laboratories, USA) was used for the detection and enumeration of *Enterococci* and the Colilert-18™ system (IDEXX) for *Total coliforms* and *Escherichia coli* detection and enumeration. The results were analysed to:

- Determine water quality compliance at Hataitai Beach.
- Ascertain the impact of duck numbers on indicator organism levels.
- Identify the principal sources of faecal pollution at Hataitai Beach.

Six marine samples (130-500 litres) were also collected for the capture of *Giardia* and *Cryptosporidium* on filter cartridges for subsequent purification of (oo)cysts by the DYNAL® immunomagnetic bead separation technique (DYNAL A.S., Norway) and

the detection of (oo)cysts by the Merifluor® *Cryptosporidium/Giardia* (Meridian Diagnostics, USA) direct immunofluorescent monoclonal antibody (IFA) procedure.

In addition, 279 duck faecal samples were collected from Hataitai Beach for the detection of *Giardia* and *Cryptosporidium* by the Merifluor® *Cryptosporidium/Giardia* IFA procedure and subsequent DNA analysis of oo(cysts) by the Polymerase Chain Reaction (PCR) amplification procedure.

The results from this study indicate that despite the stormwater and sewer upgrades by the Wellington City Council there is still a pollution problem at Hataitai Beach and that this pollution is primarily caused by a combination of duck droppings and meteorological events. The microbiological water quality can be degraded directly by the resident mallard ducks defaecating in the water or indirectly from droppings deposited on the beach sands. Faecal bacteria in the droppings on the beach sands can be carried into the water by tides, rain, and wind erosion or unwittingly by the action of beach users.

On 64 occasions during this study there were exceedences of the water quality guidelines at Hataitai Beach, especially in water samples collected from the B3 site where ducks were frequently seen loafing on the beach or swimming in the water. Moreover, on 41 occasions the *Enterococci* levels in samples from the B3 site were strikingly over the *Action Red* mode guideline value of 277 *Enterococci* per 100 ml - exceedences that require local authorities and health authorities to warn the public that the beach is unsafe for recreational activities and erect warning signs. The median value of 63 *Enterococci* per 100 ml for samples collected from the B3 site was well above the *Green* – "safe for bathing" guideline value of less than 35 *Enterococci* per 100 ml.

While no *Giardia* cysts or *Cryptosporidium* oocysts were detected in the marine water samples, protozoan cysts morphologically resembling *Giardia* spp. were detected in 29% of the duck faecal samples. However, since no detectable PCR amplification products were obtained in any cysts of the Merifluor®*C/G* IFA positive samples tested, attempts to genotype the *Giardia* cysts proved unsuccessful. After closer inspections and measurements of these Merifluor®*C/G* IFA positive protozoan cysts it was

established that the organisms were in fact *Caryospora* spp. and not *Giardia* cysts. Since *Caryospora* are phylogenetically related to *Cryptosporidiuim*, it appears that *Caryospora* can cross-react with *Cryptosporidiuim* antibody preparations but have the appearance of *Giardia* cysts when viewed under fluorescence microscopy.

Because of their aquatic lifestyle and highly mobile behaviour, ducks may be exposed to a diverse array of potentially pathogenic organisms such as *Campylobacter jejuni*, *Escherichia coli O157:H7*, *Salmonella* spp., *Pasteurella multocida*, *Giardia* and *Cryptosporidiuim* spp., and *Cercarial* trematodes. These organisms can originate from several sources including human sewage, agricultural runoff, and animal faecal matter. The microbiological health risk of humans acquiring infections from the ducks at Hataitai Beach depends on several factors, including the presence and survival of pathogenic organisms in the droppings after their deposition on the beach sands and in the water, as well as the types of recreational activities that expose humans to these droppings. In order for the public to be adequately informed of the risk so that they can make informed personal choices about engaging in recreational activities at Hataitai Beach, it seems prudent that efforts should be made to grade this beach so that the beach's suitability for recreation can be established. This should include a catchment risk assessment that considers the potential sources and transmission routes of faecally derived pollution at Hataitai Beach with an assessment of the microbiological data.

In the meantime common sense dictates that at Hataitai Beach the authorities should limit the food sources of ducks in the areas surrounding the beach by officially prohibiting people from feeding the ducks.

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DEDICATION

This thesis is dedicated to the memory of
my late mother and father, Jo and Stan Abbott,
and also to my daughters Julie and Katherine who
have remained remarkably patient during all the time
that I have spent pursuing matters microbiological.

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CHAPTER ONE: INTRODUCTION

1.1 THE WATER QUALITY OF HATAITAI BEACH

1.1.1 History

Between 50 and 100 ducks frequent Hataitai Beach, a small Evans Bay beach that is a popular Wellington Harbour location for swimming, canoeing, kayaking and fishing. Since 1994 the water quality at Hataitai beach had deteriorated to the extent where the compliance values for indicator organisms were regularly exceeded resulting in sporadic beach closures (Murphy & Zatorski, 1996; *Dominion,* 1996; *Contact,* 1997). Berry states in the *Annual Coastal Water Quality Report for the Wellington Region 1995/1996* that between 1994 and 1995 the median *Faecal coliform* level at Hataitai Beach rose from 135 *Faecal coliforms* per 100 ml to 256 *Faecal coliforms* per 100 ml (Berry, 1996). This meant that the water at Hataitai Beach did not meet the assigned SB classification since the site was deemed unsuitable for bathing and other contact recreation. Furthermore, from March 1995 to February 1996 the *Enterococci* single sample maxima guideline value for Hataitai Beach was exceeded in 31% of samples tested.

During 1996 and 1997 Hataitai Beach was again reported as exceeding the *Enterococci* guideline value for bathing for the annual and summer sampling periods. This resulted in Hataitai Beach being closed for bathing between February 1996 and January 1997 (Figures 1 and 2).

Figure 1: Pollution warning sign and a "Do not feed the ducks" sign at Hataitai Beach in February 1996. (Wellington City Council and Ministry of Health).

In January 1998 both the Wellington City Council and Wellington Regional Council recorded high single sample *Enterococci* results at Hataitai Beach (Berry 1998). Wellington City Council, under the guidance of the Ministry Health, subsequently closed Hataitai Beach for bathing in January 1998 (Berry 1998; Samson 1998).

Figure 2: Pollution warning sign at Hataitai Beach in January 1997. (Wellington City Council).

1.1.2 Reasons for the poor water quality at Hataitai Beach

The ducks that frequent the Hataitai Beach area were originally thought to be the main reason for the elevated bacterial counts in the water samples collected from this area. In the *Annual Coastal Water Quality Report for the Wellington Region 1995/1996* Berry (1996, p. II) states that "Hataitai Beach's poor water quality is possibly due to a growing duck population in the area". Wellington City Council did not believe that local sewer mains, stormwater drains or bathing sheds were responsible for the poor water quality at Hataitai Beach (Murphy & Zatorski 1996). Local residents were requested to stop feeding the ducks because their numbers had been steadily increasing and there had been complaints about the duck droppings and decaying, uneaten bread on the beach.

Towards the end of 1996 however, it became apparent that the bacterial counts remained high even when there were no or low numbers of ducks at the beach. Therefore, because laboratory tests were unable to determine the source of the faecal bacteria, the authorities decided that the ducks alone were not responsible for the poor water quality at Hataitai Beach. Instead, private drain faults, leaking sewers and stormwater flows were now suspected as being the primary cause of the high bacterial counts (*Dominion* 1996; *Contact* 1997; Baker 1998; Samson 1998).

1.1.3 Drainage improvements at Hataitai Beach

During 1996 and 1997 the Wellington City Council carried out preliminary remedial work on sewers and stormwater drains in the Hataitai Beach area. Leaks were sealed in the foreshore sewer main connection, local drains were pressure tested and the dry weather stormwater flows at the northern and southern ends of the beach were diverted to the sewer system (Berry, 1997).

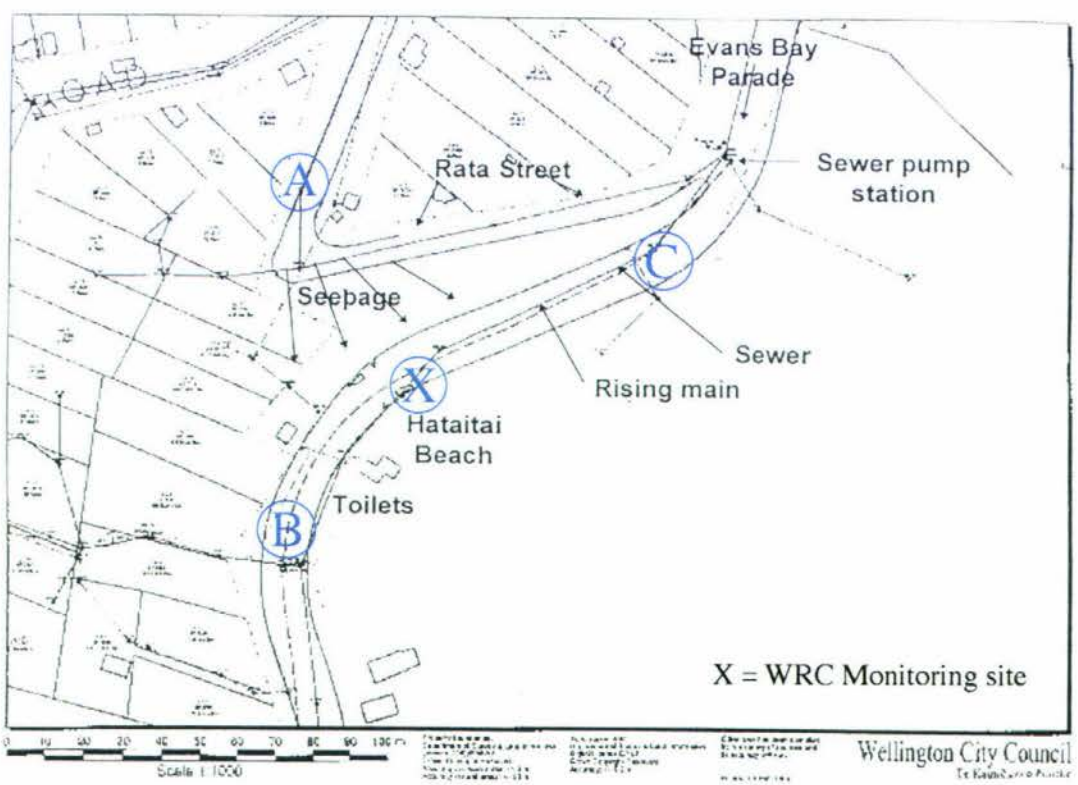

Figure 3: Sewage leakages in the Hataitai Beach area (1996-1998).

Subsequent to the beach closure in January 1998 the Wellington City Council's Sewage Pollution Elimination team investigated the Evans Bay area for sewer and stormwater

leaks (Berry, 1998). A broken sewer pipe under Rata Street was believed to be a major cause of the beach pollution (Figure 3). The City Council found that at this point sewage was seeping down the bank and running directly into Hataitai Beach (Point A). The City Council repaired this sewer pipe and also upgraded the pump station at the corner of Rata Street and Evans Bay Parade. Following these repairs the resulting increased pressure caused a break in the rising main (Point B) and another breakage occurred when stormwater was diverted to the sewer (Point C). To resolve these problems the Wellington City Council carried out remedial work, at a cost of more than $400,000 over several months in 1998. This work included the replacement of the sewer and the relocation of the rising main from the Hataitai Beach area to Cobham Drive at the end of Evans Bay.

Following the stormwater and sewer improvements Hataitai Beach was re-opened for swimming in December 1998 (Figures 4 and 5) and remained open throughout the 1999 bathing season (Lucas 1998; Baker, 1998; Berry, 1999).

Figure 4: 1998 "Beach reopened" sign at Hataitai Beach (Wellington City Council).

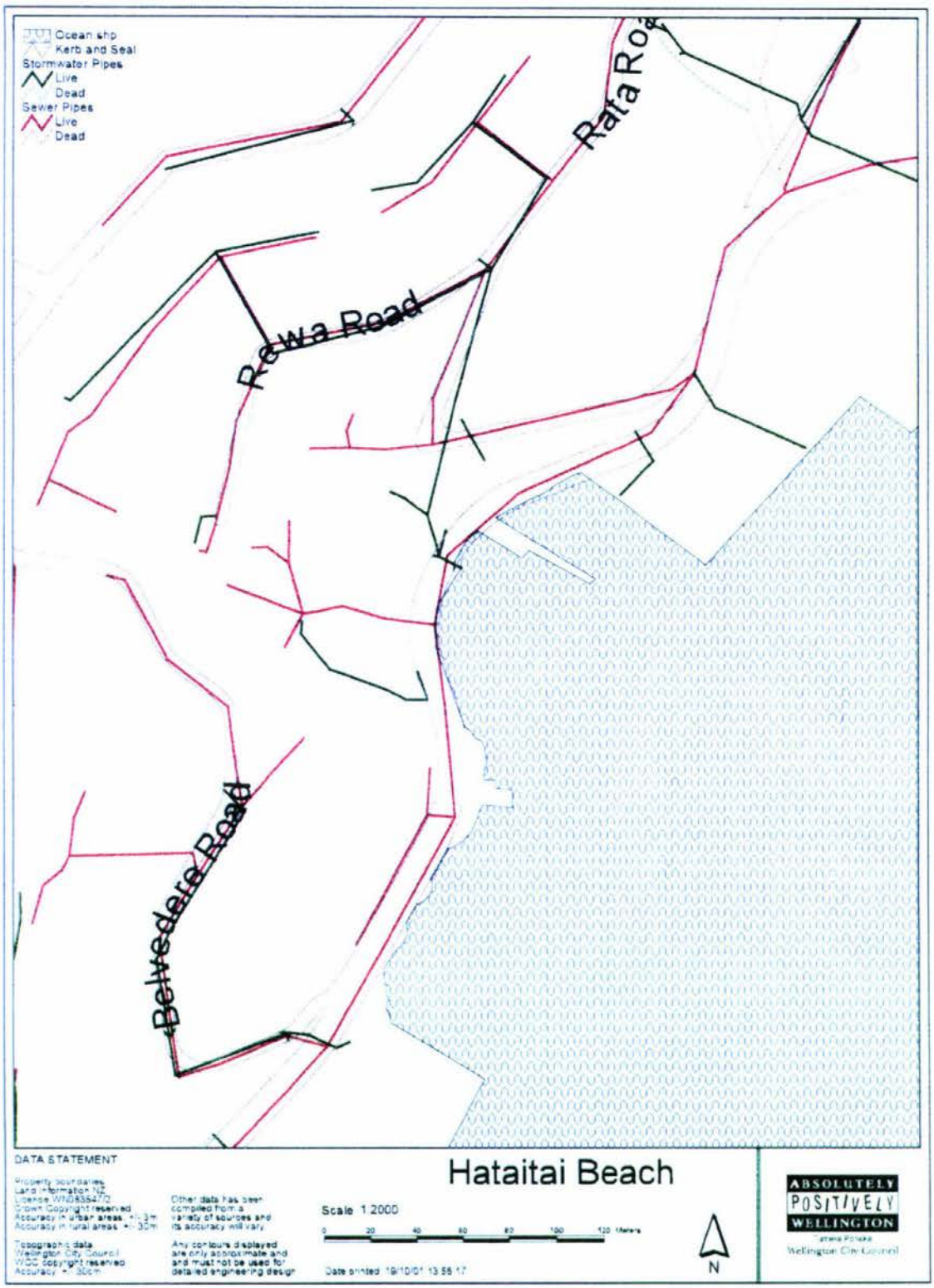

Figure 5: 1:2000 map of the Hataitai Beach area showing roads, stormwater and sewer pipes. Map produced after the Wellington City Council's stormwater and sewer improvements in 1998.

1.2 WATERFOWL AND RECREATIONAL WATER QUALITY

1.2.1 Effect of waterfowl on indicator bacteria

In a study on the effects of waterfowl on indicator bacterial populations in a recreational lake in Madison, Wisconsin (USA), Standridge *et al.*, (1979) showed that faecal coliforms that were deposited by mallard ducks multiplied in the beach sand and that these bacteria were subsequently transported to the water resulting in high faecal coliform counts in the swimming areas. A catchment study on the Island of Jersey (Wyer *et al.*, 1995) found that waterfowl, such as ducks and swans, can influence water quality compliance significantly by markedly increasing the total coliform, *Escherichia coli*, and *Enterococci* concentrations. A study (Levesque *et al.*, 1993) on ringed - billed gull droppings, found that these birds contribute to the microbiological degradation of recreational water. These authors found that once food was spread on the beach sand the number of gulls increased and subsequently a marked increase in the faecal coliform counts in the water as well.

A study on the impact of Canada geese and whistling swans on aquatic ecosystems in Baltimore (Hussong *et al.*, 1979) showed that large flocks of waterfowl can cause elevated faecal coliform densities in the water column and that it is possible to predict the microbial influence of migratory waterfowl upon aquatic roosting sites. An Alderisio and DeLuca (1999) study on the enumeration of faecal coliforms from ring-billed gulls and Canada geese showed that the faeces of these birds contained considerable numbers of faecal coliforms per gram of faeces and that the microbial impact of these birds was relative to the numbers and types of birds, as well as the duration and time of day that the birds roosted on the surface of the water and their defecation rates. These investigators also found significant numbers of viable faecal coliforms in old, sun-dried faecal samples and showed that runoff from bird faeces can also impact on water bodies, even if the birds do not roost on the water.

Studies on roosting waterfowl and the effects of feeding ducks on water quality, have demonstrated that waterfowl can degrade the quality of the waters they frequent, both in terms of indicator bacteria, particularly total coliforms, faecal coliforms, and *Enterococci*, and to a lesser extent, the nutrients in the water (Gould & Fletcher, 1978; Gere & Andrikovics 1992; Gere & Andrikovics 1994). A study by Ricca and Cooney (1998) found 10^6 somatic coliphages, 10^8 *Enterococci*, 10^9 thermotolertant coliforms

and 10^2 F-specific coliphages in waterfowl droppings from the urban/suburban environment around Boston Harbour. The authors suggest that the indicators of faecal pollution originating from bird droppings may be mistaken for indicators that come from humans. This may cause an overestimate of the hazard from human pathogens in water and confound attempts to locate the sources of faecal pollution.

Analysis of domestic and feral faeces in a New Zealand study found *Enterococci* in the range of 10^1 to 10^6 cfu per gram (1.45×10^4 to 7.90×10^6 in duck faeces) with considerable variation between species (Anderson, *et al.*, 1997). The study concludes that a considerable proportion of the load in urban/rural catchments and waterways (typically 10^2 to 10^3 *Enterococci* cfu per 100 ml) is derived from non-human sources.

A multidisciplinary study at Huntington State and City Beaches in California found that while urban runoff, sediment and vegetation contribute to increased *Enterococci* counts in the water, bird droppings were also a significant source of the *Enterococci.* (Grant *et al.*, 2001).

1.2.2 Waterfowl droppings as a potential source of pathogenic bacteria

Feare *et al.*, (1999) demonstrated that bacteria present in waterfowl droppings constitute a potential health risk to humans exposed to the droppings during recreational activities. These authors screened waterfowl droppings for a range of bacteria that could be pathogenic to humans. A wide variety of *Enterobacteriaceae*, including *Salmonella* spp., *Escherichia coli*, *Enterobacter aerogenes* and *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Proteus mirablis*, and *Providencia alcalifaciens*, were isolated from the droppings. The study also found that these bacteria can survive in the droppings for up to four weeks, and possibly longer, under conditions prevailing in summer.

In a survey of faecal shedding of selected bacterial pathogens from free-living waterfowl Fallacara *et al.*, (2001) isolated *Escherichia coli*, *Salmonella* spp., and *Campylobacter jejuni* from mallard ducks, domestic hybrid ducks, and Canada geese. The survey concluded that these waterfowl can serve as potential reservoirs for a variety of pathogens and that human contact with these birds or their droppings can result in cross-transmission of the pathogens.

Several studies have been conducted to detect the presence of bacterial pathogens in faecal material of waterfowl. *Campylobacter jejuni*, which causes acute diarrhoea in humans, was isolated from the caeca of 154 (35%) of 445 ducks killed by hunters in Colorado (Luechtefeld *et al.*, 1980). During a banding study in Washington, Pacha *et al.*, (1988) collected cloacal swabs from ducks and recovered *Campylobacter* spp. from 82 (73%) of 133 samples. An earlier study by Hill and Grimes (1984), in the Wisconsin-Minnesota region of the upper Mississippi River, found no *Campylobacter* spp. in 50 caecal samples from ducks killed by local hunters.

Seven *Salmonella* serotypes that are potentially pathogenic in humans and animals, namely, *S.brandenburg*, *S.agona*, *S.hadar*, *S.stanley*, *S.anatum*, and *S.typhimurium* have been isolated in waterfowl droppings (Levesque *et al.*, 1993). The authors estimated that in their study sites 4.6 *Salmonella* organisms per 100 ml could have been present in the water close to where the waterfowl were feeding. In 1991 there was an outbreak in America of 22 cases of *S.hadar* associated with pet ducklings (MMWR No. 41, 1992a). Although the prevalence of *Salmonella* in ducks is well recognised internationally, the degree to which the duck population itself suffers as a consequence, appears to be minimal (Henry, 2000). A survey in the USA by Price *et al.*, (1962) on 7029 duck carcasses over a ten-year period isolated 491 *Salmonella* spp. Of these 457 (93%) isolates were *S.typhimurium* and there were fewer than ten each of a variety of other serovars. In a report by Neilson (1960) ducks were implicated as carriers and disseminators of *Salmonella* spp. that could cause infection in domestic animals and humans.

In an outbreak in Vancouver, *E.coli* 0157:H7 (Samadpour *et al.*, 2002) was implicated as the cause of contaminated recreational water. Although *E. coli* 0157:H7 was also isolated from duck faecal samples the authors were unable to ascertain if the resident population of ducks at the lake was the source of the contamination. They suggest that the ducks may have been transiently infected by contaminated water and may have helped to sustain contamination levels at the lake over a period of time.

Escherichia coli from duck faeces was also implicated in the faecal contamination of the Lower Rincon Creek Watershed (Anonymous, 1999). Sources of faecal coliforms present in the water samples were determined by comparing the *E.coli* genetic material extracted from the water samples to previously established ribotypes of *E.coli* bacteria.

E.coli ribotype analogues to duck species showed the widest distribution among the sampling locations. Duck species were the second most prevalent match (where human species showed the highest) and matches were distributed over all the sampling locations.

1.2.3 Occurrence of *Giardia* and *Cryptosporidium*

Although *Giardia* and *Cryptosporidium* are distant relatives among parasitic protozoa, their epidemiology and zoonotic significance are similar. *Giardia* is a flagellated protozoan while *Cryptosporidium* is a coccidian parasite. Both parasites colonise the intestine of many mammals, birds, reptiles and amphibians. *Giardia* is recognised as the most common human intestinal parasite worldwide. *Cryptosporidium* has traditionally been considered an animal pathogen but it is now recognised as an important human pathogen, especially among immunocompromised individuals. Both *Giardia* and *Cryptosporidium* are leading causes of persistent diarrhoea in developing countries and are a major cause worldwide of waterborne disease in humans (Olson, 2002).

Giardia species have a diphasic asexual life cycle that includes a binucleate flagellated trophozoite stage and quadri-nucleate resistant cyst stage. In the environment infectious cysts are introduced into the host's body by oral ingestion of faecal contaminated food or water. Host ingestion initiates excystation in the small intestine, a process that degrades the thick cyst wall and results in the release of two vegetative trophozoites from each ingested cyst. Multiplication of *Giardia* trophozoites occurs by asexual longitudinal binary fission in the large intestine of the infected host. The binucleate trophozoites are unilaterally symmetrical and are approximately 11 µm to 15 µm long and 6µm to 8µm wide, giving them a pear-shaped appearance. Four pairs of flagella provide the trophozoite with motility while a large ovoid sucking disc on the anterior ventral surface facilitates adherence of the trophozoite to the lining of the small intestine. *Giardia* cysts are oval in shape and approximately 5 µm by 10 µm in size with a cyst wall varying in thickness from 0.3 µm to 0.5 µm (Hunt, 1999a).

The life cycle of *Cryptosporidium* resembles that of other coccidia. Sporulated oocysts are shed in the faeces of an infected host and then through contamination of the environment, food or water, other suitable hosts ingest oocysts. In the gastrointestinal or respiratory tracts of such hosts, sporozoites excyst from the oocyst and parasitise

epithelial cells. The sporozoite then differentiates into a spherical trophozoite with a single prominent nucleus. Asexual and sexual multiplication results from nuclear division and leads to the development of a fertilized macrogamont and then an oocyst which sporulates *in situ*. Upon completion of sporogony the oocyst contains four infective sporozoites. *Cryptosporidium* oocysts are spherical in shape and vary in size from 2 μm to 5 μm in diameter (Ionas, 1999).

Cryptosporidium and *Giardia* contamination of drinking and recreational waters can occur through the introduction of animal or human faecal material containing these parasites. Of the waterways tested throughout New Zealand over a six-year period, *Giardia* cysts and *Cryptosporidium* oocysts were detected in 19.2% and 12.6% of the waters tested respectively (Brown *et al.*, 1998). Although no particular pattern was evident, extensively populated areas, both urban and rural, contained a high proportion of the positive locations.

In New Zealand large numbers of animal reservoirs for *Cryptosporidium* and *Giardia* have been identified among wild animals surveyed on farms (Marino, 1993; Chilvers *et al.*, 1998). The overall prevalence of *Cryptosporidium* was much lower than *Giardia*, possibly because the duration of infection with *Cryptosporidium* is much shorter. *Giardia* cysts were detected in faecal samples from all six mammalian species and six out of nine samples from bird species; no cysts were detected in any of the five duck samples tested. *Cryptosporidium* oocysts were detected in three out of six samples from mammalian species and three out of seven samples from bird species; no ducks were tested for *Cryptosporidium*.

Graczyk *et al.*, (1996) reported that experimentally inoculated Peking ducks may serve as mechanical vectors for oocysts of *Cryptosporidium parvum*. These authors found that while *C.parvum* was unable to establish gastrointestinal or respiratory infection in the ducks, the viability and infectivity of *C.parvum* oocysts was however, retained after passage through the intestines of these refractory avian hosts. This study concludes that birds, particularly waterfowl, residing in watershed areas should be considered a potential factor for enhancing the contamination of water with *Cryptosporidium* oocysts.

Faecal droppings of Canada geese collected from nine sites near the Chesapeake Bay (Maryland, USA) were examined for the presence of *C.parvum* and *Giardia* spp. (Graczyk *et al.*, 1998). *Cryptosporidium* oocysts were found in faeces at seven of nine sites, and *Giardia* cysts were found at all nine sites. The study provides clear evidence that waterfowl can act as carriers of these organisms and disseminate them into the aquatic environment.

1.2.4 Public health implications of waterfowl droppings

Many of the organisms isolated from the waterfowl droppings have the potential for human pathogenicity, which under certain conditions could lead to infection and disease outbreaks, notably diarrhoea and gastroenteritis from organisms such as *Salmonella* and *Campylobacter* species, and wound infections and dermatitis from organisms such as *Aeromonas hydrophila* and *Pseudomonas aeruginosa* (Levesque *et al.*, 1993; Feare *et al.*, 1999). These bacterial infections could occur after exposure to a contaminated environment, for example through swimming in contaminated water, or exposure to contaminated beach sands, or the ingestion of food inadvertently contaminated with waterfowl droppings. Waterfowl faeces have been implicated in eutrophication of small ponds and lakes as well as contamination of school yards, parks and boating and swimming areas. An overriding concern of the public in most areas with high densities of waterfowl is the possibility of disease transmission to humans from direct contact with faecal material or contaminated water (Converse *et al.*, 1999).

Avian influenza virus infection occurs in a variety of wild and domestic bird species with the outcome ranging from no obvious clinical signs to 100% mortality (Swane *et al.*, 1998). A 1997 occurrence of avian influenza in Hong Kong involved 18 human cases (Snacken *et al.*, 1999) and raised concerns about transmission of avian influenza from birds to humans (Webster *et al.*, 1993).

Giardia intestinalis and *Cryptosporidium parvum* are ubiquitous intestinal parasites of animals and humans and these organisms are frequent aetiologic agents of waterborne outbreaks of intestinal disease. *Giardia* and *Cryptosporidium* are very common in farm animals and most wild animals, including waterfowl, can be infected with these organisms (Olson, 2002). Waterborne transmission in recreational aquatic environments

can occur through direct exposure of individuals to animal faecal matter containing these parasites (Marshall *et al.*, 1997). The retention of infectivity of *Giardia* and *Cryptosporidium* after intestinal passage through aquatic birds has serious epidemiological implications (Graczyk *et al.*, 1996; Graczyk *et al.*, 1998)

Publications on cercarial dermatitis (swimmers itch or duck itch) indicate that this condition, which is associated with ducks and human recreational water activities, should be considered as another emerging global parasitic disease (MMWR No. 41, 1992b; De Gentile *et al.*, 1996; Chamot *et al.*, 1998).

Clearly, waterfowl can cause a serious deterioration in water quality and in the absence of any treatment or disinfection this represents a major public health hazard. The greatest threats to public health arise when waterfowl reside or feed at the same habitats used by humans for recreation and feeding (Hatch, 1996). As mentioned earlier, ducks have been implicated as carriers and disseminators of a variety of pathogenic organisms including *Salmonella* and *Campylobacter*. Because of their aquatic lifestyle and highly mobile behaviour, ducks may be exposed to a diverse array of potentially pathogenic organisms that may have a significant microbiological impact on water quality and in turn public safety. Bidwell and Kelly (1950) showed that faceal contamination of water from ducks is a human heath hazard and that beach closings based on the presence of high indicator bacteria are warranted. In order for the public to be protected and informed of the microbiological health risks of contaminated beaches when the water quality is compromised, appropriate water quality monitoring programmes need to be in place. This includes the proper interpretation of monitoring results from surveys for bacteriological indicators of faecal contamination so that the cause of the contamination can be determined and appropriate and timely management actions be implemented.

1.3 WATER QUALITY MONITORING

1.3.1 Legislative responsibilities

One of the main reasons for monitoring water quality is to establish the suitability of a body of water for its intended purposes. Monitoring the quality of recreational water environments is usually undertaken to provide a means of assessing impacts and to check whether unexpected change is occurring. In regulatory monitoring programmes, factors such as sampling frequency, analytical methods, data analysis, interpretation and reporting, sample site selection, criteria for recreational water-use, and remedial measures are generally defined by the regulatory agency (Pond *et al.*, 2000).

Although section 35 of the *Resource Management* Act (RMA) 1991 and section 23 of the *Health Act* 1956 define a requirement for recreational water quality monitoring, neither Act explicitly defines responsibilities for beach water quality monitoring and reporting. Section 57 of the RMA however, provides for a *New Zealand Coastal Policy Statement* (NZCPS) with policies that are mandatory. Policy 5.1.1 of the NZCPS directs regional councils to make rules with the object of enhancing water quality. Policy 5.1.7 of the NZCPS directs regional councils and territorial authorities to make provisions to warn the public about poor water quality.

The Wellington Regional Council has a number of legislative requirements relating to the coastal marine area. Section 30 of the RMA requires regional councils (in conjunction with the Department of Conservation) to be responsible for the control of discharges to the marine environment. Section 35 of the RMA requires regional councils and territorial authorities to monitor the state of the whole or any part of the environment of their region or district to the extent that is appropriate for them to effectively carry out their functions under the Act. Section 64 of the RMA sets out a requirement for the preparation of a *Regional Coastal Plan*.

The Wellington Regional Council's *Regional Coastal Plan* outlines a number of environmental objectives for the coast (Robertson, 2000). These include:

- The intrinsic values of the coastal marine area and its components are preserved and protected from inappropriate use and development.

- The adverse effects new activities may have on existing legitimate activities in the coastal marine area are avoided, remedied or mitigated as far as is practicable.
- Land, water and air in the coastal marine area retain its life supporting capacity.
- Public health is not endangered through the effects of previous, present or future activities in the coastal marine area.

The *Regional Coastal Plan* also designates specific areas of the coastline to be managed for contact recreation or the recreational gathering of shellfish for human consumption. The Regional Council monitors coastal water quality at seventy sites around the Regions's coast and sampling is usually carried out on a monthly basis outside of the bathing season (1st November to 31st March). During the bathing season sampling is increased to fortnightly intervals with the territorial authorities also sampling at fortnightly intervals in the weeks between the Regional Council's sampling. In this way weekly sampling results are achieved in the bathing season (Robertson 2000).

1.3.2 The New Zealand recreational water quality guidelines

Since the 1950s a number of epidemiological studies have investigated the relationship between health risk and swimming. These studies have investigated mainly gastrointestinal symptoms, eye infections, skin complaints, ear, nose and throat infections and respiratory illness. Overall conclusions of these studies were that the rates of several symptoms were increased in swimmers compared with non-swimmers and that there was a causal dose-related relationship between gastrointestinal symptoms and recreational water quality measured by bacterial indicator counts (Cabelli *et al.* 1982; Dufour 1984; Kay *et al.*, 1994; Fleisher *et al.*, 1998; Pruss 1998).

The 1992 provisional New Zealand marine bathing guidelines (McBride *et al.*, 1992) were developed using the results of international studies (Cabelli *et al.*, 1982; Cabelli 1983). The 1998 guidelines (MFE & MOH, 1998) were developed because of the concern that overseas studies might not be applicable to New Zealand because of this country's different environmental conditions such as a much higher proportion of contamination from animal sources compared to that from humans. The new guideline values are therefore based on a *1994/1995 New Zealand Marine Bathing Health Effects Study* (McBride *et al.*, 1998). The key findings of this study were:

- The relationships in New Zealand between indicator bacteria and health effects are consistent with those found overseas (Cabelli *et al.*, 1982). *Enterococci* are the indicators most closely correlated with health effects in marine waters. Importantly, the New Zealand study found that health risks from water contaminated with animal and human faecal material were not significantly different and that *Enterococci* are good indicators for health risks associated with both these sources of contamination.

- The guideline values should apply irrespective of location or weather conditions. This is a significant change from the previous guidelines since the exclusion of results that are influenced by rainfall does not reflect the actual risks to bathers. For example, there is no justification, from a health perspective, for not sampling after heavy rainfall if people are swimming or likely to be swimming. That is, sampling programmes should be based on the number of people (likely to be) swimming, whether or not there has been a heavy rainfall event.

- *Enterococci* levels in shallow water correlate well with health risks. Therefore, sampling can take place at 0.5 m depth (mid-calf) rather than at chest depth, as proposed in the previous guidelines (McBride *et al.*, 1992). Sampling at chest depth can be hazardous to those collecting samples.

The purpose of the *New Zealand Recreational Water Quality Guidelines* is to assist authorities with the implementation of the Resource Management (RMA) 1991 and the Health Act 1956. The guidelines cover the interpretation of monitoring results from surveys for bacteriological indicators of faecal contamination. The framework used in the guidelines is a three tier management system analogous to traffic lights:

- Green (Clean) – "Safe" water for bathing requiring continued surveillance (routine monitoring).
- Amber (Potentially contaminated) - "Potentially unsafe" water requiring more detailed monitoring to asses the safety of the water
- Red (Highly likely to be contaminated) – "Highly likely to be unsafe" requiring urgent action such as public warnings or closing a beach.

The guidelines use a combination of annual (seasonal) median values as well as single sample values to assess safety.

- **Surveillance/Green mode** – Running median less than 35 *Enterococci* per 100 ml - which involves routine (eg. weekly) sampling.
- **Alert/Amber mode I** – Running median greater than 35 *Enterococci* per 100 ml and no single sample greater than 136 *Enterococci* per 100 ml – exceedences require investigations into the causes of the elevated levels and increased sampling (twice weekly) to enable the risks to bathers to be more accurately assessed.
- **Alert/Amber mode II –** Single sample between 136 and 277 *Enterococci* per 100 ml (irrespective of running median) – again, exceedences require investigations into the causes of the elevated levels and increased sampling (daily), and sanitary surveys to identify sources of contamination. (In 1999 this Alert/Amber mode II was changed to – Single sample greater than 136 *Enterococci* per 100 ml).
- **Action/Red mode** – Single sample greater than greater than 277 *Enterococci* per 100 ml (irrespective of running median) - exceedences require the local authority and health authorities to warn the public that the beach is considered to be unsafe, erect warning signs, conduct sanitary surveys and undertake daily sampling. (In 1999 this Action/Red mode was changed to – Two consecutive single samples within 24 hours greater than 277 *Enterococci* per 100 ml).

The guidelines use "maximum acceptable" swimming-associated illness risks of 19 per 1,000 bathers for marine water. These risk levels have been adopted by the US Environmental Protection Agency (USEPA) and and have been used internationally for years and generally accepted without question. Therefore the acceptance of these risk levels in New Zealand means that these guidelines are consistent with international practice (Cabelli, 1983).

In marine waters, adherence to the guideline values and use of the management framework should ensure that people using the water for recreational purposes are not exposed to significant health risks. However, because these guidelines were developed

from studies relating bacteriological indicators to illness in the general public, water conforming to the guideline values may still pose a potential health risk to high-risk user groups such as the very young, the elderly and those with impaired immunity.

As stated on page 8 of the guidelines "These guidelines must not be used as measure of suitability for bathing when there is a major outbreak of a potentially waterborne disease in the community and that community's sewage contributes to the microbiological contamination of the water. These guidelines do not apply then because the relationship between indicator organisms and disease was derived when there was no known disease in the community. When there is an outbreak of disease in the community, health risks may be increased because of a higher-than-usual level of concentration of pathogens in the water"

1.3.3 Characteristics of indicator organisms

The quality of recreational water is determined largely by bacteriological analyses in order to establish whether the water contains faecal organisms (not necessarily pathogens) whose presence is indicative of pollution by animal or human wastes. Analysis of water samples on a routine basis for each individual type of pathogen is not practical and pathogen isolation and identification is therefore usually only attempted following a waterborne disease outbreak. There are a number of reasons why pathogen identification is rarely attempted during routine assessment of water quality (Loutit, 1990):

- Pathogens may be emitted intermittently from the diseased host so that the testing programme may miss them.
- Pathogen numbers may be diluted in the receiving waters making their detection more difficult.
- The particular pathogen being tested may not be easily culturable.
- The test methodology may not be available for pathogen testing.
- Multiple strains of the pathogen may have to be targeted for satisfactory detection.
- Isolation and identification of the pathogen may involve many tests and can be expensive and time-consuming.

By implication, indicator organisms should signal the potential presence of faecal organisms that can cause gastrointestinal disease (Bonde, 1977). The purpose of a bacterial indicator is to allow prediction of the health risk associated with the water under consideration. To ensure the predictive value of indicator tests, the indicator organism must meet certain criteria:

- Must always be present in the presence of pathogens.
- Must be derived exclusively from the same source as the pathogen.
- Must occur in high numbers to allow for a safety margin.
- Must not multiply appreciably in the water.
- Must behave and survive like the pathogen in the environment.
- Must resist treatment and disinfection to a similar or slightly greater extent than the pathogen.
- Must be easily detected by simple, reliable and rapid methods.
- Must be easily detected, even in the presence of other organisms.

The search for the ideal indicator for microbiological water quality has been elusive. In no case is any indicator entirely suitable for all pathogens and no single indicator or approach is likely to represent all the facets and issues associated with contamination of water with faecal matter. For example, protozoa and viruses have different survival properties; enteric viruses and protozoan cysts are relatively stable in moist conditions. Generally, if indicator organism levels are high, then there is good prediction that virus and protozoan levels are high also; but low levels of indicator organisms do not necessarily indicate an absence of viruses and protozoa (Loutit, 1990).

Commonly used indicator organisms of water quality are organisms originating in the human and animal gastrointestinal tract and includes aerobic and facultative anaerobic organisms such as faecal coliforms and organisms belonging to the genus *Enterococcus.*

Coliform bacteria as a group, are characterised as Gram-negative, non-spore forming, facultative rod - shaped bacteria that ferment lactose with the production of acid and gas within 48 hours at 35 ± 1°C (Orskov, 1984). Since *Escherichia coli* is a normal inhabitant of the intestinal tract of humans and other warm-blooded animals it is regarded as a typical faecal type of coliform. Other members of the coliform group, for

example, *Enterobacter aerogenes*, are widely distributed in nature and found in soil, water, grain, and also in the intestinal tract of humans and other animals and are regarded as non-faecal or atypical coliforms. Total coliforms refer to both faecal and environmental coliform bacteria. Thermotolertant faecal coliforms constitute the subset of total coliforms that have historically been used as faecal indicators since they appeared to possess a more direct and closer relationship with faecal pollution (Geldreich, 1967). These bacteria conform to all the criteria used to define coliform bacteria as a group and also ferment lactose with the production of acid and gas within 48 hours but at the elevated temperature of 44.5 ± 0.2°C. Because thermotolerant coliforms include strains of *Klebsiella* and *Escherichia* and certain *Enterobacter* and *Citrobacter* strains are also able to grow under the conditions defined, it has been suggested that thermotolerant coliforms be replaced by *E.coli* as an indicator of faecal pollution (Dufour, 1977; Figueras *et al.*, 2000). *E.coli* is the only biotype of the family *Enterobacteriaceae* that is almost always of faecal origin and it is also a more appropriate indicator since faecal coliforms may replicate in the environment and falsely elevate indicator organism levels (McLellan *et al.*, 2001). Most *E.coli* can be confirmed by a positive indole test and their inability to use citrate as the sole carbon source when incorporated in traditional culture media.

Faecal streptococci have been widely accepted as convenient, and in many cases, better indicators of faecal pollution of recreational waters than faecal coliforms or *Esherichia coli*. A number of epidemiological studies have shown that these organisms have a close relationship with health risks associated with bathing in marine and freshwater environments (Cabelli *et al.*, 1982; Dufour 1984; Kay *et al.*, 1994; Fleisher *et al.*, 1998; Pruss 1998; McBride *et al.*, 1998). Major characteristics of faecal streptococci in the environment are (Sinton *et al.*, 1993a; 1993b):

- not as widespread as coliforms.
- always present in the faeces of warm-blooded animals.
- do not multiply appreciably in contaminated waters, especially if the organic matter content is low.
- die-off rate is slower than faecal coliforms in seawater.
- persistence patterns are similar to those of waterborne pathogenic bacteria.

Sinton and Donnison (1994) have reported that the faecal streptococci group of organisms includes species of different sanitary significance and survival characteristics. Rutkowski and Sjogren (1987) and Poucher *et al.*, (1991) have demonstrated that the proportion of the species of this group is not the same in animal and human faeces. The taxonomy of the faecal streptococci group, comprising species of two genera *Enterococcus* and *Streptococcus*, has been subject to extensive revision since the 1980's (Mundt, 1989; Facklam *et al.*, 1995; Leclerc *et al.*, 1996). Although several species of both genera are included under the term *Enterococci*, the species most prevalent in polluted waters are *Enterococcus faecalis, E.faecium*, and *E.durans*. (Sinton and Donnison, 1994; Figueras *et al.*, 2000). Although *Enterococci* are often regarded as being of faecal origin, some strains appear to be common on plants, insects and in soils (Sinton *et al.*, 1993b).

Enterococci includes all the species described as members of the genus *Enterococcus* that fulfil the following criteria:

- Growth at 10°C and 45°C.
- Survival at 60°C for at least 30 minutes.
- Growth at pH 9.6 and in the presence of 6.5% sodium chloride.
- Ability to reduce 0.1% methylene blue in milk.

The most common environmental species fulfil the above criteria and therefore in practice the terms faecal streptococci, *Enterococci*, intestinal *Enterococci* and *Enterococcus* group can be considered synonymous (Figueras *et al.*, 2000).

1.3.4 Methods for detecting and enumerating indicator organisms

There are a number of methods that can be used for the detection and enumeration of bacterial indicator organisms in water samples. These include traditional techniques such as the membrane filtration technique and the multiple tube fermentation technique for the most probable number (Abbott, 2001).

Coliforms and *Escherichia coli* have for many years been detected in water samples with membrane filtration (MF) techniques incorporating various differential and selective media and incubation temperatures (Donnison, 1992; APHA, 1999). mEndo

agar, incubated at 35 ± 1°C, can be used for *Total coliform* detection. On this medium the coliform bacteria ferment lactose to produce acids and aldehydes; the aldehydes react with the basic fuchsin - sulphite complex in the medium to release free fuchsin, which stains the colonies red with a characteristic metallic sheen. mFC agar, incubated at 44.5 ± 0.2°C can be used to detect faecal (thermotolerant) coliforms such as *Escherichia coli*; faecal coliforms ferment the lactose in the medium which changes the pH so that these organisms appear as characteristic blue colonies. The 24 hour mTEC method (USEPA, 1985) is the recommended membrane filtration (MF) method for monitoring *Escherichia coli* in New Zealand fresh water environments (MFE & MOH, 1998). In this medium *E.coli* ferment lactose at 44.5°C. A post-incubation test for the lack of urease confirms yellow or yellow-brown colonies as *E.coli*.

A variety of MF techniques have also been employed to detect faecal streptococci and *Enterococci* in fresh and marine waters. The 48 hour mE procedure was the previously approved New Zealand method for *Enterococci* (McBride *et al.*, 1992). Incubation of the membrane filter at 41 ± 0.5°C on a selective and differential agar medium (mE) for 48 hours was followed by transfer of the membrane to an *in situ* esculin-iron agar (EIA) substrate medium and incubation for 20 minutes at 41°C. Pink - red colonies on the membrane producing a brownish black precipitate on EIA were identified as *Enterococci*. The brownish black precipitate was the result of the hydrolysis of esculin to glucose and coumarin by the enzyme β-glucosidase. Coumarin forms a black precipitate in the presence of ferric citrate (Donnison 1992). Although this MF method has been shown to efficiently recover *Enterococci* from marine and estuarine waters, the false positive and negative rates are reported to be 10.0 and 11.7% respectively (Levin *et al.*, 1975; Budnick *et al.*, 1996). The MF method requires the dilution of water samples that contain high bacterial numbers in order to obtain counts in the acceptable counting range of 20 - 60 colonies. Turbid samples can block the membranes, and the growth of competing bacteria can often cause inhibition of growth of target organisms or mask the colour changes associated with carbohydrate fermentation (Fricker & Fricker, 1996).

Since 1998 New Zealand recreational water quality guidelines (MFE & MOH, 1998) have adopted the 24 hour EPA 1600 MF Method (USEPA, 1997) - also called the mEI method – because the 'Action/Red' mode compliance monitoring requires two

consecutive single samples analyses within 24 hours. This mEI membrane filtration medium contains the chromogenic substrate indoxyl-β-D-glucoside for use in a single step procedure for enumeration of *Enterococci* in 24 hours. Like the mE medium, the mEI medium also contains nalidixic acid, cycloheximide, but a reduced amount of triphenyltetrazolium chloride. *Enterococci* colonies produce an insoluble indigo blue complex, which diffuses into the surrounding medium forming a blue halo around the colony. Based on the recovery of *Enterococci* from environmental samples the mEI medium shows analytical performance (false positive rate 6.0%; false negative rate 6.5%) characteristics similar to those of the standard 48 hour mE MF method (Messer & Dufour, 1998).

Multiple tube fermentation techniques for the most probable number (MPN) have been used in the microbiological monitoring of water quality for many years (McGrady, 1915) and its validity and suitability for estimating bacterial numbers is explained in Cochran's classic paper (1950) and by Woodward (1957). MPN methods have been found to give statistically equivalent results to membrane filtration methods even though the techniques are significantly different (Fullerton, 1995). These methods are particularly useful for turbid water samples that require dilution, sediments and effluents. The MPN method for *Enterococci* is a three-stage test that takes 4 days to complete. The first (presumptive) stage uses liquid medium containing azide dextrose to inhibit non-target bacteria. All azide dextrose broth tubes showing turbidity after 24 or 48 hours incubation are subjected to confirmation on the same media used for the membrane filtration method (Donnison 1992). The traditional MPN test for total coliforms, faecal coliforms and *E.coli* uses lauryl tryptose broth for the presumptive identification of coliforms, brilliant green lactose broth for selective enrichment, and EC medium for confirmation of *E.coli* (APHA, 1999). MPN methods are generally time consuming because of the number of tubes and sample manipulations required and are therefore unsuitable when it is necessary to process large numbers of water samples (Abbott, 2001).

The ability to detect the presence of specific enzymes using chromogenic and fluorogenic enzyme substrates has led to the development of a variety of new methods for the rapid identification of indicator organisms in water (Edberg *et al.*, 1988; Fiksdal *et al.*, 1994; Manafi, 2000). Measurement of β-D-galactosidase, and β-D-glucuronidase,

and β-D-glucosidase activity are increasingly being used in new media and methods for detecting and enumerating total coliforms, *E.coli* and *Enterococci* respectively. These defined substrate technologies are now used to monitor faecal pollution of drinking water and recreational waters in significantly less time than membrane filtration procedures (Edberg *et al.*, 1988; Palmer *et al.*, 1993; Eckner, 1998; Solo-Gabrielle, 2000).

The Colilert™ system (IDEXX Laboratories, Westbrook, Maine, United States) is based on a defined substrate medium containing 4-methylumbelliferyl-β-D-glucuronide (MUG) and ortho-nitrophenyl β-D-galactopyronoside (ONPG) and is used for the one-step detection of total coliforms and *E.coli* in water samples. ONPG is a colourless lactose analog that is hydrolysed by β-D-galactosidase, an enzyme common to all coliforms, to form a yellow product. β-D-glucuronidase, which is present in relatively few bacterial species but is found in most *E.coli* strains, will hydrolyse MUG to form a fluorescent product that is visible under UV_{365} light. Discrepancies, including *E.coli* false positives and false negatives, interferences of plant and algal matter, and lactose fermenting marine vibrios have been published for various Colilert formulations (Davies *et al.*, 1994; Davies *et al.*, 1995a; Edberg *et al.*, 1989; Landre *et al*, 1998).

The Enterolert™ system (IDEXX Laboratories, Westbrook, Maine, United States) was designed for the rapid detection and enumeration of *Enterococci*. Enterolert utilises 4-methylumbelliferyl-β-D-glucoside as the defined substrate nutrient indicator. This compound, when hydrolysed by enterococcal-β-glucosidase, releases 4-methylumbelliferone that exhibits fluorescence under a UV_{365} lamp. The system has been shown to have a number of advantages over membrane filtration for rapid water quality monitoring (Abbott *et al.*, 1998). These authors evaluated Enterolert™ in a large study involving 343 marine water samples from selected beaches in the Wellington area. Statistical analysis of parallel test results showed a strong linear correlation (r = 0.927) and no significant difference between Enterolert™ and the 48 hour mE membrane filtration method by paired t-test analysis (P = 0.39). Based on the 2.4% false positive and 0.3% false negative rates obtained, the study found Enterolert™ to have a sensitivity of 99.8% and a specificity of 97.0%. These results were consistent with the

results of studies undertaken in the United States, Britain and Sweden where Enterolert's false positive rate was found to be ~5.0% and the false negative rate ~0.6% (Budnick *et al.*, 1996; Fricker & Fricker 1996; Eckner, 1998).

In the Colilert™ and Enterolert™ systems the most probable number (MPN) estimate of organism numbers is achieved through the use of a plastic Quanti-Tray™ partitioned into 48 small (0.16 ml) and 49 large (1.88 ml) wells. The Quanti-Tray™ allows for the inoculation, with minimum sample manipulation, of a greater number of wells than would be possible using a standard MPN method. Sealing of the sample-filled tray is accomplished by the use of a specially designed Quanti-Tray sealer that automatically distributes and seals a 100 ml sample.

Colilert™ and Enterolert™ are the defined substrate methods of choice in the new New Zealand recreational water quality guidelines; Colilert™ for monitoring total coliforms and *Escherichia coli* in fresh water environments and Enterolert™ for monitoring *Enterococci* in marine waters (MFE & MOH, 1998; MFE & MOH, 1999).

1.3.5 Distinguishing human from animal sources of faecal pollution

In order to implement proper management plans for the continuance of safe water quality it is important to be able to identify the sources of faecal pollution when this occurs. Common sources of pollution of coastal waters include point discharges of treated and untreated sewage from shoreline outfalls and boats and a variety of non-point sources, such as:

- runoff from naturally vegetated areas, including wetlands
- agricultural runoff
- stormwater runoff from impervious surfaces associated with urban, commercial, or industrial land uses
- malfunctioning or poorly sited septic tanks
- direct deposition of waterfowl faeces.

Each of the above mentioned pollution sources consists of a human or animal microbial contributor in association with a direct surface water or groundwater pathway. While a visual on-site sanitary survey can sometimes identify the principal faecal pollution

source, it is often necessary to conduct rigorous microbiological analysis of the water to establish if the pollution is of human or animal origin.

Although the indicator organism concept has been used successfully for a long time in water quality monitoring (Geldreich, 1967), there are still many questions concerning the effectiveness of indicator organisms in distinguishing animal from human sources of faecal pollution (Figueras *et al.*, 2000). The most commonly used faecal indicator organisms; coliforms, faecal coliforms (*E.coli*) and *Enterococci* are found in both human and animal faeces. A number of environmental and physical factors may influence the survival of indicator organisms in water (Sinton *et al.*, 1993b; Davies *et al.*, 1995b; Davies-Colley *et al.*, 1994; Sinton, 1995). Of several factors that considerably reduce the survival rates of faecal bacteria in the aquatic environment, sunlight is thought to be the single most important factor contributing to the death of these bacteria in marine water. Other factors include high salinity, pH, temperature, the presence of toxic agents, predation and parasitism and low nutrient concentrations.

Whereas the available information on the survival of indicator bacteria in fresh and marine water is often confusing and contradictory, it has consistently been shown that *Enterococci* exhibit superior survival characteristics in seawater than coliforms and *Escherichia coli.* Chamberlain and Mitchell (1978) showed that in marine waters the mean T_{90} (the time taken for 90% of organisms to die) for total coliforms and *E.coli* is about 2.2 hours, while in fresh waters the mean T_{90} for these organisms is about 58 hours. Although the study did not include work on *Enterococci*, Hanes and Fragala (1967) report that *Enterococci* also die off more rapidly in marine water than in freshwater environments (T_{90} = 3 days in freshwater and 2.4 days in marine water).

Using ratios between thermotolerent coliforms and faecal streptococci as a means of distinguishing between human and animal derived faecal matter was proposed by Geldreich in 1976. Since there is a sharp contrast in density relationships between faecal coliforms and faecal streptococci in the faeces of humans versus the faeces of other warm-blooded animals, it seems logical to use this concept to identify sources of faecal contamination discharged to receiving waters. Streptococcal concentrations in human faeces are generally less than faecal coliforms, 3,000,000 *vs* 13,000,000 per gram respectively.

In contrast, faecal streptococci in animal faeces generally outnumber faecal coliforms (Geldreich 1976; Jones & White 1984), for example:

- 1,300,000 *vs* 230,000 in cows
- 38,000,000 *vs* 16,000,000 in sheep
- 84,000,000 *vs* 3,300,000 in pigs
- 6,300,000 *vs* 13,000 in horses
- 3,400,000 *vs* 1,300,000 in chickens
- 54,000,000 *vs* 33,000,000 in ducks

Because the faecal coliform:faecal streptococci (FC:FS) ratio has been reported as >4.0 in human faeces and <0.7 in animal faeces it is theoretically possible to attribute a human or animal source to faecal pollution based on the evidence of FC:FS ratios of >4.0 or <0.7 (Feachem, 1975; Geldreich 1976; Mara & Oragui, 1985). These studies have used the FC:FS ratio concept to identify a variety of different faecal pollution sources in rivers, stormwater from urban and suburban areas, runoff from rural areas and cattle feedlots, and effluents from various food processing industries. In a study on the effects of mallard ducks on indicator bacteria in recreational water, Standrige *et al.*, (1979) found FC:FS ratios frequently below 0.7 that indicated that livestock or poultry (mallards) were the most likely source of pollution. However, the application of FC:FS ratios to environmental samples has been considered to be too unreliable for characterising pollution sources by Sinton and Donnison (1994). In their study these researchers found that FC:FS ratios were similar for domestic and meat-works effluents and that ratios were around or above 3. The frequently reported <0.7 in animal faeces was found not to apply to any of the meat-works samples tested. The authors did however, suggest that the alternative FC:FS ratio shift approach used by Feachem (1975) appeared to be worthy of a more detailed evaluation. This approach involves interpretation of the differential die-off rates of faecal coliforms and faecal streptococci in stored samples. In theory, a predominantly human source should exhibit an initially high FC:FS ratio (>4) which falls on storage, whereas a non-human source should exhibit an initially low FC:FS ratio, which subsequently rises.

Howell *et al.*, (1995) found that the FC:FS ratio appeared to distinguish between wild and domestic animal faecal contamination of water but the FC:FS ratio could not be

used to unambiguously distinguish between domestic and animal sources of faecal contamination.

Ratios of total coliforms to faecal coliforms (TC:FC) were used to identify faecal pollution sources in a 1998 survey of the water quality of beaches along the shoreline of the Southern Californian Bight (Noble *et al.*, 2000). In this survey faecal coliform thresholds were exceeded five times more often along Mexican than United States beaches. The likely presence of human faecal contamination along the Mexican beaches, where much of the sewage reaches the beach untreated, was supported by the results of the TC:FC ratios.

After many years of research no single microorganism or chemical has yet been identified which will reliably distinguish human from animal sources of faecal contamination of water on all occasions. In a comprehensive review of the subject Sinton *et al.*, (1998) found that a number of bacterial and non-bacterial indicators such as Bifidobacteria, *Bacteroides fragilis* bacteriophages, F-specific RNA coliphages, *Rhodococcus coprophilus*, faecal sterols, fluorescent whitening agents, sodium tripolyphospate, and linear alkyl benzenes have been used with varying success.

The use of faecal biomarkers in conjunction with existing bacterial indicators offers another way to distinguish sources of faecal contamination. Leeming and Coleman (2000) have shown that the principal faecal human sterol is coprostanol, which constitutes approximately 60% of the total sterols, found in human faeces. The coprostanol homologue 24-ethylcoprostanol, is the principal faecal sterol excreted by herbivores. Other animals that are ubiquitous in urban environments such as dogs and birds, either do not have coprostanol in their faeces or it is present in insignificant amounts relative to other sterols. Based on an empirical formula applied to combinations of concentrations of coprostanol, 24-ethylcoprostanol, and counts of faecal coliforms, *Escherichia coli*, and *Enterococci*, Leeming and Coleman were able to discriminate the sources of faecal contamination of drains in Melbourne.

Antibiotic resistance profiles appear to hold promise also as a method that may be useful for determining the sources of faecal pollution in waters. Studies on the discriminant analysis of antibiotic resistant patterns in faecal streptococci have reported measurable and consistent differences in antibiotic resistance patterns of faecal

streptococci from various sources of faecal pollution (Wiggins *et al.*, 1996; Wiggins *et. al.*, 1999; Hagedorn *et al.*, 1999; Harwood *et al.*, 2000) These studies have shown that the information contained in the antibiotic resistance patterns appear strong enough to be able to be used for the classification of unknown isolates from polluted waters, especially since the waters would likely contain unknown mixtures from different sources.

Rapid advances in nucleic acid-based technologies such as Polymerase Chain Reaction (PCR), have resulted in the appearance of a number of DNA methods for distinguishing faecal pollution sources. Dombek *et al.*, (2000) used repetitive intergenic DNA sequences to differentiate *Escherichia coli* strains obtained from human and animal sources. This type of genotypic analysis was less affected by environmental factors than some of the phenotypic methods mentioned above. Work on Amplified Fragment Length Polymorphism (AFLP) of animal and human isolates of *Escherichia coli,* is another DNA research method that is based on selective amplification of a subset of DNA fragments from a digest of total genomic DNA (Guan *et al.*, 2002). ALFP analysis is a sensitive fingerprinting technique that generates highly reproducible results and is reported as having better discriminating powers than conventional methods.

Ribotyping of faecal *Escherichia coli* from humans and animals is also being used increasingly for identifying pollution sources (Parveen *et al.*, 1999; Carson *et al.*, 2001; Samadpour *et al.*, 2002). In ribotyping the DNA patterns of collections of *Escherichia coli* isolates, known as ribotypes, are used to match specific strains of *Escherichia coli* from a contaminated site to potential sources. Riboprints appear to be associated with (if not unique) to certain host classes and as with other DNA fingerprinting methods, ribotyping produces various patterns that represent the genomic similarity or dissimilarity between isolates.

1.4 OBJECTIVES AND APPROACHES

The objectives of this thesis were to:

1. Determine if there was a significant improvement in recreational water quality at Hataitai Beach since the 1998 stormwater and sewer upgrades were carried out by the Wellington City Council.
2. Establish if the ducks at Hataitai beach have an impact on the recreational water quality.
3. Identify the principal sources of faecal pollution at Hataitai Beach.
4. Detect *Giardia* and *Crytosporidium* in recreational water samples from Hataitai Beach.
5. Perform DNA analysis on *Giardia* and *Crytosporidium* in duck faecal samples collected from Hataitai Beach.

In order to determine if there was a significant improvement in the water quality it was decided for this study to collect and analyse a large number of marine water samples for compliance monitoring from a variety of sites at Hataitai Beach from July 1998 to April 2000. This approach was in contrast to that of the Wellington City and Regional Council who usually only sampled one site fortnightly in their routine beach monitoring programme (Robertson, 2000).

For the detection and enumeration of *Enterococci* the Enterolert™ system was used primarily because of the system's advantages over the membrane filtration technique and because of this author's previous research and expertise with the Enterolert™ system (Abbott *et al.*, 1998). Furthermore, Enterolert™ is one of the preferred methods in the *New Zealand Recreational Water Quality Guidelines* (MFE & MOH, 1998; MFE & MOH, 1999) and is a method that has been used successfully overseas for monitoring marine recreational waters (Budnick *et al.*,1996; Fricker & Fricker 1996; Eckner, 1998; Noble *et al.*, 2000; Desmarais *et al.*, 2002).

A major component of this thesis was to determine if the duck droppings at Hataitai Beach were the main cause of increased indicator organism loading in the beach waters or whether the duck droppings were in fact secondary contributors to faecal pollution.

Therefore, it was decided to record the number of ducks and their location at each sampling event. Correlations were then determined between duck numbers and indicator organism counts in samples obtained from the various sites. As described below, additional methods were also employed to identify the sources of pollution that could have had a significant impact on indicator organism counts including methods for distinguishing human from animal sources of pollution.

To identify the principal sources of faecal pollution at Hataitai Beach it was decided to also detect and enumerate total coliforms and *Escherichia coli* in the marine water samples. While these are not the recommended indicator organisms for marine recreational water quality monitoring in New Zealand, for this thesis it was felt that total coliforms and *Escherichia coli* data could contribute to the identification of faecal pollution sources. Since total coliforms refers to both faecal and environmental coliform bacteria and *Escherichia coli* is a normal inhabitant of the intestinal tract of humans and other warm-blooded animals, data on these indicator organisms are especially useful when studying the influence of sewer overflows and stormwater on indicator organism levels.

In their study on faecal indicator bacteria and beach contamination (Hartley *et al.*, 2000) found that sewer overflows and stormwater were the most significant contributors of elevated bacterial concentrations in beach bathing water; higher bacterial counts were associated with proximity to point sources of stormwater discharges and *Enterococci* concentrations were significantly elevated in samples collected within 24 hours of a rain event. Rainfall can have a significant effect on indicator organism levels because animal wastes are washed from forests, pasture land and urban settings or human wastes from sewer overflow sources (Calderon, 1990 as cited in Bartram & Rees, 2000, page 180). In a study on the sources and pathways of bacterial contamination in coastal waters Weiskel *et al.* (1996) showed that when more than 2.5 cm rainfall fell in 96 hours prior to sampling there were six times higher faecal coliform densities than dry weather sample densities. Therefore, in this study it was decided to also determine if there was a significant association between increased indicator organism counts and rainfall on days preceding sampling.

As part of a process of distinguishing animal from human sources of pollution at Hataitai Beach it was decided to re-evaluate the FC:FS ratio concept. A 1979 study by Standrige *et al.*, demonstrated that FC:FS ratios below 0.7 indicated faecal pollution by livestock or poultry (mallards). More meaningful results could well be obtained by using specific *Escherichia coli*:*Enterococci* ratios of indicator organism counts obtained in samples from the various sites surveyed in this study. In spite of the earlier mentioned limitations (section 1.3.5) of using ratios to characterise pollution sources, the use of ratios here appeared to be warranted, especially since this would represent the first recorded use of *Escherichia coli*:*Enterococci* ratios calculated from counts generated by Colilert-18™ and Enterolert™ defined substrate technologies.

For the detection and enumeration of total coliforms and *Escherichia coli* the Colilert – 18™ system was used. Colilert-18™ has a special formulation that accommodates marine water samples and this system has been shown to correlate well with the traditional membrane filtration techniques for monitoring indicator organisms in marine waters (Palmer *et al.*, 1993; Fiksdal *et al.*, 1994; Eckner 1998; Solo-Gabriele *et al.*, 2000; Noble *et al.*, 2000; Desmarais *et al.*, 2002).

As mentioned in sections 1.2.2 and 1.2.4, numerous publications have appeared on the wide variety of bacteria that have been isolated from waterfowl droppings and on the potential these organisms have for causing disease in humans. Therefore, other than the extensive indicator organism detections and enumerations, no attempt was made in this study to isolate bacterial pathogens from water samples or duck faecal samples. However, as only a few research articles have been published on the occurrence of *Giardia* and *Cryptosporidiun* in waterfowl and since there is limited information on these parasites in ducks in New Zealand, attempts were made to detect these parasites in water samples and duck faecal samples collected from Hataitai Beach.

For the capture in marine water samples of *Giardia* cysts and *Cryptosporidium* oocysts on filter cartridges and their subsequent purification by immunomagnetic bead separation and detection by fluorescent monoclonal antibody techniques, the *New Zealand Interim Standard Method for Cryptosporidium and Giardia in Water* was used (Learmonth *et al.*, 2000). Since 1999 no standard method has been recommended by the American Public Health Association in their *Standard Methods for the Examination of Water and Wastewater* (APHA, 1999). The New Zealand interim method is a

compilation of the United States Environmental Protection Agency's methods for detecting *Giardia* cysts and *Cryptosporidium* oocysts in water by a fluorescent antibody procedure, filtration, and immunomagnetic bead separation (USEPA, 1995; USEPA 1999).

The Merifluor® *Cryptosporidium/Giardia* direct immunofluorescent procedure (Meridian Diagnostics, Inc., Cincinnati, Ohio) was used to detect *Giardia* cysts and *Cryptosporidium* oocysts microscopically in the duck faecal samples. In the Merifluor *C/G*® stain, combined fluorescein isothiocyanate-labeled monoclonal antibodies are directed against the cell wall antigens of *Giardia intestinalis* cysts and *Cryptosporidium parvum* oocysts. The Merifluor® combined stain has been designed for the rapid and accurate detection of *G. intestinalis* and *C. parvum* (oo)cysts in clinical specimens and has been reported to be markedly more sensitive and specific than conventional microscopic techniques. No cross-reactivity has been reported with a wide range of protozoa, helminth eggs, bacteria, yeasts, and yeast-like fungi tested with Merifluor®*C/G* (Garcia *et al.*, 1992; Garcia & Shimizu, 1997; Zimmerman & Needham, 1995; Fedorko *et al.*, 2000).

It was decided to perform genotyping on a selection of duck faecal samples that were strongly positive for *Giardia* and/or *Cryptosporidium* with the Merifluor® IFA staining procedure. This involved sucrose gradient purification of the (oo)cysts, nucleic acid extraction of the DNA and subsequent polymerase chain reaction (PCR) amplification of the nucleic acid in order to determine the size of the PCR fragments for establishing the genotype. These procedures were based on documented methods that have been used to differentiate human and animal isolates of *Giardia* (Ionas *et al.*, 1997; Hunt *et al.*, 1999a; Hunt, 1999b) and molecular genetic methods described in Massey University laboratory workshop manuals (Ionas, 1999; Bradshaw, 1999).

CHAPTER TWO: MATERIALS AND METHODS

2.1 COLLECTION OF SAMPLES

2.1.1 Site description

Hataitai Beach (Figure 6) is approximately 90 meters long and is enclosed in the southern end by the Evans Bay Yacht & Motor Club and at the northern end by the Evans Bay Sea Scouts Club. Public toilets and changing rooms are situated towards the southern end of the beach approximately 30 meters distant from a series of privately owned boat sheds. Hataitai Beach consists of highly permeable, medium to course sands and with no major river flowing into the beach area nor any direct sewage inputs from outfalls. As described in section 1.1.3, the dry weather stormwater flows at the northern and southern ends of the beach were diverted into the sewer system by the Wellington City Council during the drainage improvements carried out between 1996 and 1998. The upland area directly across Evans Bay Parade road consists of a small grassed park that borders a number of houses situated on elevated land along Rata, Rewa and Belvedere roads.

A total of three hundred and twenty-four marine water samples for indicator organism analyses were collected at sites designated B1, towards the southern end adjacent to the public toilets and changing rooms; B2, a point in line with the middle of the beach; B3, the northern corner of the beach. Another 27 samples were also simultaneously collected from the B1 site with 27 samples from the outlet of the Greta Point stormwater drain situated approximately 200 meters north of site B3. Twelve samples were also collected from site B4 and a total of 10 samples from sites that were situated close to the boat sheds SH1, SH2 and SH3.

Six large volume (150 to 500 litres) marine water samples were also collected at site B3 for the capture and detection of *Giardia* and *Cryptosporidium*.

Two hundred and seventy-nine duck faecal samples were collected from the beach sands, mostly at site B3, although a few samples were droppings that were deposited on the rocks in the vicinity of site B4.

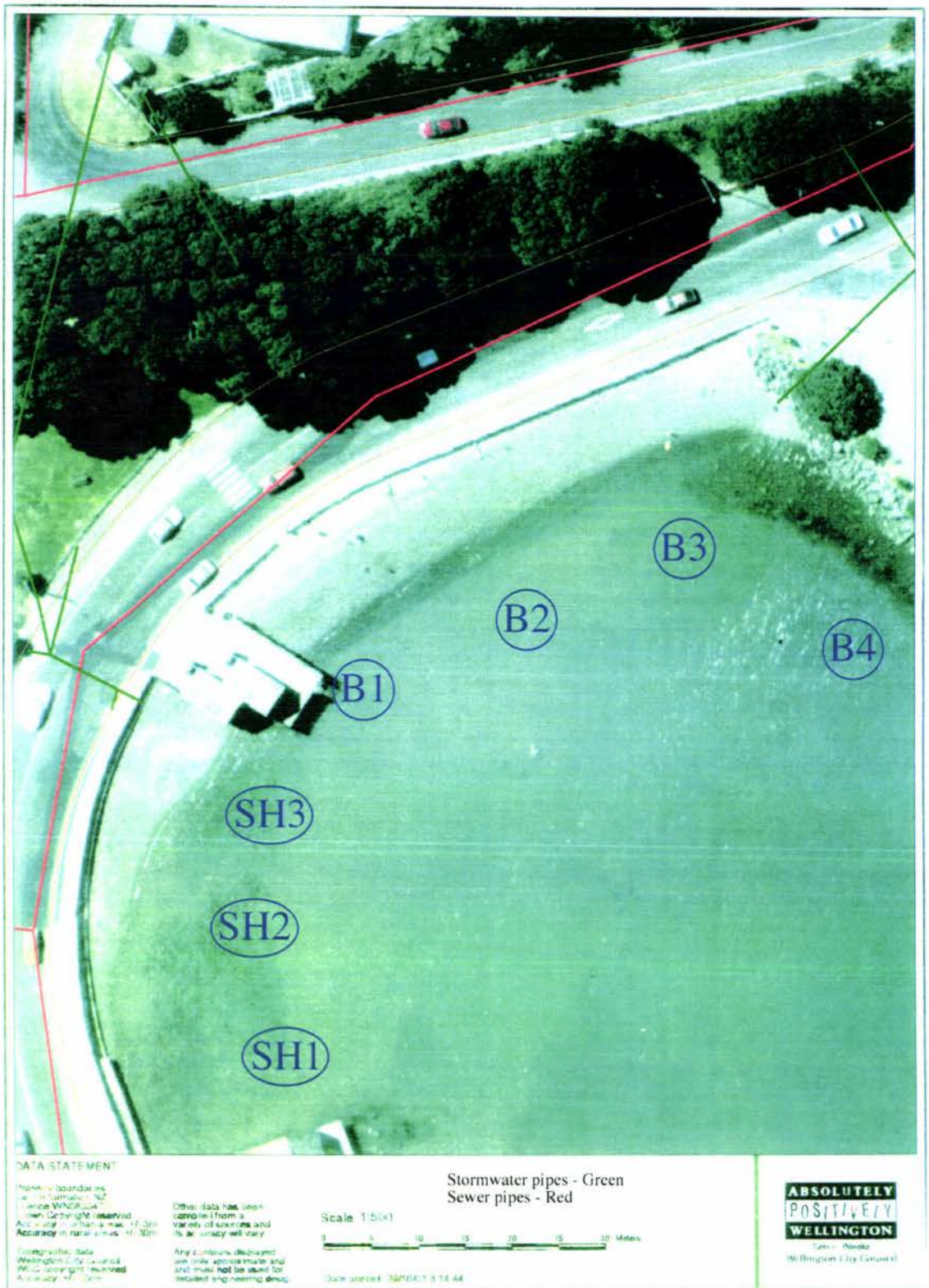

Figure 6: 1:500 aerial map of Hataitai Beach showing roads, stormwater and sewer pipes and sampling sites.

2.1.2 Rainfall data

Daily rainfall data (recorded at 0900 hours) for Wellington Airport, situated approximately 1 kilometre diagonally from Hataitai Beach, was obtained from MetService for the months December 1998 to August 1999. This MetService data included the highest daily rainfall for the month, the total rain days of 0.1 mm or more, and total wet days of 1.0 mm or more.

2.1.3 Marine water samples for indicator bacteria detection and enumeration

Materials

- Mighty Gripper Sampling device
- 500 ml Sterile screw capped glass bottles
- Large chilli bin
- Ice blocks
- Felt-tipped marker pen
- Adhesive bottle labels
- Data logging sheets
- Latex gloves

Method

The marine water samples were collected over a period of 15 months from 19/07/98 to 28/04/2000. Sample collection occurred mostly in dry weather conditions when indicator organism counts would not be influenced by rainfall or stormwater run-offs. Samples were taken at approximately adult knee depth (about 0.5 m) at 15 to 20 cm below the surface using the Mighty Gripper sampling device (Mighty Gripper Co., Wangarei, N.Z.), between 0800 and 1800 hours, and usually within 1 hour of high tide. After labelling the sterile 500 ml glass bottles (Schott Corporation, New York, USA) and putting on latex gloves, samples were collected aseptically and placed on ice in a large chilli-bin. The samples were then transported to the laboratory, usually within 30 minutes, and processed within 1 hour of arrival in the laboratory. At each sampling occasion counts were made of the number of ducks at Hataitai Beach. Ducks were more often than not seen loafing on the beach or swimming in the water in the vicinity of the B3 site (Figures 7 and 8). However, due to the duck movements across sites during sampling events, it was not practical to make the duck census site

specific. Also recorded at the time of sampling were the weather and sea conditions, as well as the presence of debris or seaweed, or any other unusual circumstances affecting the sampling site.

Figure 7: Ducks loafing on the beach adjacent to the B3 sampling site. (2/2/1999)

Figure 8: Ducks swimming in the vicinity of the B3 sampling site. (20/2/1999)

2.1.4 Marine water samples for the capture of *Giardia* and *Cryptosporidium*

Materials

- Submersible portable pump
- Water meter
- 12 volt rechargeable battery
- Garden hose connectors
- Cuno filter cartridge-Micro-Wynd //, DPPPY–1μm nominal porosity (Filtration Technology)
- Cuno AP-11 filter holder (Filtration Technology)
- Effluent hose
- Latex gloves
- Whirl-Pak plastic sampling bags (NASCO products)
- Polystyrene chilli-bin
- Ice packs
- Data logging sheets

Method

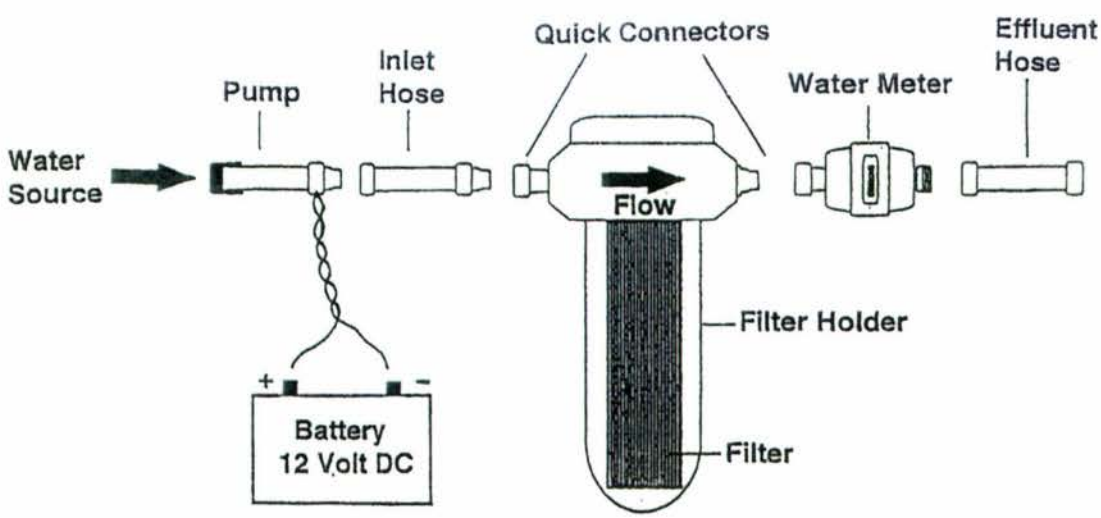

Figure 9: Assembly of water filtration apparatus for the capture of *Giardia* and *Cryptosporidium* in water samples.

The water filtration apparatus was assembled as shown in Figure 9. The pump was submerged so that the pump intake was less than 20 cm from the bottom of the water source. The pump leads were connected to the battery terminals and 50 litres of water

pumped without the filter in order to flush the apparatus. The filter holder was unscrewed, the water poured out and a filter cartridge (Filtration Technology, Auckland, N.Z.) inserted into the housing. The filter holder was re-tightened and the pump started. The meter reading was recorded at the beginning and at the end of the filtration. Following the filtration of the sample, the pump was disconnected from the battery and after putting on latex gloves the filter holder was opened. Each filter and the remaining water in the holder were placed into a Whirl-Pak plastic sampling bag (NASCO products, California). The bag was labelled and then inserted into another Whirl-Pak bag to ensure that the contents did not leak before placing the sample in a large polystyrene chilli-bin containing ice bricks. Six samples ranging from about 130 to 500 litres were filtered as described above and stored at 4°C for 24 hours prior to elution of particulates from the filters.

2.1.5 Duck faecal samples for the detection of *Giardia* and *Cryptosporidium*

Materials

- 50 ml Sterile conical centrifuge tubes (NUNC)
- Sterile wooden spatulas
- Small chilli bin
- Ice blocks
- Felt-tipped marker pen
- Latex gloves
- Small plastic biohazard waste bag (Biolab Scientific)

Method

Fresh (wet) duck droppings were usually collected opportunistically from the beach sands and occasionally as mentioned earlier, from deposits on the rocks. The droppings were carefully scooped into the sterile centrifuge tubes (NUNC, Myriad Industries, San Diego) with sterile spatulas. The used spatulas were then discarded into the biohazard waste bag (Biolab Scientific, Auckland) and the sample tubes placed on ice in a chilli bin and transported to the laboratory. The samples were refrigerated at 4°C for 24 hours prior to analysis.

2.2 ANALYSIS OF SAMPLES

2.2.1 Detection and enumeration of *Enterococci*

Materials

- Marine water samples
- *Enterococcus faecalis* (NZRM 1106 - blood agar culture)
- *Enterococcus faecium* (NZRM 1236 - blood agar culture)
- *Serratia marcescens* (NZRM 3505 - nutrient agar culture)
- 97-well Quanti-Trays (IDEXX Laboratories)
- Enterolert reagents (IDEXX Laboratories)
- 41°C Incubator
- 365 nm wavelength ultraviolet light with a 6 Watt bulb (De Saga)
- Bottles of sterile 90 ml distilled water
- Bottles of 5.0 ml sterile distilled water
- Bottles of 100 ml sterile distilled water
- IDEXX version 3.0 MPN software programme (IDEXX Laboratories)

Method

The Enterolert procedure was performed according to the manufacturer's instructions using aseptic techniques. Quanti-Trays and bottles of sterile 90 ml distilled water were labelled with the sample the time, date and sample ID numbers. A 1 in 10 dilution of the well-mixed water sample was made by adding 10 ml of the sample to 90 ml of sterile distilled water. One package of the powdered Enterolert reagent was added to the bottle and after shaking to dissolve the powder, the mixture was poured into a sterile 97-well Quanti-Tray. The tray was then mechanically sealed in a Quanti-Tray sealer, which simultaneously distributed the mixture into the wells and incubated for 24 hours at 41 ±0.5°C. After incubation the tray was viewed in a darkened room by placing it under and within 12 cm of a 365 nm wavelength ultraviolet light with a 6 Watt bulb (De Saga, Heidelberg, Germany). Blue fluorescence in a well was considered a positive reaction for that well and indicated the presence of *Enterococci* (Figure 10). The number of *Enterococci* per 100 ml was determined, based on the number of positive wells counted, by referring to a 97-well MPN table (IDEXX version 3.0 MPN software programme)

and the result multiplied by the dilution factor of 10. Wells showing no fluorescence were considered negative for *Enterococci*.

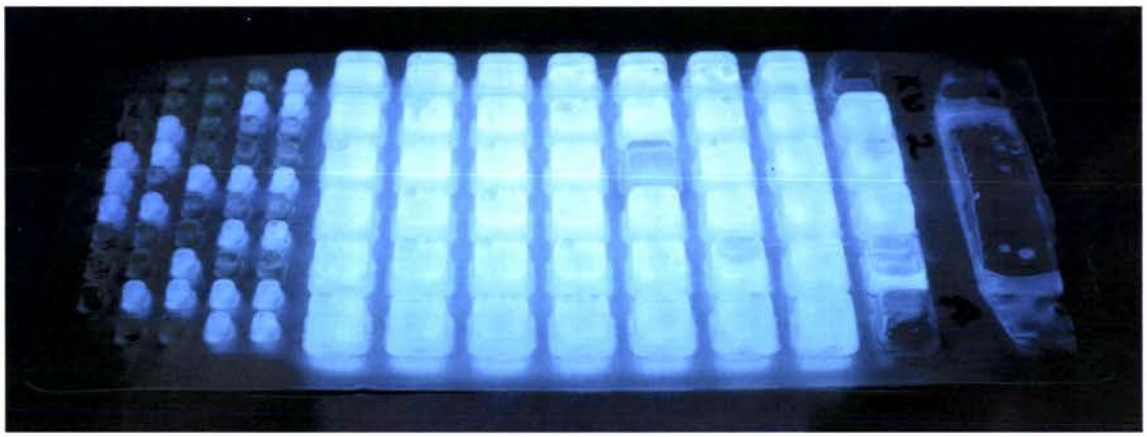

Figure 10: A 97-well Quanti-Tray filled with Enterolert reagent and water sample and viewed under a UV_{365} lamp. 45 large wells and 21 small wells exhibit fluorescence (*Enterococci* count = 1892 per 100 ml – B3 sample 13/6/1999).

Control cultures were put at regular intervals throughout the study. Blood agar cultures of *Enterococcus faecalis* and *Enterococcus faecium* were used as positive controls and a nutrient agar culture of *Serratia marcescens* as the negative control. A colony of each bacterial strain was touched with a sterile inoculating loop, transferred to 5.0 ml of sterile distilled water and thoroughly mixed. A 1.0 µl loop full of this mixture was then used to inoculate 100 ml of sterile distilled water. One package of the powdered Enterolert reagent was added to this 100 ml mixture and after shaking to dissolve the powder, the mixture was poured into a sterile 97-well Quanti-Tray. The rest of the procedure was carried out as described above for the test samples.

2.2.2 Detection and enumeration of *Total coliforms* and *Escherichia coli*

Materials

- Marine water samples
- *Escherichia coli* (NZRM 916 - nutrient agar culture)
- *Klebsiella pneumoniae* (NZRM 482 - nutrient agar culture)
- *Pseudomonas aeruginosa* (NZRM 981 - nutrient agar culture)
- 97-well Quanti-Trays (IDEXX Laboratories)

- Colilert-18 reagents (IDEXX Laboratories)
- 35°C Incubator
- 365 nm wavelength ultraviolet light with a 6 Watt bulb (De Saga)
- Bottles of sterile 90 ml distilled water
- Bottles of 5.0 ml sterile distilled water
- Bottles of 100 ml sterile distilled water
- IDEXX version 3.0 MPN software progrmme (IDEXX Laboratories)

Method

The Colilert-18 procedure was performed according to the manufacturer's instructions using aseptic techniques. Quanti-Trays and bottles of sterile 90 ml distilled were labelled with the time, date and sample ID numbers. A 1 in 10 dilution of the well-mixed water sample was made by adding 10 ml of the sample to 90 ml of sterile distilled water. One package of the powdered Colilert-18 reagent was added to the bottle and after shaking to dissolve the powder, the mixture was poured into a sterile 97-well Quanti-Tray. The tray was then mechanically sealed in a Quanti-Tray sealer, which simultaneously distributed the mixture into the wells, and incubated for 18 hours at 35 ± 0.5°C. After incubation the tray was viewed firstly for yellow wells. This was considered as a positive reaction for that well and indicated the presence of total coliforms. The tray was then viewed in a darkened room by placing it under and within 12 cm of a 365 nm wavelength ultraviolet light. Blue fluorescence in a well was considered a positive reaction for that well and indicated the presence of *Escherichia coli*. The number of total coliforms and *Escherichia coli* per 100 ml, based on the number of positive wells counted, was determined by referring to a 97-well MPN table (IDEXX version 3.0 MPN software programme) and the result multiplied by the dilution factor of 10. Wells showing no yellow colour were considered negative for total coliforms and wells showing no fluorescence were considered negative for *Escherichia coli*.

Control cultures were put up at regular intervals throughout the study. A nutrient agar culture of *Klebsiella pneumoniae* was used as a partial positive control (yellow wells but no fluorescence) and a nutrient agar culture of *Escherichia coli* as a complete positive control (yellow and blue fluorescent wells). A nutrient agar culture of

Pseudomonas aeruginosa was used as the negative control (no yellow wells and no fluorescence). A colony of each bacterial strain was touched with a sterile inoculating loop, transferred to 5.0 ml of sterile distilled water and thoroughly mixed. A 1.0 µl loop full of this mixture was then used to inoculate 100 ml of sterile distilled water. One package of the powdered Colilert-18 reagent was added to this 100 ml mixture and after shaking to dissolve the powder, the mixture was poured into a sterile 97-well Quanti-Tray. The rest of the procedure was carried out as described above for the test samples.

2.2.3 Elution and concentration of particulates from filters used for the capture of *Giardia* and *Cryptosporidium* in marine water samples

Materials

- Water samples (Filter cartridges in Whirl-Pak plastic bags)
- Scalpel with No. 22 blade.
- Graduated 3 liter plastic beaker
- 1X Phosphate Buffered Saline Eluting Solution (PBSES, pH7.4)
- Stainless Steel Tray
- Sorvall RC 3C centrifuge (6 X 1L capacity)
- Sorvall RT 7 centrifuge (20 X 50 ml capacity)
- 1 litre clear polycarbonate centrifuge bottles (Nalgene)
- 50 ml conical centrifuge tubes (NUNC)
- Vortex mixer
- Vacuum line

Method

One litre of phosphate buffered saline eluting solution (PBSES) was placed into a graduated 3 litre plastic beaker and the residual water from the Whirl-Pak sampling bag added. The filter was placed into a stainless steel tray and using a scalpel the wadding (polypropelene filter fibers) was cut lengthwise down to the core. The core was removed and rinsed with eluting solution into the beaker containing residual solution from the filter bag. The scalpel blade was then discarded carefully into a sharps container (Biolab Scientific, Auckland). The wadding was teased out and the string binding removed and discarded. A handful of wadding at a time was placed into the eluting solution in the beaker. The wadding was thoroughly washed by repeatedly

kneading and rinsing in the eluting solution for 5 minutes. The wadding was discarded and the eluted particle suspension made up to 2 litres by adding more eluting solution.

The eluted particle suspension was placed into two 1-litre polycarbonate centrifuge bottles and concentrated by centrifugation in a Sorvall RC 3C centrifuge at 5,000 x g (4150 rpm) for 12 minutes using a swinging out rotor with the brake on. Using a vacuum line 95% of the supernatant was carefully aspirated and discarded. The sediments were resuspended in the remaining supernatants and pooled into a 50 ml conical centrifuge tube. These particulates were then re-centrifuged in a Sorvall RT 7 centrifuge at 1,050 x g (2800 rpm) for 10 minutes with the brake on. At the end of this period the centrifuge tube was removed from the centrifuge and the supernatant carefully aspirated and discarded. The packed cell volume was recorded and if the volume was less than 0.5 ml, the packed pellet was resuspended by vortexing in a total volume of 1.0 ml of eluting solution. If the pellet volume was greater than 0.5 ml, the pellet was resuspended by vortexing in an equal volume of eluting solution. 1.0 ml of this suspension was used for sample purification by the immunomagnetic bead separation technique described below.

2.2.4 Separation of *Giardia* cysts and *Cryptosporidium* oocysts from filter concentrates by immunomagnetic bead separation

Materials

- Eluted water sample concentrates
- Dynabeads® GC-Combo (DYNAL)
- 10X SL™ buffer-A – clear, colourless solution (DYNAL)
- 10X SL™ buffer-B - magenta solution (DYNAL)
- Magnetic particle concentrators - MPC-1 (DYNAL)
- Sterile distilled water
- 1.5 ml Microcentrifuge tubes
- 0.1 M Hydrochloric acid
- 1.0 M Sodium hydroxide
- Vortex mixer
- Rotating mixer
- Adjustable micro-pipettes (10 µl to 100 µl)

- Sterile micro-pipette tips
- Coated microscope slides (Waterborne, Inc)

Method

Part 1. (Oo)cyst capture:
A 1X solution of SL buffer-A was prepared by diluting 100 µl of 10X SL buffer-A to 1.0 ml with sterile distilled water and put aside. 1.0 ml of the resuspended water sample concentrate was put into a micro-centrifuge tube and 100 µl of the 10X SL buffer-A, and 100 µl of the 10X SL buffer-B added to the sample. The Dynabeads® GC-Combo (DYNAL, A.S., Norway) vial was mixed by vortex to ensure that the beads were thoroughly resuspended and then 10 µl of these resuspended beads were added to the sample tube. The sample was fixed in the rotating mixer and rotated for one hour at approximately eight revolutions per minute at room temperature. The sample tube was then removed from the mixer and placed in the magnetic particle concentrator (MPC-1). The sample tube was gently rocked in the MPC-1 by hand, end-to-end, through approximately 90 degrees by tilting the cap-end and base-end of the tube up and down in turn. This tilting action was continued for two minutes with approximately one tilt per second. The MPC-1 was returned to the upright position, with cap of the tube at the top. The supernatant was poured off into a waste container without removing the tube from the MPC-1. The sample tube was then removed from the MPC-1 and the sample resuspended by adding 1.0 ml of 1X SL buffer-A. All the material in the tube was then resuspended by very gentle mixing. The sample tube was again placed in the MPC-1 and rocked for another two minutes as described above. The MPC-1 was returned to the upright position and the supernatant was then gently aspirated from the tube while still in the MPC-1 with a 10 µl micro-pipette. Care was taken not to disturb the material attached to the wall of the tube adjacent to the magnet.

Part 2. Dissociation of bead/(oo) cyst complex:
The sample tube was removed from the MPC-1. A 50 µl aliquot of 0.1M hydrochloric was added to the tube and mixed vigorously for 10 to 15 seconds. The tube was placed in a vertical position for 10 minutes and then mixed vigorously again for 10 to 15 seconds. The sample tube was placed back in the MPC-1 and gently rocked by hand, end to end, through 90 degrees for two minutes. The supernatant (containing (oo)cysts) was removed and placed into a fresh centrifuge tube. A 5 µl aliquot of 1.0 M sodium

hydroxide was added and gently mixed. Without removing the tube from the MPC-1, the entire sample was removed from the tube with a micro-pipette and transferred to the well on the slide (Waterborne, Inc, USA) containing the sodium hydroxide. The sample was then allowed to air-dry on the slide prior to immunofluorescent antibody staining.

2.2.5 Detection of *Giardia* cysts and *Cryptosporidium* oocysts by immunofluorescence antibody staining

Materials

- Air-dried slides of water sample concentrates (prepared in section 2.2.4)
- Duck faecal samples
- Sterile distilled water
- 1.6 ml Eppendorf tubes
- Adjustable micro-pipettes (10 µl to 50 µl)
- Sterile micro-pipette tips
- Merifluor® *Cryptosporidium/Giardia* staining kit (Meridian Diagnostics)
- Wooden applicator sticks
- 1X Phosphate buffered saline (pH 7.4)
- 37°C Incubator
- 1X Wash buffer (Meridian Diagnostics)
- 1% Evans blue solution
- Moist chamber
- Coplin jars
- Coated microscope slides (Waterborne, Inc)
- Fluoroprep mounting fluid (bioMerieux, sa)
- Cover slips (25 mm X 60 mm)
- Clear fingernail polish
- BX60 epifluorescent microscope (Olympus)

Method

Using a wooden applicator stick approximately 3 to 5 g (a pea size portion) of the faecal sample was added to 1 ml of sterile distilled water in an Eppendorf tube. The sample was emulsified and thoroughly mixed in the tube with the applicator stick and then left to sit for 1 minute at room temperature. This facilitated the settling of larger debris from

the suspension. A 50 µl aliquot of supernatant was removed and spread over one well of a coated microscope slide (Waterborne, Inc). Three samples per slide were prepared. A 10 µl aliquot each positive and negative control suspension (provided with the Merifluor® *C/G* staining kit) was spread over separate wells on another slide. The slides were left to air dry at 37°C for one hour. A 50 µl aliquot of Merifluor® *C/G* combined fluorescein isothiocyanate (FITC)-labelled monoclonal antibody preparation was diluted 10-fold in 1X phosphate buffered saline and added to the dried suspensions (including the air-dried water concentrates prepared in section 2.2.4). The slides were incubated in the dark for 30 minutes in a moist chamber. The FITC labelled monoclonal antibody was removed by rinsing the slides briefly in two Coplin jars filled with 1X wash buffer (2 drops of 1% Evans blue was added to the first Coplin jar as a counterstain). Excess wash buffer was drained off the slides by holding the bottom edge of the slides briefly against a piece of filter paper. A small drop of fluoroprep mounting fluid was added to each well and the slides then overlaid with a coverslips taking care to avoid the formation of any air bubbles. To eliminate coverslip movement over the slide, the coverslip edges were sealed by applying clear nail polish. The slide preparations were viewed in a darkened room using an epifluorescent microscope with an excitation wavelength of 450-490 nm. Each well was scanned thoroughly using 200X magnification and confirmations were made using 400X magnification. Using the control preparations (Waterborne, Inc) for comparison the presence of *Giardia* cysts was recorded if any apple-green fluorescent ovoid cysts (approximately 5 µm x 8 µm in size) were seen and the presence of *Cryptosporidium* oocysts if any apple-green fluorescent round oocysts (approximately 4 µm x 5 µm in size) were seen. These controls were prepared with each batch of samples tested.

2.2.6 Measurement of Merifluor®*C/G* IFA positive protozoan cysts.

Materials

- Phase contrast microscope
- Eye-piece graticule (1 cm divided into 100 divisions)
- Slide micrometer (1 mm divided into 0.01 mm divisions)
- Duck faecal samples
- Sterile distilled water
- Lugol's iodine

- Eppendorf tubes (1.6 ml)
- Micro-pipettes (10µl to 50µl)
- Sterile micro-pipette tips
- Wooden applicator sticks
- Microscope slides (76.2 x 25.4 mm)
- Cover slips (22 x 22 mm)

Method

Before calibrating the 40X objective, the light intensity of phase contrast microscope was adjusted for optimal brightness by rotating the annulus under the condenser into position. The condenser was then focused so that the image of the annulus was superimposed on the phase plate at the back of the objective. The annulus and phase ring were then adjusted so that they coincided by using the centring screws under the condenser. The right hand ocular lens was removed form the microscope and the eyepiece graticule placed on the focal plane diaphragm (between the eye lens and field lens) within the ocular lens. The ocular lens was placed back in the microscope and the graticule focused using the eyepiece-focusing ring. The slide micrometer was placed on the stage and the scale located and focused by using the 10X objective lens. The 40X objective was swung into the light path and the slide micrometer scale focused again. The position of the stage was adjusted so that the "0" lines of the eyepiece graticule scale and the slide micrometer scale were superimposed and clearly focused. Without moving the stage other superimposed lines were then looked for as far along the scale as was possible. The number of ocular divisions and the distance in millimetres (mm)on the slide between the superimposed sets of lines was recorded. The distance covered by the ocular graticule divisions was calculated as follows:

50 eyepiece divisions = 10 stage divisions
= 0.1 mm
Therefore 1 eyepiece division = 0.002 mm
= 2.0 µm

The stage micrometer slide was then removed and wet mounts of duck faecal samples were prepared for cyst measurements using the calibrated 40X objective. These wet mounts were made from six faecal samples that had more than 10 *Giardia* cysts per field at 400X magnification in the Merifluor® *C/G* IFA preparations (section 2.2.5).

The faecal suspensions were prepared in sterile distilled water in Eppendorf tubes as described in earlier section 2.2.5. A 20 µl aliquot of faecal suspension was placed in the centre of a clean slide. 20 µl of Lugols's iodine was added to the suspension and mixed with the pipette tip. A coverslip was carefully placed over the suspension so as to avoid the formation of air bubbles. The slide preparation was positioned on the stage of the phase contrast microscope. The preparation was then brought into focus and prior to their measurement, selected cysts in the suspension were positioned in the centre of the field of view in order to minimise any objective distortion. Cysts were measured across their minimum and maximum diameters by counting the number of eyepiece divisions. The number of divisions counted were multiplied by 2 and the cyst dimensions expressed in micrometers (µm). In this manner a total of 36 cysts were measured (6 cysts per slide) in faecal suspensions of 6 different samples.

2.2.7 Sucrose gradient recovery of *Giardia* cysts from duck faecal samples

Materials

- Positive *Giardia* faecal samples
- 0.01% Polyoxyethylene sorbitan monolaurate (Tween 20)
- 1.0 M Sucrose
- TE Buffer (TRIS-EDTA)
- 0.01% Tween 20
- 50 ml conical centrifuge tubes (NUNC)
- Sorvall RT 7 centrifuge (20 X 50 ml capacity)
- Lugol's iodine
- Micro-pipettes (10 µl to 50 µl)
- Sterile micro-pipette tips
- Wooden applicator sticks
- Vortex mixer
- Microscope slides (76.2 x 25.4 mm)

- Cover slips (22 x 22 mm)
- Phase contrast microscope (Olympus)

Method

Sucrose floatation purification was used to separate *Giardia* cysts from other debris in the faecal samples since the debris have a greater specific gravity than the cysts. Duck faecal samples that contained high numbers (15 to 30 per field of view at 400X magnification) of *Giardia* cysts in the Merifluor® *C/G* combined immunofluorescent antibody preparations (section 2.2.5) were concentrated using this sucrose gradient procedure in order to provide sufficient DNA for nucleic acid extraction (section 2.2.8) and subsequent PCR amplification and genotyping of the cysts (section 2.2.9).

Using a wooden applicator stick 5 to 10 gram of faecal sample was placed into a 50 ml conical centrifuge tube and the volume made up to 45 ml with 0.01% Tween 20. The sample was thoroughly emulsified by vortexing and the centrifuged at 1,050 x g (2800 rpm) for 10 minutes. The supernatant was discarded and the pellet washed by adding 45 ml of 0.01% Tween 20 and centrifuged three times. The pellet was resuspended in 35 ml of 0.01% Tween 20 and underlaid with 10 ml of 1.0M Sucrose taking care to avoid mixing the two solutions. The centrifuge tube was centrifuged at 1,050 x g (2800 rpm) for 10 minutes with the brake off. Fifteen millilitres of the interface solution was collected and transferred to a fresh 50 ml conical centrifuge tube. The volume was increased to 50 ml with TE buffer and the tube centrifuged at 1,050 x g (2800 rpm) for 5 minutes. The supernatant was discarded and the deposit resuspended by increasing the volume to 50 ml with TE buffer. This step was repeated once more to wash out the sucrose. The final pellet was then resupended in 0.5 ml of TE buffer.

A 20 µl aliquot of well-mixed suspension (prepared above) was placed in the center of a clean slide. Twenty microlitres of Lugols's iodine was added to the suspension and mixed with the pipette tip. A coverslip was carefully placed over suspension to avoid the formation of air bubbles. The concentration of *Giardia* cysts was then determined by viewing wet mount preparation with the phase contrast microscope at 400X magnification.

2.2.8 Nucleic acid extraction from *Giardia* cysts

Materials

- *Giardia* cysts (purified in section 2.2.7)
- 10% Chelex 100™ (Biorad)
- Phenol pH 8.0 (Tis-buffered) (APS Ajax Finechem)
- Chloroform/isoamyl alcohol (24:1)
- 3.0M Sodium acetate pH 5.5
- Absolute ethanol
- 70% Ethanol
- Sterile Milli-Q water
- Sterile 1.6 ml Eppendorf tubes
- Vortex mixer
- Liquid nitrogen
- Boiling water bath

Method

A 0.5 ml aliquot of sucrose gradient purified *Giardia* cysts (section 2.2.7) was mixed thoroughly by vortexing for 20 to 30 seconds. A 100 µl aliquot of this suspension was transferred to a 1.6 ml Eppendorf tube and centrifuged for two minutes at 1,050 x g (2800 rpm). The supernatant was discarded and the pellet resuspended in 100 µl of 10% Chelex 100, taking care to ensure the Chelex was thoroughly mixed immediately prior to use. A small hole was made in the lid of the Eppendorf tube with a needle to prevent the tube lid from opening under pressure during the nucleic acid extraction.

The Eppendorf tube was frozen in liquid nitrogen (-196°C) for 2 minutes and then immediately placed into a boiling waterbath for another 2 minutes. This freeze-thaw cycle was repeated another 4 times. During the freeze-thaw cycles the tube was occasionally agitated to disperse the Chelex granules which tended to sediment rapidly. The tube was then centrifuged at 1,050 x g (2800 rpm) for 5 minutes to pellet the cyst debris and Chelex. The nucleic acid contained in the supernatant was transferred with a micropipette to a fresh sterile 1.6 ml Eppendorf tube ensuring that no Chelex granules were also transferred since Chelex inhibits DNA amplification. The extracted nucleic acid was left to stand at 4°C for one hour before further use.

An equal volume of Tris-buffered phenol was added to the extracted nucleic acid and the solution gently mixed for 2 minutes. The tube was centrifuged at 1,050 x g (2800 rpm) for 5 minutes. Following centrifugation the nucleidc acid in the upper aqueous phase was transferred to a fresh sterile Eppendorf tube taking care to leave the interface and phenol behind. An equal volume of chloroform/isoamyl alcohol (24:1) was added to the aqueous solution. Following 2 minutes of gentle mixing the Eppendorf tube was centrifuged at 1,050 x g (2800 rpm) for 5 minutes. The aqueous phase was then transferred to a fresh sterile Eppendorf tube. The solution containing the nucleic acid was concentrated by ethanol precipitation prior to further use.

The nucleic acid was concentrated using a one-tenth volume of 3M Sodium acetate followed by the addition of 2.5 volumes of cold absolute ethanol to the extracted nucleic acid solution. After gentle mixing the solution was left overnight at –20°C. Collection of the nucleic acid involved centrifugation at 1,050 x g (2800 rpm) at 4°C for 30 minutes. The supernatant was discarded, 500 µl of 70% ethanol was added to the Eppendorf tube and gently mixed to wash the pellet containing the nucleic acid. The tube was then centrifuged at 1,050 x g (2800 rpm) at 4°C for 20 minutes to pellet the nucleic acid. The supernatant was discarded and the nucleic acid was left to air dry after which it was resuspended in 10 µl of sterile Milli-Q water. The extracted nucleic acid was stored at -20°C until required.

2.2.9 Polymerase Chain Reaction (PCR) amplification of *Giardia* nucleic acid

Materials

- Nucleic acid extracted from *Giardia* cysts

PCR Primers:

- *Giardia* genus specific primer left (GspL)
- *Giardia* genus specific primer right (GspR)
- *Giardia intestinalis* specific primer left (GiL)
- *Giardia intestinalis* specific primer right (GiR)
- *Giardia muris* specific primer left (GmL)
- *Giardia muris* specific primer right (GmR)
- *Giardia intestinalis* rDNA primer (Cyn 1)
- *Giardia intestinalis* rDNA primer (Cyn 2)

PCR Reagents:

- 10X PCR buffer pH 8.7 at 20°C (QIAGEN™)
- 5X concentrated Q-solution (QIAGEN™)
- $MgCl_2$ solution (25mM, QIAGEN™)
- *Taq* DNA polymerase (5 units/µl QIAGEN™)
- Ultra pure dNTPs (2mM each of dATP, dCTP, dGTP, dTTP, Pharmacia Biotech)
- Sterile Milli-Q H_20
- Sterile plugged micro-pipette tips
- Micro-pipettes
- Sterile 0.2 ml dome cap PCR reaction tubes (Scientific Specialities, Inc).
- Analytical grade Agarose (Biorad)
- 10X E Buffer
- 1Kb Plus DNA or 1Kb DNA molecular marker ladder (Gibco BRL)
- Ethidium bromide (5µg/ml, BDH)
- 10X SDS Loading Dye
- Gel casting tray
- Gel electrophoresis chamber (BRL Horizon)
- Gel documentation apparatus

Method

The Polymerase Chain Reaction (PCR) procedure was performed with nucleic acid extracted from *Giardia* cysts (section 2.2.8). All PCR reagents and tubes were placed on ice and in order to minimise contamination the entire PCR cocktail dispensing procedure was carried in a Biohazard Safety Cabinet using gloves throughout. For each reaction a cocktail was prepared which contained 2.0 µl of 10X PCR buffer, 8.0 µl of 5X concentrated Q-solution, 1.0 µl of dNTPS, 1.0 µl of each primer (one primer set per cocktail), 1.0 µl of $MgCl_2$ solution, 0.2 µl of *Taq* DNA polymerase, and 3.8 µl of sterile Milli-Q water. The PCR cocktail was dispensed in as an 18.0 µl aliquot in a sterile 0.2 ml dome cap PCR reaction tube. A 2.0 µl aliquot of template DNA was added to the

PCR reaction tube after the cocktail mix had been dispensed to give a total volume of 20.0 µl per reaction. In addition, instead of nucleic acid, a negative control containing 2.0 µl of sterile Milli-Q water and a positive control of 2.0 µl DNA extracted from a cultured laboratory trophozoite strain of *Giardia* was amplified with each reaction set. Further details concerning the PCR primer sequences and their expected product sizes appear at the end of this section.

The PCR protocol described below was used for all five primer combinations as every primer had a calculated melting temperature (Tm) of Tm = 4(G+C) + 2(A+T). High stringency conditions (Tm – 2°C) were applied to all reactions by use of an annealing temperature of 60°C.

All PCR amplifications were performed using a DNA thermalcycler (9600 Perkin Elmer, Cetus Corp., Norwalk, USA) using version 2.01 software. The first cycle began with 2 minutes at 98°C to ensure complete denaturation of the template DNA. The second temperature step involved 1 minute at 60°C for specific primer annealing. Generally the annealing temperatures used for all PCR amplifications was two degrees below the Tm of the primers as calculated by the GC method. To complete the cycle an extension period of one minute at 72°C was used. After the initial temperature cycle forty repeats of the following were performed: 98°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds. To complete the PCR amplification a final extension period of 72°C for 5 minutes was used. At the completion of the programme the reaction was held at 4°C until removed from the thermalcycler. Maximum temperature ramp rates were used for all PCR amplification steps.

Each amplified PCR product was detected by horizontal slab gel electrophoresis using a 1.6% agarose/1X E buffer gel. The gel was prepared adding 0.8 g of agarose powder to 50 ml of 1X E buffer in a sterile 100 ml screw-capped bottle. The agarose was dissolved by placing the bottle (loosely capped) in a microwave oven for 6 minutes. The melted agarose was cooled to about 55 to 60°C and then poured into a casting tray containing a 14 tooth-slot comb. Enough agarose was poured into the gel tray to cover the lower third (about 6.0 mm) of the comb. After the gel had set it was placed in an electrophoresis chamber (BRL Horizon 58) with the wells nearest the negative (black lead) electrode of the chamber. The electrophoresis chamber was filled with sufficient

1X E buffer to completely cover the gel and the electrodes. The comb was then carefully removed from the gel.

Using a fresh sterile micro-pipette each time a 4.0 µl aliquot of the each PCR product was mixed with 1.0 µl of 10X SDS loading dye and loaded into the appropriate well in the gel. A 4.0 µl aliquot of the 1Kb DNA or the 1Kb Plus DNA molecular marker ladder (Appendix F) was also loaded in order to determine the size of the PCR fragment. The amplified PCR products and molecular marker ladders were electrophoresed at 3-5V/cm for 2 to 3 hours.

Following electrophoresis the gel was removed carefully from the electrophoresis chamber and placed into a staining bath. The gel was stained with 0.5µg/ml ethidium bromide in 1X E buffer for twenty minutes. Next the gel was carefully removed from the staining bath and placed in 1X E buffer for 10 minutes to remove excess stain from the gel. The gel was then placed into a gel documentation apparatus and visualised with a UV transilluminator in a darkened room.

The sequences of the PCR primers used in this thesis and their expected product sizes are detailed below.

***Giardia* genus specific PCR primer sequences:**

GspL: $^{5'}$CAT AAC GAC GCC ATC GCG GCT CTC AGG AA$^{3'}$

GspR: $^{5'}$TTC GTG AGC GCT TCT GTC GTG GCA GCG CTA$^{3'}$

Expected product size = 171bp

***Giardia intestinalis* specific PCR primer sequences:**

GiL: $^{5'}$AAG TGC GTC AAC GAG CAG CT$^{3'}$

GiR: $^{5'}$TTA GTC CTT TGT GAC CAT CGA$^{3'}$

Expected product size = 218bp

***Giardia muris* specific PCR primer sequences:**

GmL: $^{5'}$GAG GAA TCA TCA GAA CCT CGC $^{3'}$

GmR: $^{5'}$CAT AAA TCA GTC CAG TGT TTC TC$^{3'}$

Expected product size = 306bp

***Giardia intestinalis* rDNA PCR primer sequences:**

Cyn 1: $^{5'}$CAG GAA TGT CTT GTA GGC GC $^{3'}$

Cyn 2: $^{5'}$CCC GGT TGG TTT CTC CTC C$^{3'}$

Expected product size = 506bp

CHAPTER THREE: RESULTS

3.1 RAINFALL DATA

No correlation was found between rainfall in the preceding 24, 48 or 72 hours of sampling and the *Enterococci* counts for period in which MetService climatological data was obtained namely, December 1998 to August 1999 (Table 1).

Table 1: Comparison of *Enterococci* counts following rainfall episodes (December 1998 to August 1999).

Rainfall (mm)	**24 hours (*Enterococci* per 100 ml)**	**48 hours (*Enterococci* per 100 ml)**	**72 hours (*Enterococci* per 100 ml)**
0	0-1334	0-1334	0-1334
10-20	0-3448	0-3448	0-3448
20-55	20-216	10-1182	0-1182

No relationship was found between rainfall and *Enterococci* counts over the 1998/1999 bathing season and neither was any relationship found between rainfall and the *Enterococci* counts in water samples from individual sites. Correlation values (r) of between 0.08 and 0.17 were found in each of the above cases. In a preliminary study to determine if stormwater run off had any significant influence on indicator organism counts, 27 water samples were collected from Hataitai Beach site B1 and another 27 samples simultaneously from the outlet of the Greta Point stormwater drain situated approximately 200 metres north of site B3. No statistical association was found between stormwater contaminations and indicator organism counts at the B1 site. However, the variable pattern of the two may have been due to the effects of currents and tides and a lag time between water leaving the stormwater outlet and reaching the beach site. The overall data for these 54 samples are detailed in Appendix B. The majority of the other 346 samples collected throughout remainder of study period (December 1998 to April 2000) were collected on rainless days and could therefore be classified as dry weather samples.

3.2 ENTEROCOCCI COUNTS

Table 2: Hataitai Beach Median *Enterococci* values - 10/12/1998 to 28/04/2000 (*Enterococci* per 100 ml - median values represented in large bold font)

Time period	All Sites	B1 Site	B2 Site	B3 Site
10/12/98 to 28/04/00 (Overall)	**31** (346 samples)	**20** (110 samples)	**20** (101 samples)	**63** (101 samples)
10/12/98 to 25/03/99 (Bathing Season)	**41** (151 samples)	**20** (45 samples)	**57** (36 samples)	**164** (36 samples)
2/02/99 to 27/02/99 (February)	**31** (90 samples)	**20** (22 samples)	**36** (22 samples)	**112** (22 samples)
4/03/99 to 25/03/99 (March)	**158** (33 samples)	**97** (11 samples)	**203** (11 samples)	**301** (11 samples)
7/11/99 to 11/03/00 (Bathing Season)	**20** (36 samples)	**20** (12 samples)	**15** (12 samples)	**63** (12 samples)
3/08/99 to 28/04/00 (October to April)	**20** (89 samples)	**20** (29 samples)	**10** (29 samples)	**31** (29 samples)

Table 3: Number of exceedences of the New Zealand Water Quality Guidelines at Hataitai Beach 10/12/1998 to 28/04/2000.

Sites	**Alert Amber Mode 2** (Single sample greater than 136 *Enterococci* per 100 ml)	**Action Red Mode** (Single sample greater than 277 *Enterococci* per 100 ml)
All (346 samples)	**23**	**41**
B1 (110 samples)	**5**	**8**
B2 (101 samples)	**7**	**10**
B3 (101 samples)	**11**	**21**

3.2.1 All sites

The composite data for the 346 marine water samples collected from December 1998 to April 2000 are shown in Appendix A. Although samples were also collected initially from the SH (boat sheds) sites and B4 sites, the majority of the samples were collected from sites B1, B2, and B3. As mentioned in section 2.1.3 although ducks were more often than not seen loafing on the beach or swimming in the water in the vicinity of the B3 site, due to the duck movements across sites during sampling events, it was not practical to make the duck census site specific. Therefore at each sampling occasion the numbers of ducks counted were recorded as the count for all 3 sites.

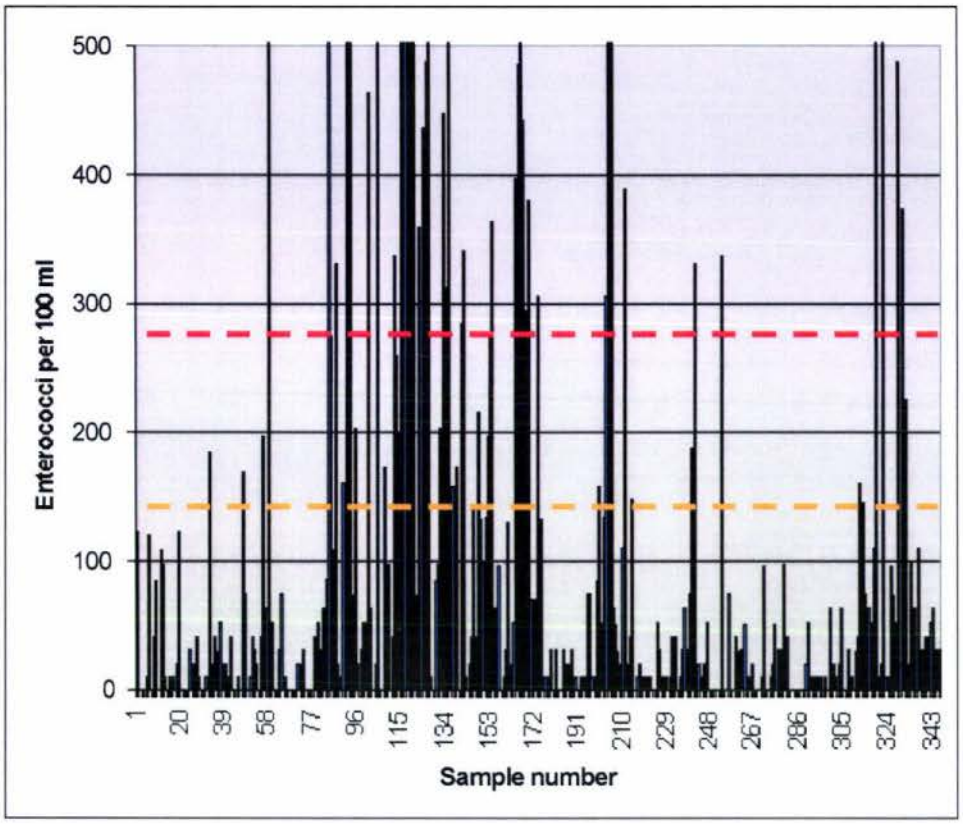

Figure 11: *Enterococci* counts of all sites for December 1998 to April 2000 (Counts in excess of 500 not shown; >136 Amber/Alert Mode; >277 Action/RedMode).

For the 346 samples collected from December 1998 to April 2000 a median of 31 *Enterococci* per 100 ml was obtained but for 151 samples collected during the bathing season (10/12/98 to 25/3/99) the median was 41 *Enterococci* per 100 ml. (A median of less than 35 *Enterococci* per 100 ml was considered safe for bathing - 1998/1999 New Zealand recreational water quality guidelines). For the period 4/03/99 to 25/03/99 (33 samples) the median was 158 *Enterococci* per 100 ml. For the bathing season (07/11/99 to 11/03/99) the median for 36 samples was 20 *Enterococci* per 100 ml. The median values for all samples from all the sites over the 6 time periods are shown in Table 2.

During the study the 346 samples from all sites yielded results in the Alert/Amber Mode II (single greater than 136 *Enterococci* per 100 ml) on 23 occasions and in the Action/Red Mode (single sample greater than 277 *Enterococci* per 100 ml) on 41 occasions. The category and number of exceedences for all samples are shown in Table 3 and illustrated in Figure 11.

3.2.2 B1 sites

An overall median of 31 *Enterococci* per 100 ml was obtained for the 110 samples collected from the B1 site from December 1998 to April 2000. A value of 20 *Enterococci* per 100 ml value was obtained for samples collected during the time periods except for the 11 samples collected in the period 4/03/99 to 25/03/99 when a median of 97 *Enterococci* per 100 ml was obtained (Table 2). Samples from the B1 sites yielded results in the Alert/Amber Mode II on 5 occasions and in the Action/Red Mode on 8 occasions (Table 3 and Figure 12).

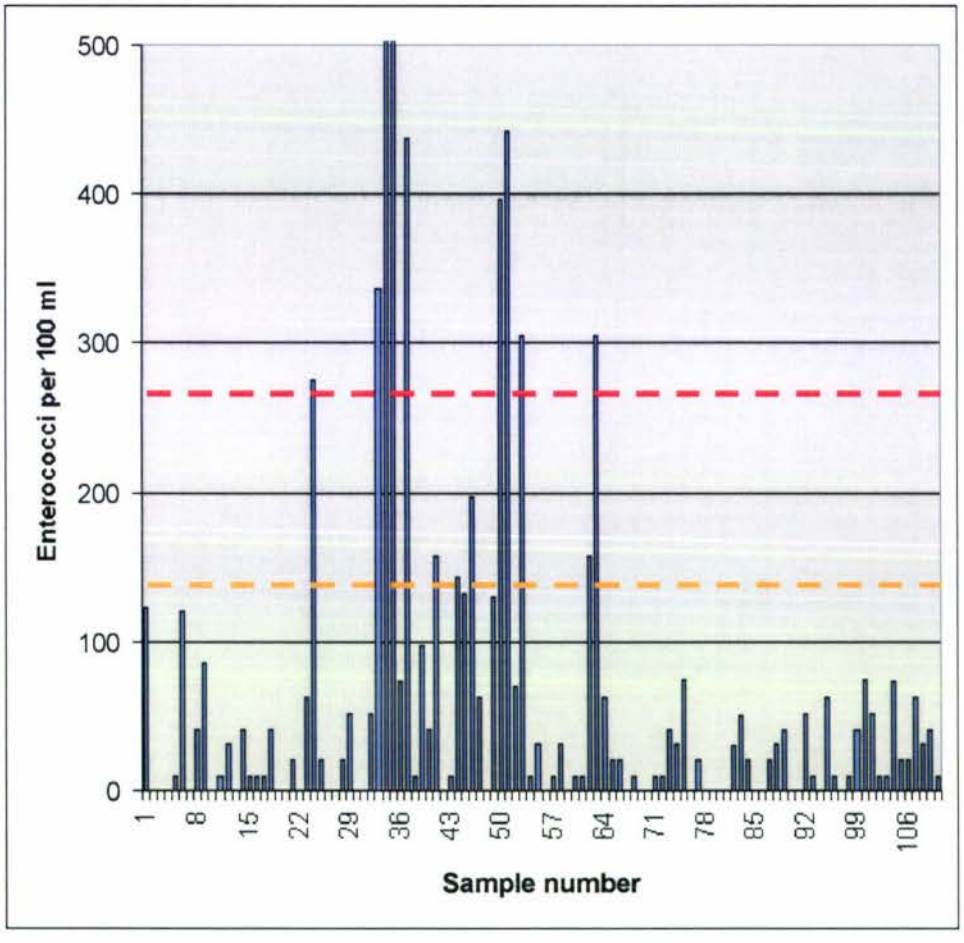

Figure 12: *Enterococci* counts of the B1 site for December 1998 to April 2000 (Counts in excess of 500 not shown; > 136 Amber/Alert Mode; >277 Action/Red Mode).

3.2.3 B2 sites

An overall median of 20 *Enterococci* per 100 ml was obtained for the samples collected from the B2 site from December 1998 to April 2000. As shown in Table 2 the medians for all but one of the time periods were above 35 *Enterococci* per 100 ml with March 1999 median as high as 203 *Enterococci* per 100 ml. Samples from the B2 sites yielded results in the Alert/Amber Mode II on 7 occasions and in the Action/Red Mode on 10 occasions (Table 3 and Figure 13).

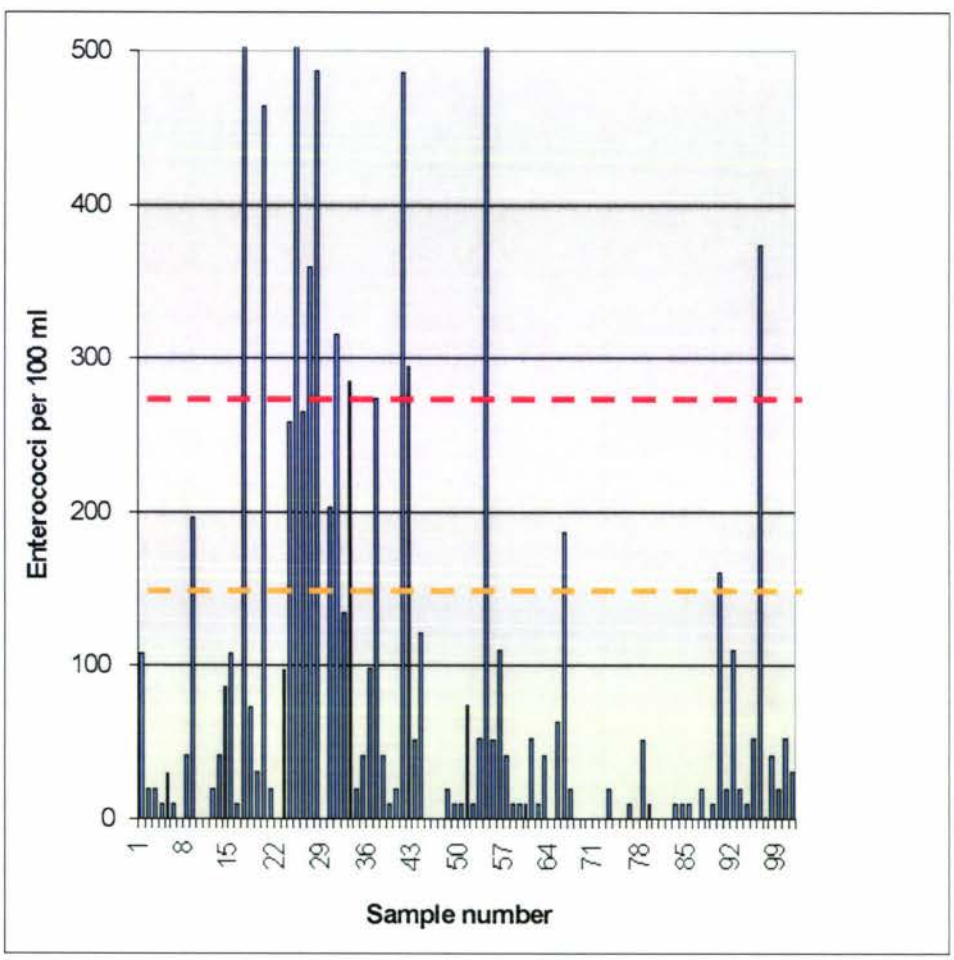

Figure 13: *Enterococci* counts of the B2 site for December 1998 to April 2000 (Counts in excess of 500 not shown; > 136 Amber/Alert Mode; >277 Action/Red Mode).

3.2.4 B3 sites

An elevated median of 63 *Enterococci* per 100ml was obtained for the samples collected from the B3 site from December 1998 to April 2000. As shown in Table 2 the medians for all but one of the time periods were markedly above 35 *Enterococci* per 100 ml. The 1998/1999 bathing season median was 164 *Enterococci* per 100 ml and a median of 301 was obtained for the March 1999 time period. B3 site samples yielded results in the Alert/Amber Mode II on 11 occasions and in the Action/Red Mode on 21 occasions (Table 3 and Figure 14).

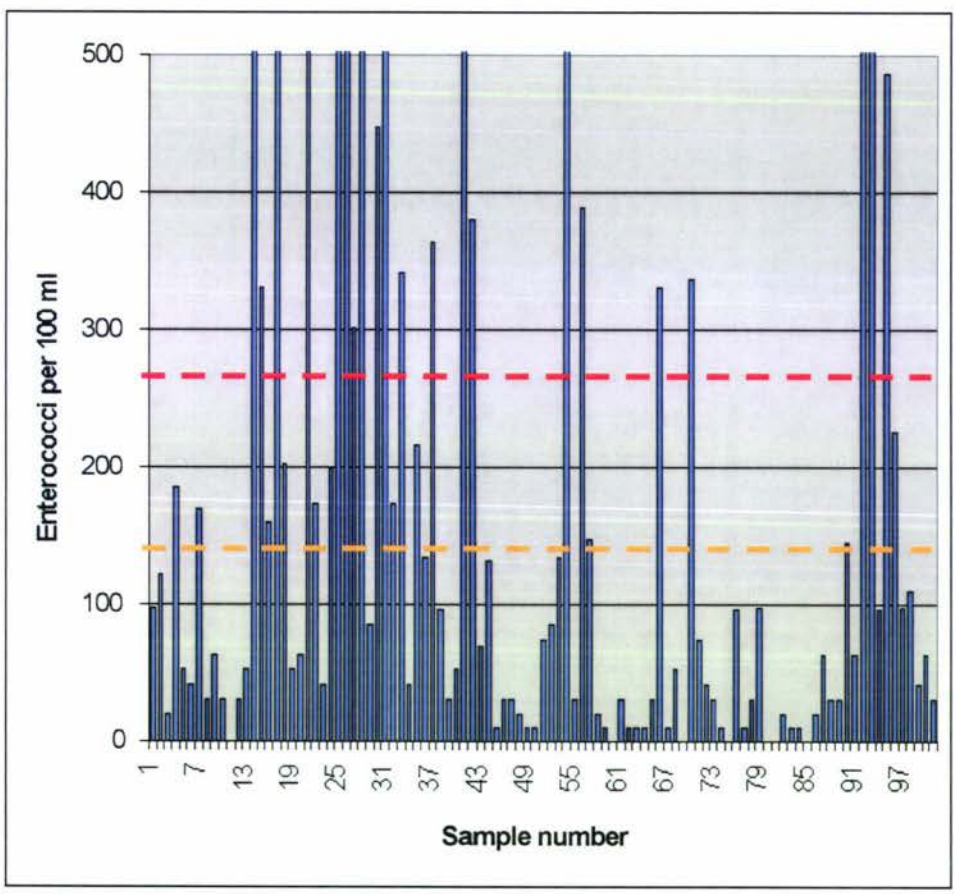

Figure 14: *Enterococci* counts of the B3 site for December 1998 to August 2000 (Counts in excess of 500 not shown; > 136 Amber/Alert Mode; >277 Action/Red Mode).

3.3 TOTAL COLIFORMS AND *ESCHERICHIA COLI* COUNTS

3.3.1 Overall results for all sites

To identify the sources of faecal pollution at Hataitai Beach it was decided, as mentioned in section 1.4, to also detect and enumerate total coliforms and *Escherichia coli* in the marine water samples despite the fact that these organisms are no longer used in New Zealand for marine recreational water quality monitoring.

While high counts for both total coliforms and *Escherichia coli* were obtained throughout the study on a number of occasions in samples from all sites, there is no guideline value against which these counts could be compared with. However, as with the *Enterococci* counts, markedly increased total coliform and *Escherichia coli* median values were obtained in samples collected from the B3 site. These median values for samples from each site were as follows:

Total coliforms	**B1** = 98 per 100 ml	**B2** =134 per 100 ml	**B3** = 341 per 100 ml
Escherichia coli	**B1** =20 per 100 ml	**B2** = 20 per 100 ml	**B3** = 52 per 100 ml

3.3.2 Correlations between total coliforms, *E.coli* and *Enterococci*

No significant statistical association was found between total coliforms, *E.coli* and *Enterococci* counts at any level or any site. Only very weak correlations between *E.coli* and *Enterococci* were found at counts of less than 100 per 100 ml ($r = 0.40$) and counts between 500 and 600 per ml ($r = 0.46$).

3.3.3 Correlations between duck counts and indicator organism counts

No correlation was found between the number of ducks and either the total coliform, *E.coli*, or *Enterococci* counts. The duck numbers on the beach or in the water at the time of sampling during this study ranged from 0 to 96. While several very high counts of the indicator organisms were obtained from water samples that were collected when large numbers of ducks were present, high counts were also obtained from samples when few or no ducks were present at the sampling sites. However, as mentioned earlier,

strikingly higher counts of all three indicator organisms were obtained in samples from the B3 site where the ducks were inclined to congregate.

3.3.4 Ratios of *Escherichia coli* to *Enteroccocci*

In order to determine the sources (animal or human) of the indicator organisms the ratios of *E.coli* to *Enterococci* were calculated and plotted as shown in Figure 15. The ratios for samples from all sites varied between 0.08 and 19.5. Ratios for samples from the B3 site varied between 0.08 and 4.1 with a median of 0.8. Because ratios were above and below 0.7 (<0.7 indicates animal faecal pollution) a specific faecal pollution source could not be categorically established.

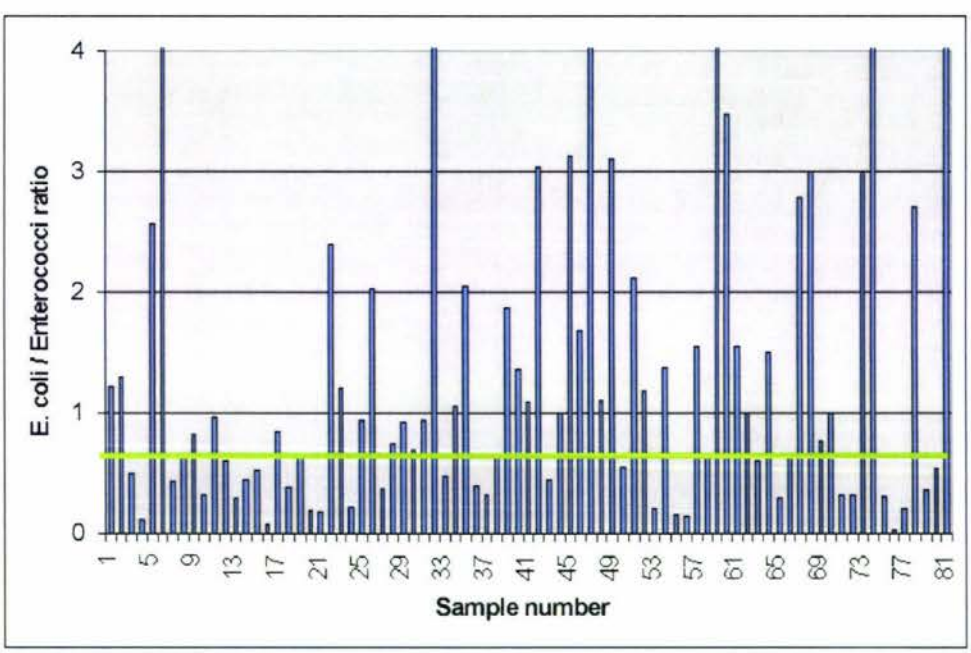

Figure 15: Ratios of *E.coli* to *Enterococci* for 81 sample results from the B3 site (Ratios above 4.0 not shown; green horizontal line represents the 0.7 ratio mark).

3.4 *GIARDIA* AND *CRYPTOSPORIDIUM*

3.4.1 *Giardia* and *Cryptosporidium* in marine water samples.

A total of 6 large volume marine water samples were filtered for the capture of *Giardia* cysts and *Cryptosporidium* oocysts. As described in sections 2.2.3 and 2.2.4. the particulates from the filters were eluted and then purified by immunomagetic bead separation (IMS). The resulting air-dried slide concentrates were stained with Merifluor® *C/G* combined immunofluorescent antibody (IFA) and examined as described in section 2.2.5. The number of (oo)cysts observed in an entire slide well was recorded and the (oo)cyst count per 100 liters calculated as follows:

$$\frac{X}{100\ L} = \frac{TG \text{ or } TC\ (100)}{FVR}$$

For samples in which no (oo)cysts were detected, the detection limit was calculated as follows:

$$\frac{<X}{100\ L} = \frac{(<1)\ (100)}{FVR}$$

The following values were used for the above calculations:

V = Volume (liters) of original water sample filtered.

P = Eluate packed pellet volume.

F = Fraction of eluate packed pellet volume (P) subjected to immunomagnetic bead separation (IMS). Determined by the equation:

$$F = \frac{ml\ P \text{ subjected to IMS}}{P}$$

R = Percentage (expressed as a decimal) of IMS sediment examined.

TG = Total *Giardia* cyst count.

TC = Total *Cryptosporidium* oocyst count

No *Giardia* cysts or *Cryptosporidium* (oo)cysts were detected in any of the 6 slide preparations. The data of the 6 marine water samples were as follows:

Sample M1

V = 132.4 litres, P = 6.0 ml, F = 0.125, R = 0.5

Result = <0.12 (oo)cysts per 100 liters of water.

Sample M2

V = 102.7 litres, P = 2.0 ml, F = 0.45, R = 1.0

Result = <0.02 (oo)cysts per 100 liters of water.

Sample M3

V = 243.3 litres, P = 5.0 ml, F = 0.5, R = 0.5

Result = <0.01 (oo)cysts per 100 liters of water.

Sample M4

V = 350.0 litres, P = 3.0 ml, F = 0.125, R = 1.0

Result = <0.02 (oo)cysts per 100 liters of water.

Sample M5

V = 440.0 litres, P = 8.0 ml, F = 0.125, R = 0.5

Result = <0.02 (oo)cysts per 100 liters of water.

Sample M6

V = 500.0 litres, P = 2.0 ml, F = 0.5, R = 1.0

Result = <0.004 (oo)cysts per 100 liters of water.

3.4.2 *Giardia* and *Cryptosporidium* in duck faecal samples

A total of 279 duck faecal samples were collected and examined for the presence of *Cryptosporidium* oocysts and *Giardia* cysts. The samples were IFA screened as described in section 2.2.5 and graded according to the numbers of (oo)cysts seen at 400X magnification in 5 to 6 randomly selected microscopic fields as follows:

>10 (oo)cysts per field = 3+

5-10 (oo)cysts per field = 2+

< 5 (oo)cysts per field = 1+

Oocysts morphologically resembling *Cryptosporidium* were not detected in any of the samples. Eighty-one (29%) of the total 279 samples were positive for cysts morphologically resembling *Giardia* spp. (Figure 16).

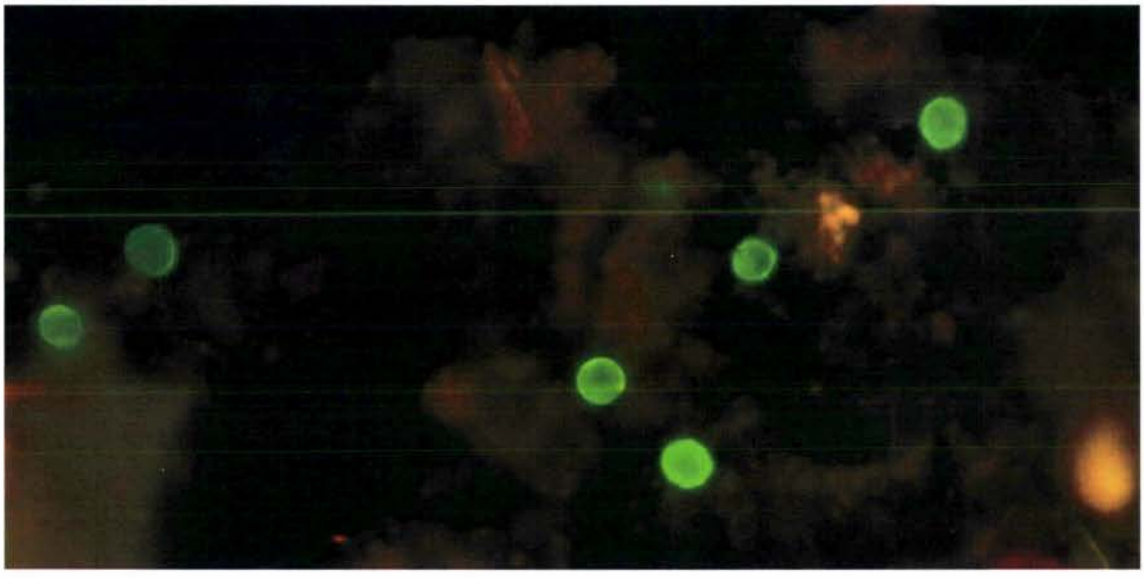

Figure 16: Cysts morphologically resembling *Giardia* spp. but identified as *Caryospora* (stained with Merifluor G/C direct immunofluorescent reagent – Epifluorescence, 400 X magnification).

Of these 31 samples had more than 10 cysts (3+) per field of view at 400X magnification, 21 samples had 5 to 10 cysts (2+) per field and 29 samples had less than 5 cysts (1+) per field (Appendix E). In the remaining 198 (71%) samples cysts morphologically resembling *Giardia* were not detected. Only samples with greater than 10^5 cysts per gram of faeces (greater than 10 cysts per field) were subjected to sucrose gradient purification and subsequent nucleic acid extraction and PCR amplification.

3.4.3 Measurements of Merifluor®*C/G* IFA positive protozoan cysts.

Wet mounts of duck faecal samples for cyst measurements were prepared (section 2.2.6) from 6 of the samples that had more than 10 cysts morphologically resembling *Giardia* per field at 400X magnification in the Merifluor® *C/G* IFA preparations (section 2.2.5) but yielded negative *Giardia* PCR products.

At 400X magnification with Phase Contrast microscopy these cysts were observed as being spherical to slightly subspherical in shape (Figure 17).

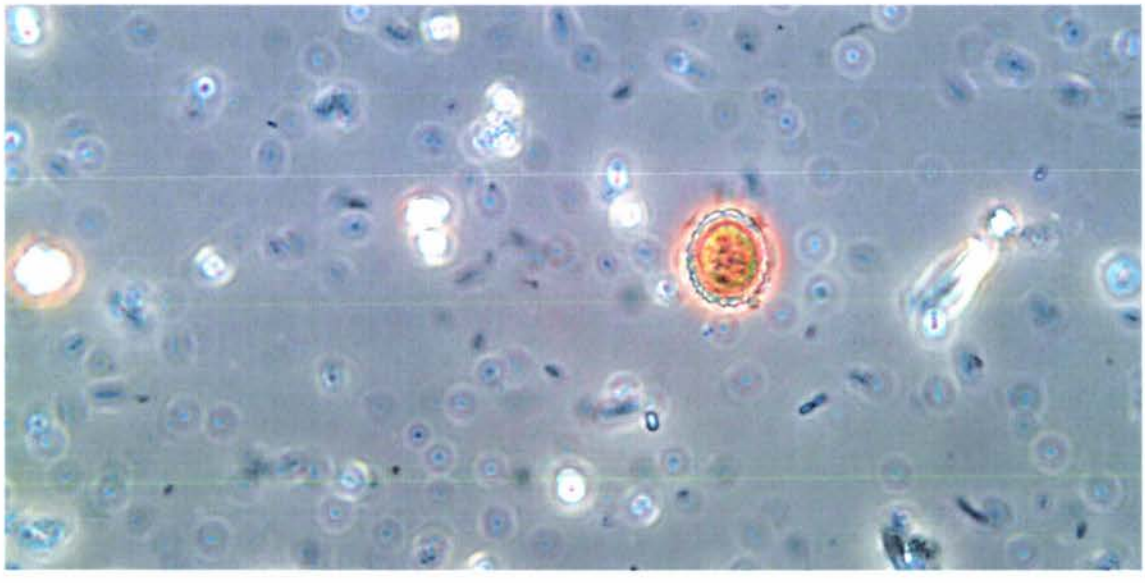

Figure 17: A *Caryospora* spp. oocyst (Phase contrast microscopy – 400 X magnification).

The mean dimensions of the cysts (6 cysts per wet mount) were as follows:

No. 55: 10 µm x 13 µm (Smallest = 10 µm x 12 µm / Largest = 11µm x16 µm)

No. 115: 10 µm x 13 µm (Smallest = 10 µm x 10 µm / Largest = 8 µm x 15 µm)

No. 133: 13 µm x 15 µm (Smallest = 12 µm x12 µm / Largest = 18 µm x 11 µm)

No. 171: 9 µm x 14 µm (Smallest = 8 µm x 10 µm / Largest = 8 µm x 20 µm)

No. 189: 10 µm x 14 µm (Smallest = 6 µm x 8 µm / Largest = 10 µm x 20 µm)

No. 207: 12 µm x 13 µm (Smallest = 8 µm x 9 µm / Largest = 10 µm x 15 µm)

The cysts were on average larger than ovoid *Giardia* cysts that are usually measure 7 µm to 10 µm by 8 µm to 14 µm in size (Markell *et.al.*, 1992). The cysts were distinctly larger than spherical *Cryptospridium* oocysts (approximately 4 µm x 5 µm).

The dimensions of the measured cysts and colour photographs of the apple-green fluorescent ovoid cysts observed in the Merifluor® *C/G* IFA preparations were sent to the Department of Biological Sciences at Macquarie University in Sydney for identification. It was subsequently confirmed that the Merifluor® *C/G* IFA positive cysts were in fact *Caryospora* oocysts (Personal communication: D.Veal, November 5,

2001; M.Slade, November 7, 2001). Since *Caryospora* are phylogenetically related to *Cryptosporidiuim,* these organisms cross react with many *Cryptospridium* antibody preparations but have the appearance *Giardia* cysts when viewed by fluorescence microscopy. *Caryospora* were reported as confounding organisms isolated from duck faeces during Australian *Cryptosporidium* and *Giardia* proficiency testing programs (NATA/PTAC Report, 2001). A brief description of the genus *Caryospora* appears in the discussion section of this thesis.

In view of the false positives (i.e. fluorescent cysts morphologically resembling *Giardia* spp.) observed in the Merifluor® *C/G* IFA stained faecal preparations these findings were communicated to the manufacturers, Meridian Diagnostics Inc. in Cincinnati, Ohio. Meridian Diagnostics informed this author that Merifluor® *C/G* was essentially designed for clinical (human) use and that no cross-reactivity had been reported with a wide range of microorganisms tested with Merifluor®*C/G* (Personal communication, G.Workman, November 8, 2001).

3.4.4 Polymerase Chain Reaction (PCR) amplification of *Giardia* cysts

Screened faecal speicmens that yielded high numbers of *Giardia* cysts (more than 10 cysts per field at 400X magnification) with the Merifluor® *C/G* IFA were subjected to sucrose gradient purification (section 2.2.7) and nucleic acid extraction (section 2.2.8) for the PCR amplifications. Due the inherent poor cyst recovery following sucrose gradient purification, samples that yielded less than 10 cysts per field at 400X magnification by IFA were not subjected to nucleic acid extraction. PCR amplifications were therefore carried out on nucleic extracts of only 12 samples.

While the *Giardia* positive controls produced strong visual bands (Figure 18), none of the 12 samples tested positive with any of the 4 primer sets used in the PCR amplifications. In an attempt to increase the level of detection of the PCR assay two different faecal cyst concentration methods as well a variation in the nucleic acid extraction procedure were performed before additional PCR amplifications were carried out. These were formal-ether concentration/sucrose gradient purification and sodium chloride flotation concentration followed by nucleic acid extraction with 1% Nonidet-NP40/TE buffer and 20% Chelex prior to the freeze – thawing cycles. The nucleic acid

conditions described in section 2.2.9. In spite of employing these variations no PCR products were yet again detected with any of the primer sets.

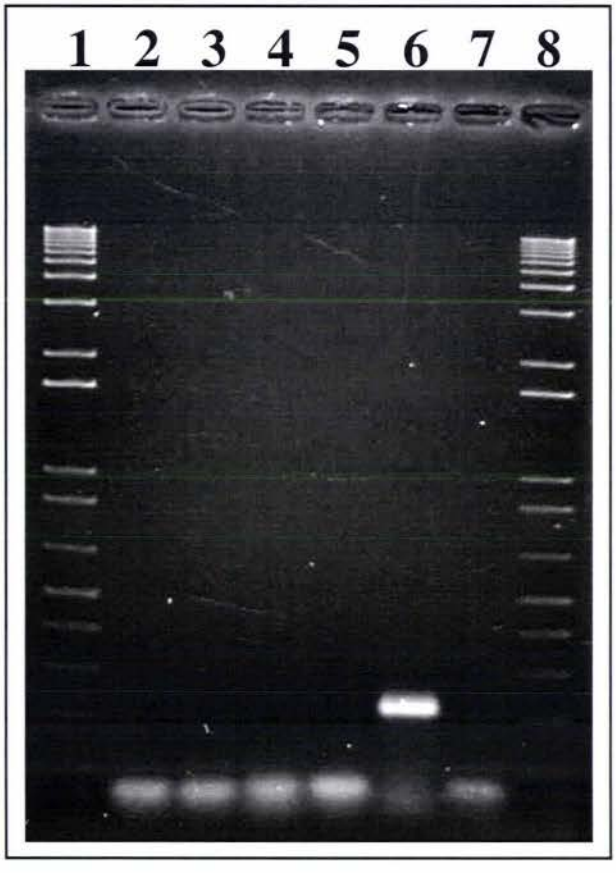

Figure 18: PCR amplification of a *Giardia intestinalis* islolate. Lanes 1 and 8, 1Kb DNA Ladder; Lane 2 Sample 71; Lane 3 Sample 80; Lane 4 Sample 119, Lane 5 Sample 177, Lane 6 Positive control (*G. intestinalis*); Lane 7 Negative control (*G. muris*).

CHAPTER FOUR: DISCUSSION

4.1 Water Quality Results at Hataitai Beach

4.1.1 Indicator organism counts

In this study a very large number of marine water samples were collected from multiple sites at Hataitai Beach from July 1998 to April 2000. The results of indicator organism counts clearly indicate that despite the stormwater and sewer upgrades there is still a pollution problem at Hataitai Beach. On 64 occasions during the study there were exceedences of the water quality guidelines at Hataitai Beach, especially in water samples collected from the B3 site where ducks were frequently seen loafing on the beach or swimming in the water. Moreover, on 41 occasions the *Enterococci* levels in samples from the B3 site were strikingly over the *Action Red* mode guideline value of 277 *Enterococci* per 100 ml - exceedences that require local authorities and health authorities to warn the public that the beach is unsafe for recreational activities and erect warning signs. The median value of 63 *Enterococci* per 100 ml for samples collected from the B3 site was well above the *Green* – "safe for bathing" guideline value of less than 35 *Enterococci* per 100 ml.

These results are in direct contrast to the results that were obtained in samples collected fortnightly (usually from the B1 site only) by the Wellington City and Regional Councils as part of their routine beach-monitoring programme. The *Annual Coastal Water Quality Report for the Wellington Region* 1998/1999 shows only 4 exceedences in 17 samples from the B1 site over a 12-month period with a median of 12 *Enterococci* per 100 ml (Berry 1999). The report states "Water Quality has improved significantly at Hataitai Beach as a result of sewer and stormwater upgrades". However, in January and February 1999, when the Wellington City Council did collect samples from the B1, B2 and B3 sites the results of 27 samples showed a median of 56 *Enterococci* per 100 ml. Furthermore, site B1 *Enterococci* values of 270 and 540 per 100 ml and site B3 values of 150, 380, 1000 and 1000 per 100 ml were obtained. (Personal communication, I.Idris, WCC, March 7, 1999). Robertson (2000) reported an increase in the median *Enterococci* count from 12 to 42 per 100 ml for Hataitai Beach from February 1999 to March 2000.

Conflicting media reports regarding the water quality of Hataitai Beach appeared in 2000 (Johnson, 2000a) when Hataitai Beach's bacterial levels were reported on the one hand (WRC) to be above guideline values and on the other hand (WCC) as "fortnightly testing had confirmed Hataitai Beach was safe and people should have no fears of about swimming at Hataitai" (Mallon 2000, quoted in Johnson, 2000b). In the latter article the WRC sampling site was incorrectly identified by Mallon as a stormwater outfall site at the intersection of Evans Bay Parade and Cobham Drive situated approximately 600 metres to the north of Hataitai Beach (Personal communication, G.Robertson, WRC, September 14, 2000). The WRC sampling site in this instance was in fact close to the B3 site sampled in this study and similarly also showed a number of high *Enterococci* counts (Abbott *et al.* 2000, as cited in Robertson 2000; Robertson, 2000).

From March 2000 to June 2001 the WRC annual coastal report showed only a slight decrease in the Hataitai Beach median value; from 42 to 40 *Enterococci* per 100 ml (Stephenson 2001). Examples of Hataitai Beach results published on the Internet appear in Appendix C (01/11/99 to 26/03/01- MFE/WCC) and Appendix D (05/11/01 to 17/06/02 - WRC).

4.1.2 Causes of the elevated indicator organism counts.

In this study no significant relationship between rainfall and high indicator organism counts was found thus ruling out the likelihood of the influence of stormwater run-off. Most samplings occurred in dry weather and from the Metservice data no association was found between increased counts and rain on days preceding sampling. Further more, no statistical association was found between indicator organism counts in samples collected from the stormwater outlet at Greta Point (about 200 meters north of the B3 site) and the counts in samples collected simultaneously from the B1 site. However, as mentioned earlier, this may have been due to the effects of currents and tides and a lag time between water leaving the stormwater outlet and reaching the beach site.

The combined results of all three bacterial indicators have not been particularly useful for identifying the principal faecal sources responsible for the high counts obtained in this study. While the median values of total coliform, *Escherichia coli* and *Enterococci* were all markedly higher in the samples collected from the B3 site, no correlations were found between any of the three indicators. In their study on faecal indicator bacteria and

beach contamination in Auckland, Hartley *et al.*, (2000) found that the strong correlations between all three indicators tested by conventional MPN methods (APHA, 1995) suggested co-occurrence in the dominant source of contamination. Using Colilert-18™ could explain why no significant statistical association was obtained between any of the three indicators tested in this study. Although Colilert-18 has been shown to correlate well with the traditional membrane filtration techniques for monitoring indicator organisms in marine waters (Palmer *et al.*, 1993; Fiksdal *et al.*, 1994; Eckner 1998; Solo-Gabriele *et al.*, 2000; Noble *et al.*, 2000; Desmarais *et al.*, 2002), Manafi (2000) reported that *Aeromonas* spp. could cause an overestimation of coliforms detected by Colilert formulations and conversely, Colilert was found to gave false negative results in samples with low numbers of coliforms and *E.coli* (Schets *et al.*, 1993, cited in Manafi, 2000). A study by Pisciotta *et al.*, (2002) on the use Colilert-18 indicated that the detection and enumeration of *E.coli* by Colilert-18 is not accurate when the recommended 1 in 10 dilution is used for marine water. The results of this study suggest that greater dilution may diminish the false positive problem but recommend that further study of this possibility needs to be carried out.

Using the ratios of *Escherichia coli* to *Enterococci* counts to determine the likely sources of pollution has been of limited value since ratios of below and above 0.7 were obtained. While Standrige *et. al.*, (1979) found FC:FS ratios useful for assessing mallard duck contamination of recreational waters, in this study the ratios of *Escherichia coli* to *Enterococci* calculated from results produced by the Colilert-18 and Enterolert methods respectively, could not categorically establish a pollution source, even though the median ratio for the B3 site was 0.8 (range 0.08 to 4.1). Since the application of ratios to environmental sample results needs many qualifications, newer methods for distinguishing sources of faecal pollution (reviewed in section 1.3.5), although more expensive, have distinct advantages over the limitations of using ratios. Antibiotic resistance patterns and molecular techniques such as ribotyping in particular, should be more reliable in discriminating pollution sources.

Whereas the results from the B3 site indicate that ducks could be responsible for the high counts that were frequently obtained at this site, no significant statistical association was found between the duck counts and either the total coliform, *E.coli*, or *Enterococci* counts. However, despite these findings a combination of duck droppings

and meteorological events appears be the most likely reason for the high indicator organism counts at Hataitai Beach. The work by Standrige *et al.*, (1979) could explain why in this study high indicator organisms were obtained when not only when large numbers of ducks were present at the sampling sites but also when few or no ducks were present. The Standrige study found that ducks frequently defaecate onto the beach sands and that the faecal organisms in the droppings not only survived in the sand but multiplied rapidly during the first week. The authors demonstrated the presence of sufficient nutrients in the beach sand and near shore sediments to support microbial growth. Furthermore, the study showed that faecal bacterial organisms introduced into the sands could be carried into the water by tides, rain or wind erosion.

Although testing of the Hataitai Beach sands was not, for practical reasons, carried out, reports have been published on indicator organism levels and microorganisms isolated from beach sands. In an Italian study, a significant correlation was found between beach sand contamination and the adjacent waters, although the sand generally had higher bacterial counts than the water (Aulicino *et al.*, 1985). A similar correlation was found in Barcelona beaches, but in contrast to the Italian study, the level of contamination did not show any significant difference in sand and seawater (Roses-Codinachs *et al.*, 1988). Papadakis *et al.*, (1997) found no correlation between the bacterial indicators of faecal pollution counted on the wet part of the beach sand and *Staphylococcus aureus* counts, nor the with the presence of fungi. In other studies a wide variety of organisms, including potential pathogens, were isolated from beach sands. Even though these organisms could be encountered through contact with the contaminated sand, no epidemiological evidence of transmission by this route was reported in any of the studies (Ghinsberg *et al.*, 1994; De Moura Sarquis & De Olivera 1996; Mendes *et al.*, 1997). Although a draft, 1998 World Health Organization *Guidelines for Safe Recreational Water Environments* document (WHO, 1998), indicates that routine monitoring of beach sand is not justified, a study on the presence of *Campylobacter* and *Salmonella* in sand from bathing beaches (Bolton *et al.*, 1999) suggests that assessment of the microbiological quality of water alone may not be a completely satisfactory method for determining safety standards for bathing beaches.

In several countries, particularly at resort areas, machine sand cleaning are common practices that can eliminate visible garbage mixed with sand as well as reduce animal

excreta and hence diminish the survival and multiplication of microorganisms in the beach sands (Llewellyn & Shackley, 1996). Following the Standrige *et al.*, (1979) mallard duck beach contamination study, the city of Madison introduced a program of daily raking of beaches to expose more sand to sunlight and to remove organic debris from the beaches. This activity, coupled with vigorous efforts by the beach lifeguards to discourage people feeding the ducks, has resulted in a substantial reduction in the number of beach closures (Personal communication, J. Standrige, University of Wisconsin, March 28, 2000).

4.1.3 The impact of duck droppings on water quality.

While the results in this study did not support a direct relationship between duck numbers and the indicator organism counts, the bacterial load in the beach water can be estimated from calculations based on published data for animal faecal production rates and indicator organism density relationships (Geldreich 1976; Jones & White 1984). Given that ducks produce on average 336 grams of faeces per day and that each gram contains about 5.4 X 10^7 *Enterococci* and 3.3 X 10^7 *E.coli*, the daily load of these indicator organisms from one duck alone could be ~1.8 X 10^{10} *Enterococci* and ~1.1 X 10^{10} *E.coli*, and fifty ducks could produce ~9.1 X 10^{11} and ~5.6 X 10^{11} *Enterococci* and *E.coli* respectively. These figures demonstrate that the presence of even a few ducks can rapidly lead to a significant deterioration the water quality at Hataitai Beach.

In a study on coliform contamination of Buttermilk Bay in Massachusetts, Weiskel *et al.*, (1996) found that 67% of the direct faecal coliform (FC) loading of the bay waters were from waterfowl (ducks, geese, and swans). The average daily FC load to the bay from waterfowl ranged from a low of ~0.49 X 10^9 FC d^{-1} in August to ~217 X 10^9 FC d^{-1} in December when the greatest number of waterfowl was observed at the bay. Ducks were the dominant waterfowl FC source, both because of their generally higher numbers and their high faecal coliform production rates (i.e., ~10^9 FC d^{-1} *vs* ~10^7 FC d^{-1} for geese). The study found the waterfowl FC load to be highly correlated with the size of the duck population (r = 0.99). The annual FC load from all waterfowl was estimated to be ~33 X 10^{12} FC yr^{-1}.

The most obvious and easiest way to prevent ducks from polluting the water at Hataitai Beach is to limit their food sources in the areas surrounding the beach and officially

prohibit people from feeding the ducks. The seemingly kind act of artificial feeding creates a dependency on humans and produces unnaturally large congregations of ducks, a situation that not only subjects the birds to disease outbreaks, but also creates a public health hazard because of the variety of pathogens ducks have been shown to carry. Moreover, the feeding of bread to ducks can cause metabolic bone disease as well as other nutritional deficiencies in the ducks (Bills, 1999).

4.2 *Giardia and Cryptosporidium.*

4.2.1 *Giardia* and *Cryptosporidium* in water samples.

No *Giardia* or *Cryptosporidium* (oo)cysts were detected in any of the six large volume (range 132 to 500 litres) marine water samples collected from Hataitai Beach. While Brown *et al.*, (1998) detected *Giardia* cysts *Cryptosporidium* oocysts in 19.2% and 12.6% respectively of fresh water samples collected from throughout New Zealand, there is limited information regarding the detection and occurrence of these parasites in marine waters. Using excystation experiments, Brown *et al.*, (1999) showed that in comparison to cyst viability in freshwater, there was a significant reduction in the viability of *Giardia* after exposure to seawater. Both low and wide variation in the recovery efficiency of the cartridge filtration method for detection of *Giardia* cysts *Cryptosporidium* oocysts have been reported (LeChevalier & Norton, 1995; Johnson *et.al.*, 1995). Because the concentration of these organisms could be diluted considerably in marine bathing waters, volumes of greater than 500 litres per sample may need to be filtered for their detection.

4.2.2 *Giardia* and *Cryptosporidium* in duck faecal samples.

Whereas no *Cryptosporidium* oocysts were detected in any of the 279 IFA (Merifluor® combined G/C stain) screened duck faecal samples, in 81 (29%) cysts morphologically resembling *Giardia* (but identified as *Caryospora* spp.) were detected. As mentioned earlier, since *Caryospora* are phylogenetically related to *Cryptosporidium,* these organisms cross react with many *Cryptospridium* antibody preparations but have the appearance *Giardia* cysts when viewed by fluorescence microscopy.

The genus *Caryospora* (Apicomplexa, Eimeriorina) contains 30 species of coccidia that develop primarily in reptiles and predatory birds but have a wide host range including

Herring gulls, Guillemots and Puffins (Personal communication, M.Slade, November 7, 2001). The lifecycles of *Caryospora* spp. are distinct and complex and have been succinctly described and illustrated (Upton *et al.*, 1986; Upton *et al.*, 1992; Lindsay *et al.*, 1994). From reviewing the literature it appears that the *Caryospora* oocysts identified in this study are most likely to be *Caryospora argentati*, a species that was first described in 1959 (Schwalbach 1959, as cited in Upton *et al.* 1986) and that have Herring gulls as their primary hosts. While little is known about the lifecycle of *Caryospora* involving secondary hosts, Euzeby (1991) has reported that dogs and pigs can be infected from oocysts emitted by primary hosts or by consuming infected secondary hosts harbouring caryocysts. The infected animals exhibit a severe generalised pyo-granulomatous dermatitis. At the time of writing this thesis no reports have been found on Caryosporan infection in humans.

The observation of fluorescent cysts resembling *Giardia*, highlights the importance of careful examination of shape, size and internal structures as well as measurements in making a positive identification of *Giardia*, not just fluorescence alone. Although the Merifluor® combined G/C IFA stain has been designed for use with clinical (human) specimens and while no cross reactivity has been reported with a wide range of organisms tested, it seems prudent, in view of the false positives obtained in this study, for Meridian Diagnostics to alert users of their product about *Caryospora* cross reactivity. This information could be conveyed *via* future product package inserts. Since *Giardia* and *Crypotsporidium* IFA staining procedures are used widely in research and clinical laboratories as well as for monitoring (oo)cyst presence in environmental samples, these immunodiagnostic products should be applicable to human and veterinary specimens (CDC, Division of Parasitic Diseases, 2001).

In a recent study a variety of wild duck species (predominantly mallards) surveyed on the lower Rio Grande River, New Mexico, were found to be carriers of *Cryptosporidium* and *Giardia* (Kuhn *et al.*, 2002). Sixty-nine ducks were tested for the presence of these parasites over a three-month period. Using an immunofluorescence-antibody (IFA) staining procedure (*Crypto/Giardia* IF, Techlab, Blacksburg, VA, USA), 49% of faecal samples tested positive for *Cryptospridium* spp. and 26% for *Giardia* spp. For some samples, recovered cysts and oocysts were further screened via Polymerase Chain Reaction (PCR) to determine the presence of *G.intestinalis* and

C.parvum. While 14 of the 69 samples had (oo)cyst concentrations that were considered to be above the PCR detection limit, *G.intestinalis* and *C.parvum* were not detected in any of these 14 samples. Although the authors did not report on whether any IFA false positives were detected nor provided details of the dimensions of the (oo)cysts, they concluded that either the concentration of these organisms were in fact below the PCR detection limit or that the ducks may have carried a completely new species of *Cryptosporidium* and *Giardia*.

CHAPTER FIVE: CONCLUSION

The results from this study clearly indicate that despite the stormwater and sewer upgrades by the Wellington City Council there is still a pollution problem at Hataitai Beach and that this pollution is primarily caused by a combination of duck droppings and meteorological events. The microbiological water quality can be degraded directly by the resident mallard ducks defaecating in the water or indirectly from droppings deposited on the beach sands. Faecal bacteria in the droppings on the beach sands can be carried into the water by tides, rain, and wind erosion or unwittingly by the actions of beach users.

On 64 occasions during this study there were exceedences of the water quality guidelines at Hataitai Beach, especially in water samples collected from the B3 site where ducks were frequently seen loafing on the beach or swimming in the water. Moreover, on 41 occasions the *Enterococci* levels in samples from the B3 site were strikingly over the *Action Red* mode guideline value of 277 *Enterococci* per 100 ml - exceedences that require local authorities and health authorities to warn the public that the beach is unsafe for recreational activities and erect warning signs. The median value of 63 *Enterococci* per 100 ml for samples collected from the B3 site was well above the *Green* – "safe for bathing" guideline value of less than 35 *Enterococci* per 100 ml.

Because of their aquatic lifestyle and highly mobile behaviour, ducks may be exposed to a diverse array of potentially pathogenic organisms such as *Campylobacter jejuni*, *Escherichia coli O157:H7*, *Salmonella spp.*, *Pasteurella multocida*, *Giardia* and *Cryptosporidiuim* spp., and *Cercarial* trematodes. These organisms can originate from several sources including human sewage, agricultural runoff, and animal faecal matter.

The microbiological health risk of humans acquiring infections from the ducks Hataitai Beach depends on several factors, including the presence and survival of pathogenic organisms in the droppings after their deposition on the beach sands and in the water, as well as the types of recreational activities that expose humans to these droppings. In order for the public to be adequately informed of the risk so that they can make informed personal choices about engaging in recreational activities at Hataitai Beach, it seems prudent that efforts should be made to grade this beach so that the beach's suitability for recreation can be established. This should include a catchment risk

assessment that considers the potential sources and transmission routes of faecally derived pollution at Hataitai Beach with an assessment of the microbiological data. The grading of beaches is an improved risk-based approach to monitoring water quality that is based on the World health Organization's *'Annapolis Protocol'* (WHO, 1998) and is incorporated in the latest New Zealand Recreational Water Quality Guidelines (MFE, 2002).

In the meantime common sense dictates that at Hataitai Beach the authorities should limit the food sources of ducks in the areas surrounding the beach by officially prohibiting people from feeding the ducks.

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Appendix A:

Sampling data, numbers of ducks, tides and weather conditions, and indicator organism counts.

Sample	Site	Year	Date	Tide	Weather	No. of Ducks at Site	*Total coliforms* per 100 ml	*E.coli* per 100 ml	*Enterococci* per 100 ml
1	B1	1998	10/12pm	HT	Fine / Lw	48	95	10	122
2	B1		11/12am	HT	Fine / Lw	36	74	0	0
3	B1		11/12pm	LT	Fine / Lw	0	0	0	0
4	B1		12/12pm	HT	Fine / Lw	0	52	10	0
5	B1		13/12am	HT	Fine / Lw	15	0	0	10
6	BI		13/12pm	HT	Fine / Lw	26	74	30	120
7	B1		14/12am	HT	Fine / Lw	36	145	10	0
8	B1		14/12pm	HT	Overcast	18	31	10	41
9	B1		15/12am	LT	Fine / Lw	36	538	10	85
10	B1		15/12pm	HT	Fine / Lw	42	0	0	0
11	B2		15/12pm	HT	Fine / Lw	42	199	96	108
12	B3		15/12pm	HT	Fine / Lw	42	354	118	97
13	SHI		15/12pm	HT	Fine / Lw	0	74	41	10
14	SH2		15/12pm	HT	Fine / Lw	0	20	20	0
15	SH3		15/12pm	HT	Fine / Lw	0	0	0	10
16	SH4		15/12pm	HT	Fine / Lw	0	0	0	0
17	B1		16/12pm	HT	Fine / Lw	0	10	0	10
18	B2		16/12pm	HT	Fine / Lw	0	52	41	20
19	B3		16/12pm	HT	Fine / Lw	0	272	158	122
20	SHI		16/12pm	HT	Fine / Lw	0	0	0	0
21	SH2		16/12pm	HT	Fine / Lw	0	10	0	0
22	SH3		16/12pm	HT	Fine / Lw	0	0	0	0
23	SH4		16/12pm	HT	Fine / Lw	0	20	0	0
24	B1		18/12pm	HT	Light Rain	0	681	467	31
25	B2		18/12pm	HT	Light Rain	0	265	175	20
26	B3		18/12pm	HT	Light Rain	0	301	10	20
27	B4		18/12pm	HT	Light Rain	0	86	0	41
28	B5		18/12pm	HT	Light Rain	0	0	0	10
29	B6		18/12pm	HT	Light Rain	0	0	0	0
30	B1	1999	2/2 am	HT	Fine / No/W	75	10	0	0
31	B2		2/2 am	HT	Fine / No/W	75	20	0	10
32	B3		2/2 am	HT	Fine / No/W	75	181	20	185
33	B4		2/2 am	HT	Fine / No/W	75	2481	20	20
34	B5		2/2 am	HT	Fine / No/W	75	31	0	0
35	B1		3/2 am	HT	Fine / No/W	73	52	20	41
36	B2		3/2 am	HT	Fine / No/W	73	52	20	30
37	B3		3/2 am	HT	Fine / No/W	73	145	31	52
38	B4		3/2 am	HT	Fine / No/W	73	218	0	20
39	B5		3/2 am	HT	Fine / No/W	73	52	0	20
40	B1		4/2 am	HT	Fine / Lgt/W	85	175	0	10
41	B2		4/2 am	HT	Fine / Lgt/W	85	161	0	10
42	B3		4/2 am	HT	Fine / Lgt/W	85	379	171	41
43	B4		4/2 am	HT	Fine / Lgt/W	85	271	10	0
44	B5		4/2 am	HT	Fine / Lgt/W	85	98	0	0
45	B1		5/2 pm	LT	Fine / Lgt/W	22	419	31	10

46	B2	1999	5/2 pm	LT	Fine / Lgt/W	22	31	20	0
47	B3		5/2 pm	LT	Fine / Lgt/W	22	87	74	169
48	B4		5/2 pm	LT	Fine / Lgt/W	22	52	31	74
49	B5		5/2 pm	LT	Fine / Lgt/W	22	0	0	0
50	B1		6/2 am	HT	Ovc/Lgt/W	76	20	0	10
51	B2		6/2 am	HT	Ovc/Lgt/W	76	74	63	41
52	B3		6/2 am	HT	Ovc/Lgt/W	76	110	20	31
53	B4		6/2 am	HT	Ovc/Lgt/W	76	216	0	20
54	B5		6/2 am	HT	Ovc/Lgt/W	76	2	0	0
55	B1		7/2pm	HT	Fine/Lgt/W	48	120	20	41
56	B2		7/2pm	HT	Fine/Lgt/W	48	108	63	197
57	B3		7/2pm	HT	Fine/Lgt/W	48	109	52	63
58	B4		7/2pm	HT	Fine/Lgt/W	48	256	52	556
59	B5		7/2pm	HT	Fine/Lgt/W	48	0	0	20
60	SH1		7/2pm	HT	Fine/Lgt/W	48	41	0	52
61	B1		8/2 pm	LT	Fine/Lgt/W	0	10	0	0
62	B2		8/2 pm	LT	Fine/Lgt/W	0	41	10	0
63	B3		8/2 pm	LT	Fine/Lgt/W	0	85	10	31
64	B4		8/2 pm	LT	Fine/Lgt/W	0	243	31	74
65	B5		8/2 pm	LT	Fine/Lgt/W	0	86	0	10
66	B1		9/2 pm	LT	Fine / No/W	0	20	0	0
67	B2		9/2 pm	LT	Fine / No/W	0	0	0	0
68	B3		9/2 pm	LT	Fine / No/W	0	31	10	0
69	B4		9/2 pm	LT	Fine / No/W	0	10	0	0
70	B5		9/2 pm	LT	Fine / No/W	0	10	0	0
71	B1		14/2 pm	HT	Fine / No/W	14	97	10	20
72	B2		14/2 pm	HT	Fine / No/W	14	20	10	20
73	B3		14/2 pm	HT	Fine / No/W	14	109	30	31
74	B4		14/2 pm	HT	Fine / No/W	14	20	0	0
75	B5		14/2 pm	HT	Fine / No/W	14	20	0	0
76	SH1		14/2 pm	HT	Fine / No/W	14	0	0	0
77	B1		15/2 pm	HT	Fine/Lgt/W	4	96	0	0
78	B2		15/2 pm	HT	Fine/Lgt/W	4	240	20	41
79	B3		15/2 pm	HT	Fine/Lgt/W	4	41	30	52
80	B4		15/2 pm	HT	Fine/Lgt/W	4	74	0	31
81	B5		15/2 pm	HT	Fine/Lgt/W	4	20	10	0
82	B1		16/2 am	HT	Ovc/Lgt/W	62	41	10	63
83	B2		16/2 am	HT	Ovc/Lgt/W	62	161	41	86
84	B3		16/2 am	HT	Ovc/Lgt/W	62	354	160	529
85	B1		17/2 am	HT	Ovc/Lgt/W	81	285	187	275
86	B2		17/2 am	HT	Ovc/Lgt/W	81	354	160	108
87	B3		17/2 am	HT	Ovc/Lgt/W	81	743	148	331
88	B1		18/2am	HT	Ovc/Lgt/W	58	10	0	20
89	B2		18/2am	HT	Ovc/Lgt/W	58	134	10	10
90	B3		18/2am	HT	Ovc/Lgt/W	58	569	85	160
91	B1		18/2pm	HT	Fine/Lgt/W	0	41	10	0
92	B2		18/2pm	HT	Fine/Lgt/W	0	578	51	576
93	B3		18/2pm	HT	Fine/Lgt/W	0	1059	73	836
94	B1		19/2 pm	HT	Fine/Lgt/W	61	41	0	0
95	B2		19/2 pm	HT	Fine/Lgt/W	61	166	41	73
96	B3		19/2 pm	HT	Fine/Lgt/W	61	278	171	203
97	B1		20/am	HT	Ovc/Lgt/W	48	51	0	20
98	B2		20/2am	HT	Ovc/Lgt/W	48	62	31	31
99	B3		20/2am	HT	Ovc/Lgt/W	48	145	20	52
100	B1		21/2am	HT	Fine/Stw/Rs	86	262	41	52

101	B2	1999	21/2am	HT	Fine/Stw/Rs	86	2489	83	464
102	B3		21/2am	HT	Fine/Stw/Rs	86	226	41	63
103	B1		23/2am	HT	Fine/Stw/Rs	81	145	20	0
104	B2		23/2am	HT	Fine/Stw/Rs	81	72	10	20
105	B3		23/2am	HT	Fine/Stw/Rs	81	4106	1005	5475
106	B1		24/2pm	HT	Fine / No/W	58	98	20	0
107	B2		24/2pm	HT	Fine / No/W	58	41	0	0
108	B3		24/2pm	HT	Fine / No/W	58	230	31	173
109	B1		25/2pm	HT	Ovc / No/W	76	145	10	52
110	B2		25/2pm	HT	Ovc / No/W	76	281	41	97
111	B3		25/2pm	HT	Ovc / No/W	76	161	98	41
112	B1		26/2pm	HT	Ovc / No/W	63	314	109	336
113	B2		26/2pm	HT	Ovc / No/W	63	816	285	259
114	B3		26/2pm	HT	Ovc / No/W	63	472	238	199
115	B1		27/2pm	HT	Ovc/Lgt/W	90	987	459	657
116	B2		27/2pm	HT	Ovc/Lgt/W	90	1046	450	847
117	B3		27/2pm	HT	Ovc/Lgt/W	90	1616	754	3448
118	B4		27/2pm	HT	Ovc/Lgt/W	90	3873	1017	3076
119	B1		4/3pm	HT	Fine / No/W	65	1956	1236	520
120	B2		4/3pm	HT	Fine / No/W	65	759	324	265
121	B3		4/3pm	HT	Fine / No/W	65	2909	1259	1334
122	B1		6/3am	HT	Ovc / No/W	50	199	41	73
123	B2		6/3am	HT	Ovc / No/W	50	1274	465	359
124	B3		6/3am	HT	Ovc / No/W	50	1119	613	301
125	B1		7/3am	HT	Ovc/Lgt/W	64	2595	146	437
126	B2		7/3am	HT	Ovc/Lgt/W	64	2400	253	487
127	B3		7/3am	HT	Ovc/Lgt/W	64	6131	759	2035
128	B1		8/3pm	LT	Fine / No/W	45	119	20	10
129	B2		8/3pm	LT	Fine / No/W	45	74	20	0
130	B3		8/3pm	LT	Fine / No/W	45	259	63	85
131	B1		11/3pm	HT	Ovc / No/W	66	1050	31	97
132	B2		11/3pm	HT	Ovc / No/W	66	1259	309	203
133	B3		11/3pm	HT	Ovc / No/W	66	882	413	448
134	B1		12/3pm	HT	Fine / No/W	55	6867	51	41
135	B2		12/3pm	HT	Fine / No/W	55	3130	452	313
136	B3		12/3pm	HT	Fine / No/W	55	1483	591	855
137	B1		20/3pm	HT	Fine / No/W	7	228	63	158
138	B2		20/3pm	HT	Fine / No/W	7	122	20	134
139	B3		20/3pm	HT	Fine / No/W	7	262	161	173
140	B1		21/3pm	HT	Ovc / No/W	0	63	10	0
141	B2		21/3pm	HT	Ovc / No/W	0	209	199	285
142	B3		21/3pm	HT	Ovc / No/W	0	7701	3784	341
143	B1		22/3am	HT	Fine / No/W	55	650	20	10
144	B2		22/3am	HT	Fine / No/W	55	97	20	20
145	B3		22/3am	HT	Fine / No/W	55	122	20	41
146	B1		23/3am	HT	Ovc/Lgt/W	27	399	132	143
147	B2		23/3am	HT	Ovc/Lgt/W	27	216	52	41
148	B3		23/3am	HT	Ovc/Lgt/W	27	594	228	216
149	B1		25/3am	HT	Fine / No/W	56	609	41	132
150	B2		25/3am	HT	Fine / No/W	56	601	62	98
151	B3		25/3am	HT	Fine / No/W	56	1112	275	134
152	B1		19/4am	HT	Fine / No/W	63	399	41	197
153	B2		19/4am	HT	Fine / No/W	63	218	41	274
154	B3		19/4am	HT	Fine / No/W	63	594	146	364
155	B1		20/4am	HT	Fine/Lgt/W	7	448	31	63

156	B2	1999	20/4am	HT	Fine/Lgt/W	7	256	0	41
157	B3		20/4am	HT	Fine/Lgt/W	7	327	31	96
158	B1		21/4pm	LT	Fine/Lgt/W	12	31	20	0
159	B2		21/4pm	LT	Fine/Lgt/W	12	62	41	10
160	B3		21/4pm	LT	Fine/Lgt/W	12	86	20	31
161	B1		22/4am	HT	Fine / No/W	63	529	31	130
162	B2		22/4am	HT	Fine / No/W	63	98	10	20
163	B3		22/4am	HT	Fine / No/W	63	226	97	52
164	B1		11/5am	HT	Fine/Stw/Rs	56	717	315	397
165	B2		11/5am	HT	Fine/Stw/Rs	56	1152	358	486
166	B3		11/5am	HT	Fine/Stw/Rs	56	2809	903	663
167	B1		13/5pm	HT	Ovc/Lgt/W	72	281	253	442
168	B2		13/5pm	HT	Ovc/Lgt/W	72	206	169	295
169	B3		13/5pm	HT	Ovc/Lgt/W	72	437	414	380
170	B1		14/5pm	HT	Ovc/Lgt/W	21	187	102	70
171	B2		14/5pm	HT	Ovc/Lgt/W	21	118	40	51
172	B3		14/5pm	HT	Ovc/Lgt/W	21	249	213	70
173	B1		16/5pm	HT	Ovc/ Stw/Rs	14	1039	673	305
174	B2		16/5pm	HT	Ovc/ Stw/Rs	14	110	132	121
175	B3		16/5pm	HT	Ovc/ Stw/Rs	14	462	60	132
176	B1		17/5pm	HT	Fine/ Lgt/W	0	41	10	10
177	B2		17/5pm	HT	Fine/ Lgt/W	0	51	10	0
178	B3		17/5pm	HT	Fine/ Lgt/W	0	92	10	10
179	B1		19/5am	HT	Fine/ Lgt/W	16	52	20	31
180	B2		19/5am	HT	Fine/ Lgt/W	16	31	10	0
181	B3		19/5am	HT	Fine/ Lgt/W	16	121	97	31
182	B1		23/5am	HT	Ovc/Lgt/W	12	30	0	0
183	B2		23/5am	HT	Ovc/Lgt/W	12	41	10	0
184	B3		23/5am	HT	Ovc/Lgt/W	12	243	52	31
185	B1		28/5pm	HT	Ov/Rain/Lw	34	374	63	10
186	B2		28/5pm	HT	Ov/Rain/Lw	34	246	74	20
187	B3		28/5pm	HT	Ov/Rain/Lw	34	539	110	20
188	B1		7/6am	HT	Fine/Lgt/W	44	63	10	31
189	B2		7/6am	HT	Fine/Lgt/W	44	31	0	10
190	B3		7/6am	HT	Fine/Lgt/W	44	52	10	10
191	B1		8/6am	HT	Fine/Lgt/W	58	30	0	0
192	B2		8/6am	HT	Fine/Lgt/W	58	52	0	10
193	B3		8/6am	HT	Fine/Lgt/W	58	109	31	10
194	B1		9/6pm	HT	Ovc/Lgt/W	54	489	20	10
195	B2		9/6pm	HT	Ovc/Lgt/W	54	712	20	74
196	B3		9/6pm	HT	Ovc/Lgt/W	54	471	41	74
197	B1		10/6am	LT	Fine / No/W	57	201	10	10
198	B2		10/6am	LT	Fine / No/W	57	201	20	10
199	B3		10/6am	LT	Fine / No/W	57	759	181	85
200	B1		12/6pm	HT	Ovc/Lgt/W	0	556	110	158
201	B2		12/6pm	HT	Ovc/Lgt/W	0	364	74	52
202	B3		12/6pm	HT	Ovc/Lgt/W	0	842	158	134
203	B1		13/6pm	HT	Rain/Lgt/W	13	2098	213	305
204	B2		13/6pm	HT	Rain/Lgt/W	13	3609	583	650
205	B3		13/6pm	HT	Rain/Lgt/W	13	3873	369	1892
206	B1		19/6pm	HT	Fine / No/W	33	206	41	63
207	B2		19/6pm	HT	Fine / No/W	33	171	10	51
208	B3		19/6pm	HT	Fine / No/W	33	109	41	30
209	B1		20/6am	HT	Fine/Lgt/W	36	85	0	20
210	B2		20/6am	HT	Fine/Lgt/W	36	169	31	110

211	B3	1999	20/6am	HT	Fine/Lgt/W	36	275	63	389
212	B1		22/6am	HT	Fine / No/W	43	146	0	20
213	B2		22/6am	HT	Fine / No/W	43	216	20	41
214	B3		22/6am	HT	Fine / No/W	43	471	20	148
215	B1		23/6pm	HT	Fine / No/W	55	20	1	0
216	B2		23/6pm	HT	Fine / No/W	55	74	20	10
217	B3		23/6pm	HT	Fine / No/W	55	171	31	20
218	B1		6/7am	HT	Fine/Lgt/W	50	51	10	10
219	B2		6/7am	HT	Fine/Lgt/W	50	86	20	10
220	B3		6/7am	HT	Fine/Lgt/W	50	109	0	10
221	B1		7/7am	HT	Ovc/Lgt/W	53	41	0	0
222	B2		7/7am	HT	Ovc/Lgt/W	53	52	0	10
223	B3		7/7am	HT	Ovc/Lgt/W	53	98	10	0
224	B1		10/7pm	HT	Fine / No/W	50	73	10	0
225	B2		10/7pm	HT	Fine / No/W	50	160	41	52
226	B3		10/7pm	HT	Fine / No/W	50	41	20	31
227	B1		11/7pm	HT	Fine / No/W	50	63	0	10
228	B2		11/7pm	HT	Fine / No/W	50	109	41	10
229	B3		11/7pm	HT	Fine / No/W	50	30	0	10
230	B1		12/7pm	HT	Ovc/Lgt/W	56	2755	41	10
231	B2		12/7pm	HT	Ovc/Lgt/W	56	331	41	41
232	B3		12/7pm	HT	Ovc/Lgt/W	56	52	0	10
233	B1		17/7pm	HT	Ovc/Lgt/W	0	419	31	41
234	B2		17/7pm	HT	Ovc/Lgt/W	0	441	74	0
235	B3		17/7pm	HT	Ovc/Lgt/W	0	359	195	10
236	B1		18/7pm	HT	Ovc/Lgt/W	26	4611	134	31
237	B2		18/7pm	HT	Ovc/Lgt/W	26	3255	31	63
238	B3		18/7pm	HT	Ovc/Lgt/W	26	11119	108	31
239	B1		19/7am	HT	Rain/Lgt/W	20	2755	275	74
240	B2		19/7am	HT	Rain/Lgt/W	20	1723	295	187
241	B3		19/7am	HT	Rain/Lgt/W	20	4106	512	331
242	B1		20/7am	HT	Fine / No/W	26	98	0	0
243	B2		20/7am	HT	Fine / No/W	26	393	10	20
244	B3		20/7am	HT	Fine / No/W	26	213	10	10
245	B1		21/7pm	HT	Fine / No/W	0	41	10	20
246	B2		21/7pm	HT	Fine / No/W	0	122	0	0
247	B3		21/7pm	HT	Fine / No/W	0	189	31	52
248	B1		22/7pm	HT	Fine / Lgt/W	22	41	0	0
249	B2		227pm	HT	Fine / Lgtw	22	20	0	0
250	B3		22/7pm	HT	Fine / Lgtw	22	41	0	0
251	B1		23/7pm	HT	Ovc/ Lgt/W	33	2590	20	0
252	B2		237pm	HT	Ovc/ Lgtw	33	8330	20	0
253	B3		23/7pm	HT	Ovc/ Lgtw	33	12997	504	336
254	B1		24/7pm	HT	Fine / No/W	22	20	0	0
255	B2		24/7pm	HT	Fine / No/W	22	41	10	0
256	B3		24/7pm	HT	Fine / No/W	22	41	0	74
257	B1		25/7pm	HT	Fine / Lgt/W	26	41	20	0
258	B2		25/7pm	HT	Fine / Lgtw	26	52	0	0
259	B3		25/7pm	HT	Fine / Lgtw	26	73	0	41
260	B1		3/8am	HT	Fine / Lgt/W	22	121	31	30
261	B2		3/8am	HT	Fine / Lgtw	22	275	31	20
262	B3		3/8am	HT	Fine / Lgtw	22	309	86	31
263	B1		4/8am	HT	Fine / Lgt/W	21	441	41	51
264	B2		4/8am	HT	Fine / Lgtw	21	108	10	0
265	B3		4/8am	HT	Fine / Lgtw	21	228	30	10

266	B1	1999	8/8pm	HT	Fine / No/W	16	31	0	20
267	B2		8/8pm	HT	Fine / No/W	16	31	0	0
268	B3		8/8pm	HT	Fine / No/W	16	41	0	0
269	B1		9/8pm	HT	Fine / No/W	18	31	0	0
270	B2		9/8pm	HT	Fine / No/W	18	201	63	10
271	B3		9/8pm	HT	Fine / No/W	18	471	74	96
272	B1		12/8pm	HT	Ovc/ Lgt/W	8	318	86	0
273	B2		12/8pm	HT	Ovc/ Lgtw	8	63	20	0
274	B3		12/8pm	HT	Ovc/ Lgtw	8	52	10	10
275	B1		22/8am	HT	Fine / Lgtw	16	275	41	20
276	B2		22/8am	HT	Fine / Lgtw	16	240	52	51
277	B3		22/8am	HT	Fine / Lgtw	16	189	10	31
278	B1		22/9pm	HT	Fine / No/W	2	85	0	31
279	B2		22/9pm	HT	Fine / No/W	0	0	0	10
280	B3		22/9pm	HT	Fine / No/W	0	175	31	97
281	B1		28/9am	HT	Fine / No/W	5	73	0	41
282	B2		28/9am	HT	Fine / No/W	5	41	0	0
283	B3		28/9am	HT	Fine / No/W	5	41	0	0
284	B1		29/9am	HT	Fine / No/W	5	10	0	0
285	B2		29/9am	HT	Fine / No/W	5	41	10	0
286	B3		29/9am	HT	Fine / No/W	5	10	0	0
287	B1		30/9am	HT	Fine / Lgtw	7	10	0	0
288	B2		30/9am	HT	Fine / Lgtw	7	31	0	0
289	B3		30/9am	HT	Fine / Lgtw	7	74	0	20
290	B1		3/10pm	HT	Fine / Lgtw	0	63	0	52
291	B2		3/10pm	HT	Fine / Lgtw	0	41	0	10
292	B3		3/10pm	HT	Fine / Lgtw	0	10	0	10
293	B1		7/11am	HT	Fine / Lgtw	0	52	0	10
294	B2		7/11am	HT	Fine / Lgtw	0	63	10	10
295	B3		7/11am	HT	Ovc/ Lgtw	0	31	0	10
296	B1		8/11am	HT	Ovc/ Lgtw	0	74	20	0
297	B2		8/11am	HT	Ovc / Lgtw	0	10	0	10
298	B3		8/11am	HT	Ovc / Lgtw	0	30	0	0
299	B1		9/11am	HT	Fine / Lgtw	0	10	0	63
300	B2		9/11am	HT	Fine / Lgtw	0	31	0	0
301	B3		9/11am	HT	Fine / Lgtw	0	41	0	20
302	B1	2000	22/2am	HT	Fine / Lgtw	22	75	31	10
303	B2		22/2am	HT	Fine / Lgtw	22	11	10	20
304	B3		22/2am	HT	Fine / Lgtw	22	282	189	63
305	B1		23/2am	HT	Fine / No/W	32	134	10	0
306	B2		23/2am	HT	Fine / No/W	32	98	10	0
307	B3		23/2am	HT	Fine / No/W	32	175	0	31
308	B1		24/2am	HT	Fine / No/W	32	52	52	10
309	B2		24/2am	HT	Fine / No/W	32	20	10	10
310	B3		24/2am	HT	Fine / No/W	32	108	0	30
311	B1		28/2/pm	HT	Fine / Lgtw	22	231	63	41
312	B2		28/2/pm	HT	Fine / Lgtw	22	432	97	160
313	B3		28/2/pm	HT	Fine / Lgtw	22	762	624	145
314	B1		3/3/pm	HT	Fine / No/W	30	108	74	74
315	B2		3/3/pm	HT	Fine / No/W	30	0	0	20
316	B3		3/3/pm	HT	Fine / No/W	30	41	20	63
317	B1		4/3/pm	HT	Fine / No/W	56	20	10	52
318	B2		4/3/pm	HT	Fine / No/W	56	52	31	110
319	B3		4/3/pm	HT	Fine / No/W	56	62	10	548
320	B1		4/3/pm	HT	Fine / No/W	58	31	20	10

321	B2	2000	4/3/pm	HT	Fine / No/W	58	20	0	20
322	B3		4/3/pm	HT	Fine / No/W	58	373	160	776
323	B1		8/3/am	HT	Fine / No/W	8	275	97	10
324	B2		8/3/am	HT	Fine / No/W	8	171	41	10
325	B3		8/3/am	HT	Fine / No/W	8	1054	259	96
326	B1		11/3/pm	HT	Fine / No/W	22	213	31	73
327	B2		11/3/pm	HT	Fine / No/W	22	171	41	52
328	B3		11/3/pm	HT	Fine / No/W	22	448	175	487
329	B1		9/4/pm	HT	Ovc/ Lgtw	38	109	20	20
330	B2		9/4/pm	HT	Ovc/ Lgtw	38	712	203	373
331	B3		9/4/pm	HT	Ovc / Lgtw	38	241	121	226
332	B1		13/4pm	HT	Fine / No/W	56	85	0	20
333	B2		13/4pm	HT	Fine / No/W	56	301	169	10
334	B3		13/4pm	HT	Fine / No/W	56	512	435	98
335	B1		15/4pm	HT	Fine / No/W	59	119	51	63
336	B2		15/4pm	HT	Fine / No/W	59	226	62	41
337	B3		15/4pm	HT	Fine / No/W	59	281	169	110
338	B1		26/4pm	HT	Fine / No/W	61	121	0	31
339	B2		26/4pm	HT	Fine / No/W	61	282	20	20
340	B3		26/4pm	HT	Fine / No/W	61	146	20	41
341	B1		30/4pm	HT	Fine / Lgt/W	57	62	10	41
342	B2		30/4pm	HT	Fine / Lgt/W	57	30	20	52
343	B3		30/4pm	HT	Fine / Lgt/W	57	135	31	63
344	B1		28/4pm	HT	Fine / No/W	35	3448	41	10
345	B2		28/4pm	HT	Fine / No/W	35	1553	309	31
346	B3		28/4pm	HT	Fine / No/W	35	759	21	31

Appendix B:

Comparison of indicator organism counts of Hataitai Beach and Greta Point stormwater outfall – July and August 1998

Date	Sample	Site	Weather	Tide	*Total coliforms* (per 100ml)/	*E.coli* (per 100ml)	*Enterococci* (per 100ml)
July							
19/07/1998	SW1	HB	Dry	Low	11	8	30
	SW2	GP			>2419	>2419	240
20/07/1998	SW3	HB	Dry	Low	45	12	370
	SW4	GP			>2419	>2419	108
20/07/1998	SW5	HB	Dry	High	13	6	20
	SW6	GP			15	9	52
20/07/1998	SW7	HB	Dry	Low	4	1	84
	SW8	GP			>2419	416	0
21/07/1998	SW9	HB	Dry	Low	365	132	30
	SW10	GP			>2419	914	51
21/07/1998	SW11	HB	Dry	High	25	17	20
	SW12	GP			20	15	52
21/071998	SW13	HB	Rain	Low	35	23	759
	SW14	GP			>2419	>2419	14136
22/07/1998	SW15	HB	Rain	Low	11	7	1246
	SW16	GP			>2419	275	2755
22/07/1998	SW17	HB	Rain	High	11	9	135
	SW18	GP			134	91	368
23/07/1998	SW19	HB	Rain	High	66	44	20
	SW20	GP			>2419	>2419	5748
23/07/1998	SW21	HB	Dry	Low	2382	310	146
	SW22	GP			67	34	41
24/07/1998	SW23	HB	Dry	High	2909	238	933
	SW24	GP			>2419	921	728
24/07/1998	SW25	HB	Dry	Low	305	121	122
	SW26	GP			>2419	416	24192

25/07/1998	SW27	HB	Dry	High	121	86	10
	SW28	GP			>2419	607	677
25/07/1998	SW29	HB	Dry	Low	20	10	10
	SW30	GP			17	10	313
26/07/1998	SW31	HB	Dry	High	97	20	130
	SW32	GP			1533	193	1948
August							
24/08/1998	SW33	HB	Rain	High	108	10	10
	SW34	GP			6131	3654	173
24/08/1998	SW35	HB	Dry	Low	11199	958	134
	SW36	GP			41	0	0
25/08/1998	SW37	HB	Rain	High	1616	135	292
	SW38	GP			1664	798	1396
25/08/1998	SW39	HB	Dry	High	313	31	1119
	SW40	GP			1935	122	249
26/08/1998	SW41	HB	Dry	Low	109	74	52
	SW42	GP			1274	422	158
26/08/1998	SW43	HB	Rain	Low	31	0	0
	SW44	GP			3448	359	262
27/08/1998	SW45	HB	Rain	High	216	137	198
	SW46	GP			>24192	6867	14136
27/08/1998	SW47	HB	Rain	Low	31	0	119
	SW48	GP			>24192	43512	17329
28/08/1998	SW49	HB	Dry	High	156	10	480
	SW50	GP			1187	794	213
28/08/1998	SW51	HB	Dry	Low	96	51	0
	SW52	GP			6867	878	402
29//08/1998	SW53	HB	Dry	High	185	30	245
	SW54	GP			959	487	20

Appendix C:

Ministry for the Environment results 01/11/99 to 26/03/01

Retrieved 02/04/2000 from: *World Wide Web:*
http://www.marine.mfe.govt.nz/beach-water/wellington

http://marine.mfe.govt.nz/marine/beach-water/wellington/seasons/season1999-2000.html

Retrieved 02/04/2000 from: *World Wide Web:*
http://www.marine.mfe.govt.nz/beach-water/wellington

MfE Beach Water Quality Indicator -- Hataitai Beach

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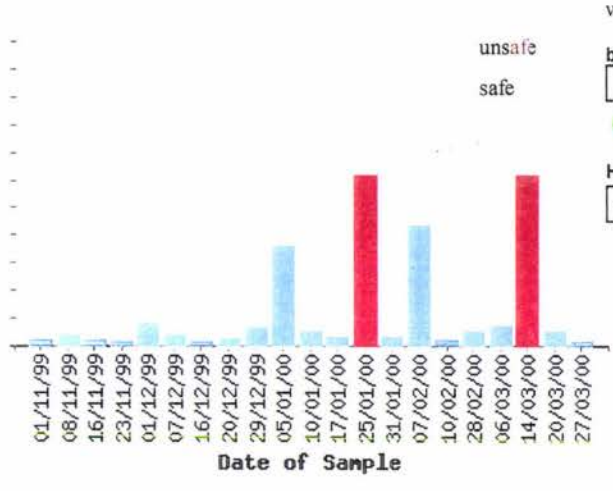
ENVIRONMENTAL PERFORMANCE INDICATORS PROGRAMME

Beach Water Quality

Hataitai Beach

27/03/00	4
20/03/00	24
14/03/00	308
06/03/00	36
28/02/00	24
10/02/00	8
07/02/00	216
31/01/00	16
25/01/00	308
17/01/00	16
10/01/00	24
05/01/00	180
29/12/99	32
20/12/99	12
16/12/99	4
07/12/99	20
01/12/99	40
23/11/99	5
16/11/99	10
08/11/99	20
01/11/99	8

Microbiological readings at Hataitai Beach



wellington

beaches in wellington

Balaena Bay

Go

Hataitai Beach

All Seasons

Go

NZ Map

Other Indicators

Retrieved 10/05/2001 from: *World Wide Web:*
http://www.marine.mfe.govt.nz/beach-water/wellington

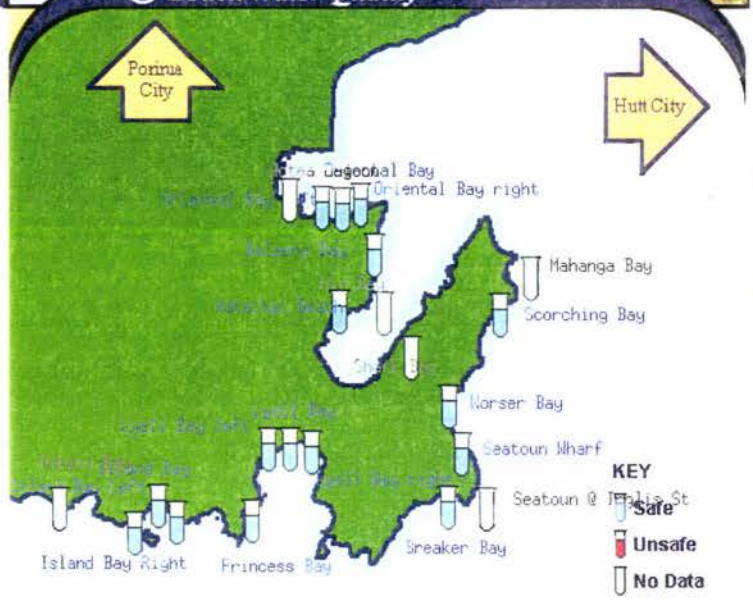
ENVIRONMENTAL PERFORMANCE INDICATORS PROGRAMME

Beach Water Quality

Seasonal medians 2000/2001
wellington

Balaena Bay		2.0
Hataitai Beach		7.0
Island Bay		2.0
Lyall Bay		2.0
Oriental Bay		3.0
Princess Bay		2.0
Scorching Bay		2.0
Seatoun Wharf		2.0
Worser Bay		2.0
Breaker Bay		2.0
Island Bay Left		4.0
Island Bay Right		3.0
Oriental Bay left		3.0
Oriental Bay right		3.0
Lyall Bay left		2.0
Lyall Bay right		2.0
Aotea Lagoon	-	no data
Kio Bay	-	no data
Shark Bay	-	no data
Mahanga Bay	-	no data
Seatoun @ Inglis St	-	no data
Owhiro Bay	-	no data

(Enterococci per 100ml)
Enterococci levels greater than 277 indicate that beach failed to meet the guidelines on the date of the sample.



Click an area of the map for data on beach water quality.

Available Results

Latest season 26/11/02

Previous Seasons 2001-2002

Information used in this Indicator was supplied by:

Wellington City Council

NZ Map

Other Indicators

Retrieved 10/05/2001 from: *World Wide Web:*
http://www.marine.mfe.govt.nz/beach-water/wellington

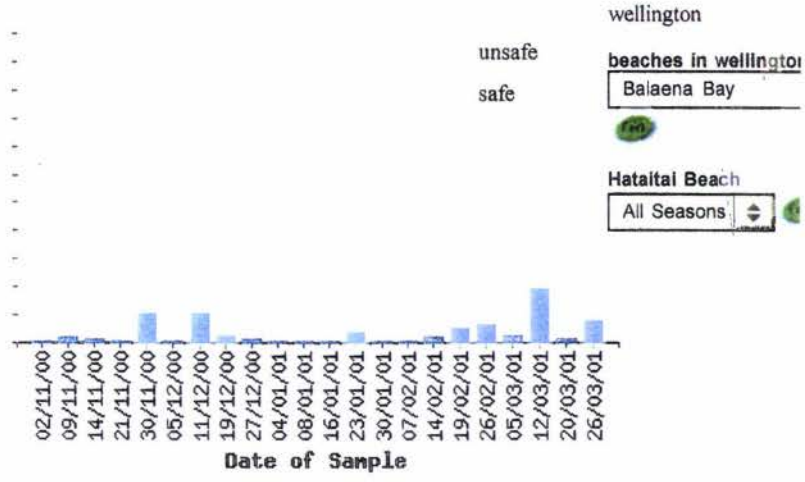
ENVIRONMENTAL PERFORMANCE INDICATORS PROGRAMME

Beach Water Quality

Hataitai Beach

26/03/01	40
20/03/01	4
12/03/01	96
05/03/01	12
26/02/01	32
19/02/01	24
14/02/01	8
07/02/01	2
30/01/01	2
23/01/01	20
16/01/01	2
08/01/01	2
04/01/01	2
27/12/00	6
19/12/00	12
11/12/00	52
05/12/00	2
30/11/00	52
21/11/00	2
14/11/00	4
09/11/00	8
02/11/00	2

Microbiological readings at Hataitai Beach



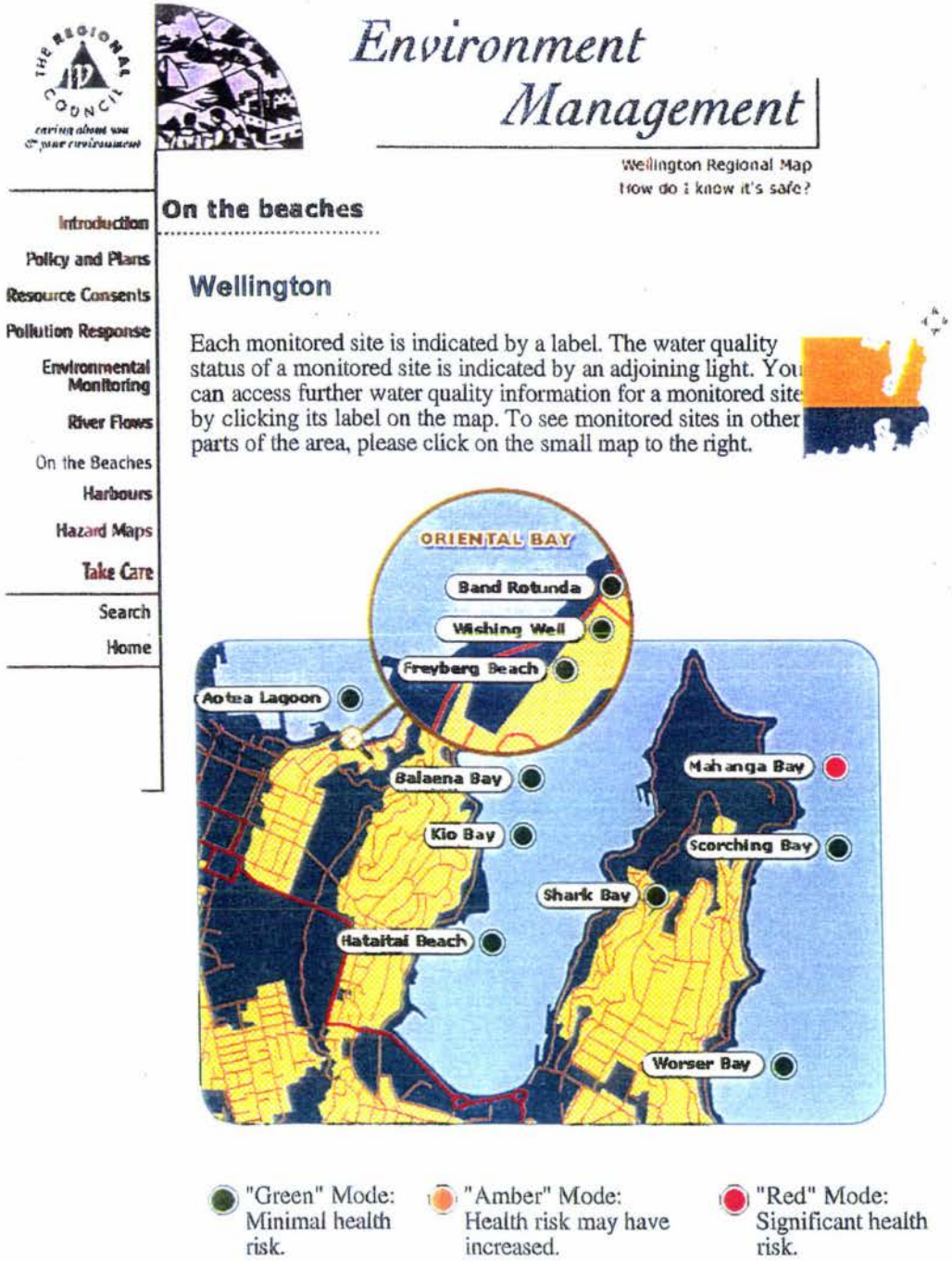
NZ Map

Other Indicators

Appendix D:

Wellington Regional Council results 05/11/01 to 17/06/02

Retrieved 22/06/2002 from: *World Wide Web:*
http://www.wrc.govt.nz/EDC/MON/Bathingsites/index.cfm



Retrieved 22/06/2002 from: *World Wide Web:*
http://www.wrc.govt.nz/EDC/MON/Bathingsites/index.cfm

Environment Management

Wellington Regional Map
How do I know it's safe?

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On the beaches

Site: Hataitai Beach

Sample Date **17 June 2002**
Parameter Analysed **Enterococci**
Result **124 CFU/100mL**

17 ◆ JUN ◆ 2002 ◆ Week ○ Month ○ Year ◉

Results for the period **05-NOV-2001 to 17-JUN-2002**

Sample Date	Parameter Analysed	Result (cfu/100 mL)
05-Nov-2001	Enterococci	4
12-Nov-2001	Enterococci	4
20-Nov-2001	Enterococci	2
27-Nov-2001	Enterococci	4
04-Dec-2001	Enterococci	230
06-Dec-2001	Enterococci	72
07-Dec-2001	Enterococci	100
11-Dec-2001	Enterococci	4
18-Dec-2001	Enterococci	12
24-Dec-2001	Enterococci	24
31-Dec-2001	Enterococci	16
07-Jan-2002	Enterococci	4
14-Jan-2002	Enterococci	4
22-Jan-2002	Enterococci	20
29-Jan-2002	Enterococci	20
04-Feb-2002	Enterococci	24
12-Feb-2002	Enterococci	8
18-Feb-2002	Enterococci	140
19-Feb-2002	Enterococci	6
26-Feb-2002	Enterococci	4
04-Mar-2002	Enterococci	990
05-Mar-2002	Enterococci	8
07-Mar-2002	Enterococci	2
08-Mar-2002	Enterococci	3
11-Mar-2002	Enterococci	12
18-Mar-2002	Enterococci	170
19-Mar-2002	Enterococci	360
21-Mar-2002	Enterococci	56
25-Mar-2002	Enterococci	8
22-Apr-2002	Enterococci	120
06-May-2002	Enterococci	140
20-May-2002	Enterococci	4
05-Jun-2002	Enterococci	130
17-Jun-2002	Enterococci	124

Appendix E:

Data for cysts* in duck faecal samples morphologically resembling *Giardia* spp. but identified as *Caryospora* spp. (After staining with Merifluor® direct immunofluorescent reagent – Epifluorescence 200 X magnification)

*In 5 to 6 randomly selected fields: >10 cysts = 3+; 5-10 cysts = 2+; <5cysts = 1+

Sample	Cysts per field	Grading		Sample	Cysts per field	Grading
2	6	2+		149	<3	1+
3	3	1+		150	<5	1+
4	2-3	1+		153	3	1+
6	8	2+		162	4	1+
9	6-8	2+		164	2	1+
19	3	1+		167	12	3+
26	3	1+		168	3	1+
30	1	1+		171	10-12	3+
32	12	3+		177	>15	3+
33	1	1+		180	8	2+
35	>10	3+		181	7-8	2+
43	2	1+		182	<3	1+
45	2	1+		187	22	3+
55	>30	3+		188	6	2+
56	2	1+		189	10-13	3+
67	>20	3+		200	20-30	3+
71	>10	3+		201	2-3	1+
72	3	1+		203	5-6	2+
73	>10	3+		205	5-6	2+
76	5	2+		206	8	2+
77	>30	3+		207	>20	3+
78	6	2+		214	8	2+
79	12	3+		216	10	2+
80	>15	3+		217	16	3+
93	18	3+		218	6	2+
94	20	3+		221	<5	1+
99	3	1+		225	10	2+
104	3	1+		226	6	2+
110	6-8	2+		231	20	3+
112	15	3+		234	5-6	2+
115	15	3+		246	2-3	1+
116	12	3+		249	2-3	1+
117	<5	1+		252	<5	1+
119	20	3+		253	<5	1+
122	3	1+		263	12-15	3+
131	10-12	3+		264	5-6	2+
133	12	3+		266	15-20	3+
136	11	2+		269	12	3+
138	6	2+		276	20-25	3+
145	12	3+		277	2-3	1+
148	<3	1+				

Appendix F:

DNA ladders

The DNA ladders used in this thesis to size and /or quantify the PCR fragments were supplied by Gibco BRL and are described below (taken from the Gibco BRL 1998-1999 Product Catalogue).

1Kb DNA Ladder (Gibco BRL)

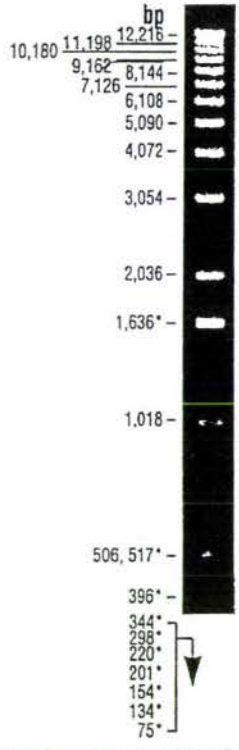
1 Kb DNA Ladder (U.S. Patent No. 4,403,036) is suitable for sizing linear double-stranded DNA fragments from 500 bp to 12 kb. Prepared from a plasmid containing repeats of a 1,018-bp DNA fragment (1), the ladder consists of 12 fragments ranging from 1,018 bp to 12,216 bp. In addition to these 12 bands, the ladder contains vector DNA fragments that range from 75 to 1,636 bp. The double-stranded ladder can be visualized on 0.5 to 1% agarose gels after ethidium bromide staining. This ladder may be radiolabeled using T4 polynucleotide kinase, T4 DNA polymerase, DNA polymerase I, or the large fragment of DNA polymerase I (Klenow fragment).

Concentration in storage buffer: 1 µg/µl in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.1 mM EDTA.

Recommended storage condition: −20°C.

Reference(s):

1. Hartley, J.L. and Donelson, J.E. (1980) *Nature (Lond.) 286*. 860.



0.5 µg/lane; 1.0% agarose gel stained with ethidium bromide.* *H* fragments of the vector

1 Kb Plus DNA Ladder (Gibco BRL)

The 1 Kb PLUS DNA LADDER is suitable for sizing linear double-stranded DNA fragments from 100 bp to 12 kb. The ladder contains a total of twenty bands: twelve bands ranging in size from 1000 bp to 12,000 bp in 1000-bp increments and eight bands ranging in size from 100 to 1650 bp. The 1650-bp band contains approximately 8% of the mass applied to the gel. The ladder may be radioactively labeled by one of the following methods: (i) Partial exonucleolytic degradation and resynthesis with T4 DNA polymerase. This method is preferred because higher specific activity is achieved with less ^{32}P input; (ii) Labeling the 5′ ends with T4 polynucleotide kinase; (iii) Filling in the 3′ recessed ends with *E. coli* DNA polymerase I or the large fragment of DNA polymerase I.

Storage Buffer:
10 mM Tris-HCl (pH 7.5)
1 mM EDTA
50 mM NaCl

1 Kb PLUS DNA LADDER
0.7 µg/lane
0.9% agarose gel
stained with ethidium bromide

Low Molecular Mass Ladder (Gibco BRL)

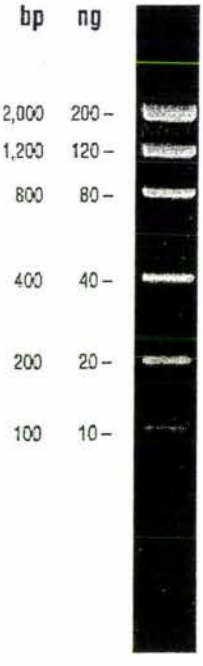
Low DNA Mass Ladder (patent pending) is suitable for estimating the mass of unknown DNA samples by ethidium bromide staining (1). Prepared from a specially constructed plasmid, the ladder consists of an equimolar mixture of six blunt-ended fragments from 100 to 2,000 bp. Electrophoresis of 4 µl of Low DNA Mass Ladder results in bands containing 200, 120, 80, 40, 20, and 10 ng (470 ng total) of DNA, respectively.

Concentration in storage buffer: 470 ng/4 µl in 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Recommended storage condition: −20°C.

Reference(s):

1. Hartley, J.L. and Xu, L. (1994) *Focus* 16, 52.



Appendix G:

Reagent formulations

BROMOPHENOL BLUE DYE SOLUTION

0.1% w:v Bromophenol blue (BDH)	0.1 g
80% v:v Glycerol	0.8 ml
1X E buffer	2.0 ml

Store at room temperature.

10% CHELEX 100™

Chelex (Biorad)	1.0 g
Milli-Q H_20 up to	10.0 ml

Store the suspension at room temperature and mix thoroughly immediately prior to use.

EVAN'S BLUE (1%)

Evan's blue (CI 23860, BDH)	1.0 g
Distilled H_20 up to	100.0 ml

Dissolve the Evan's blue in distilled water. Store the solution at room temperature and use within 6 months of preparation.

10X E BUFFER (EDTA)

Tris (Sigma)	96.88 g
EDTA	7.44 g
Sodium acetate	8.20 g
Distilled H_20 up to	2000.0 ml

Adjust to pH 7.8 with glacial acetic acid and then prepare a 1X solution by diluting one volume of the 10X E buffer in nine volumes of distilled H_20. Store the solution at room temperature until required.

LUGOL'S IODINE SOLUTION

Potassium iodide	10.0 g
Powdered iodine	5.0 g
Distilled H_20	100.0 ml

Dissolve the potassium iodide in the distilled H_20 and then slowly add with continuous stirring, the iodine until it is dissolved. Filter the solution through filter paper into a brown bottle and stopper tightly. Store the solution at room temperature and use within 3 weeks of preparation.

PHOSPHATE BUFFERED SALINE ELUTING SOLUTION (PBSES, pH7.4)

(A) 10X PBS (Phosphate Buffered Saline)

Sodium chloride (NaCl)	80.0 g
Potassium dihydrogen phosphate (KH_2PO_4)	2.0 g
Disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$)	29.0 g
Potassium chloride (KCl)	2.0 g
Distilled H_20 up to	1000.0 ml

Dissolve the reagents in about 800 ml of distilled H_20 and make up the solution to 1 liter. Prepare a 1X PBS solution by diluting one volume of the 10X PBS in nine volumes of distilled H_20. Adjust the pH to 7.4 with 0.1M HCl or 0.1M NaOH and autoclave at 121°C and 103kPa for 15 minutes. Store the solution at room temperature until required.

(B) 10% SDS (Sodium Dodecyl Sulphate)

Sodium dodecyl sulphate	100.0 g
Distilled H_20 up to	1000.0 ml

To prepare a 1% SDS solution dilute one volume of the 10X SDS solution in nine volumes of distilled H_20. Store the solution at room temperature until required.

(C) 1 % Tween 20 Solution

Polyoxyethylene sorbitan monolaurate (Tween 2, Sigma)	1.0 ml
Distilled H_20	99.0 ml

Store the solution at room temperature until required. Prepare a 0.01% solution by diluting one volume of the 1% Tween 20 solution in 99 volumes of distilled water.

(D) 10 X Eluting Solution

1% SDS	100.0 ml
1 % Tween 20 solution	1.0 ml
10X PBS	100.0 ml
Distilled H_20 up to	1000.0 ml

Add 500 ml of distilled H_20 to the reagents (SDS, Tween 20 and PBS) and adjust the pH to 7.4 with 0.1M HCl or 0.1M NaOH. Make up the solution to 1 liter with distilled H_20. The solution must be used within one week of preparation.

10X SDS LOADING DYE (Sucrose-EDTA-Dodecyl)

20% Sucrose	4.0 g
0.05M EDTA	0.2µl of 0.5M
10% SDS	2.0 ml
Bromophenol blue dye solution	40.0 µl
Distilled H_20 up to	20.0 ml

Mix the reagents and dispense in 5.0ml aliquots. Store at room temperature.

1.0M SUCROSE SOLUTION

Sucrose	102.7 g
Distilled H_20 up to	300.0 ml

Dissolve the sucrose in warm (40°C) distilled water. Store the solution at –20°C. Prior to use place the solution in a 37°C water bath until completely thawed.

TE BUFFER (TRIS-EDTA)

(A) 0.2M EDTA

EDTA (Ethylene diamine tetra-acetic acid – sodium salt)	7.44 g
Distilled H_20 up to	1000.0 ml

Adjust the solution to pH 7.2 by adding 5M NaOH. Store at room temperature

(B) 1.0M Tris-HCl (pH 7.5)

Tris	121.0 g
Distilled H_20 up to	1000.0 ml

Adjust the solution to pH 7.5 by adding 5M HCl. Store at room temperature.

(C) TE Buffer

1M Tris-HCl (pH 7.5)	1.0 ml
0.2M EDTA (pH 7.2)	5.0 ml
Distilled H_20 up to	10.0 ml

Autoclave the solution at 121°C and 103kPa for 15 minutes. Store the solution at room temperature until required.

Appendix H:

Publications

New Zealand Journal of Marine and Freshwater Research, 1998, Vol. 32: 505–513 505
0028–8330/98/3204–0505 $7.00 © The Royal Society of New Zealand 1998

Evaluation of Enterolert® for the enumeration of enterococci in the marine environment

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Abstract Current methods for the analyses of enterococcal densities include the membrane filtration (MF) technique and the multiple tube fermentation technique for the most probable number (MPN). Both techniques are labour intensive, tedious, and require a minimum of 48–72 h before results can be obtained. The Enterolert® system, designed to detect enterococci in water in 24 h, uses 4-methylumbelliferyl-ß-D-glucoside as a defined substrate nutrient indicator. This compound, when hydrolysed by enterococcal-ß-glucosidase, releases 4-methylumbelliferone which exhibits fluorescence under a UV_{365} lamp. In this study 343 marine water samples from selected sites in the Wellington area of New Zealand were tested to evaluate the sensitivity and specificity of Enterolert in parallel with the MF method. Statistical analysis of parallel test results showed a strong linear correlation ($r = 0.927$) and no significant difference between the two methods by paired t-test analysis ($P = 0.39$). Based on the 2.4% false positive and 0.3% false negative rates, Enterolert was found to have a sensitivity of 99.8% and a specificity of 97.0%. Activity-costing analyses revealed that the variable cost per test was less for Enterolert (NZ$18.33) than MF (NZ$22.79). Significant time savings are achieved because Enterolert requires less time than MF for reagent preparation, sample set-up, incubation, and reading of tests. The results from this study suggest that more widespread use of this new technology in marine water quality monitoring is warranted, since rapid tests mean that monitoring agencies can respond to sudden increases in enterococci numbers more quickly and can therefore take immediate corrective action to ensure the safety of users of recreational waters.

Keywords enumerating enterococci; marine water; Enterolert®; defined substrate technology; enterococcal-ß-glucosidase; quanti-tray™; 4-methylumbelliferone fluorescence; membrane filtration; most probable number; rapid test; linear correlation; cost analyses; recreational water quality

INTRODUCTION

In 1986 the United States Environmental Protection Agency (USEPA) developed new microbiological water quality guidelines for recreational marine water, with enterococci as the indicator organisms for faecal pollution (Anon. 1986). These guidelines were based on epidemiological studies, carried out at three United States coastal areas between 1973 and 1978, that demonstrated a strong correlation between enterococci in marine water and swimming-associated illness (Cabelli et al. 1982; Cabelli 1983).

In January 1992 the New Zealand Department of Health issued provisional microbiological water quality guidelines for recreational and shellfish-gathering waters based on the USEPA guidelines (McBride et al. 1992). These guidelines were considered provisional because of New Zealand's: (1) lack of chlorinated effluent waters; (2) higher ratio between animal and human waste; and (3) the possibility of different immunity rates in the population compared to the situation in the United States (McBride et al. 1993). The results of recent New Zealand epidemiological studies at selected beaches are consistent with the Cabelli studies in that enterococci were also found to be the most appropriate health-effects indicator (Bandaranayake et al. 1996).

M98021
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The 1991 Resource Management Act (RMA) allows regional councils to set numeric standards based on microorganisms considered suitable to meet section 5 (2) in the Third Schedule of the Act, namely, that the water shall not be rendered unsuitable for bathing by the presence of contaminants. Since 1992 several New Zealand councils have adopted enterococci as alternatives to faecal coliforms for monitoring coastal waters (Sinton & Donnison 1994). However, as these authors point out, regional councils may set numeric standards based on whatever organisms they consider suitable to meet the narrative microbiological standards specified in the Resource Management Act.

Most New Zealand water testing laboratories use conventional methods for detecting and enumerating enterococci, namely the membrane filtration (MF) technique and/or the multiple tube fermentation test which is based on the most probable number (MPN) estimate (Donnison 1992; Lewis & Donnison 1994; Anon. 1995a). However, a 1994 report on water quality states that there is a lack of consistency among regional councils in the methods used to monitor the microbiological quality of recreational water (Anon. 1994). The report further states that it will be necessary to agree on standards and methodology to enable proper nation-wide comparisons to be made. As noted by Sinton et al. (1993), if enterococcal standards are to be effectively implemented a new set of standard protocols for New Zealand laboratories may be needed since membrane filtration enumeration of enterococci is more time consuming than enumeration for faecal coliforms.

The approved MF method (Anon. 1986, 1993, 1995b) for enterococci is a 2-step procedure; incubation of the membrane filter at 41°C on a selective and differential agar medium (mE) for 48 h followed by transfer of the membrane to an *in situ* esculin-iron agar (EIA) substrate medium and incubation for 20 min at 41°C. Pink-red colonies on the membrane producing a brownish black precipitate on EIA are identified as enterococci. The brownish black precipitate is the result of the hydrolysis of esculin to glucose and coumarin by the enzyme ß-glucosidase. Coumarin forms a black precipitate in the presence of ferric citrate. Although the MF method has been shown to efficiently recover enterococci from marine and estuarine waters, the false positive and negative rates were reported to be 10.0 and 11.7% respectively (Levin et al. 1975; Budnick et al. 1996). The MF method requires the dilution of water samples which contain high bacterial numbers in order to obtain counts in the acceptable counting range of 20–60 colonies. Turbid samples can block the membranes, and the growth of competing bacteria can often cause inhibition of growth of target organisms or mask the colour changes associated with carbohydrate fermentation (Fricker & Fricker 1996).

The MPN test has been used in the microbiological monitoring of water quality for many years (McGrady 1915) and its validity and suitability for estimating bacterial numbers is explained in Cochran's classic paper (1950) and by Woodward (1957). The MPN method for enterococci is a 3-stage test which takes 4 days to complete. The first (presumptive) stage uses liquid medium containing azide dextrose to inhibit non-target bacteria. All azide dextrose broth tubes showing turbidity after 24 48 h incubation are subjected to confirmation on t... same media used for the membrane filtration method (Donnison 1992).

The MPN method has been found to give statistically equivalent results to the membrane filtration method even though the techniques are significantly different (Fullerton 1995). Although the MPN method is particularly useful for turbid water samples, sediments, and effluents, traditional MPN methods are time consuming because of the number of tubes and sample manipulations required and are therefore unsuitable when it is necessary to process large numbers of water samples.

The MPN principle, as used in the recently developed Enterolert® Quanti-Tray™ system (IDEXX Laboratories, United States), allows for the inoculation, with minimum sample manipulation, of a greater number of wells than the standard MPN method. The Quanti-Tray is a sterile, disposable tray containing 51 separate wells. Sealing of the sample-filled tray is accomplished by the use of a special designed Quanti-Tray sealer which automatically distributes and seals a 100 ml sample. The Enterolert system is based on Defined Substrate Technology (Edberg et al. 1988; Fiksdal et al. 1994) that has been used successfully to monitor faecal pollution of drinking water and recreational waters in significantly less time than membrane filtration procedures. Enterolert utilises 4-methylumbelliferyl-ß-D- glucoside as the defined substrate nutrient indicator. This compound, when hydrolysed by enterococcal-β-glucosidase, releases 4-methylumbelliferone which exhibits fluorescence under a UV_{365} lamp. The enterococci numbers per 100 ml can be determined by referring to a 51-well MPN table based on the number of positive blue fluorescing wells.

In this study, we evaluated Enterolert and compared it to the standard membrane filtration

technique using water samples collected from a variety of coastal sites in the Wellington region. The study was undertaken in order to determine the suitability and reliability of Enterolert for detecting and enumerating enterococci in New Zealand marine waters.

MATERIALS AND METHODS

Sample collection

A total of 343 marine water samples were collected over a period of 12 months during dry as well as wet weather conditions. The samples were collected from various sites including recreational bathing ıter, tidal lagoons, water from marinas, untreated sewage effluent outfalls, and marine water samples from stormwater-drainage sites. Samples obtained from bathing areas, lagoons, and stormwater sites were taken by wading out to either adult chest height or waist depth depending on the wave conditions. Samples were taken from below the surface using the Mighty Gripper (Bolton Co., Whangarei, New Zealand) sampling device. Samples obtained from sewage outfall and marina sites were taken from below the surface using the Mighty Gripper bottle clamp attached to a long cord. Samples were collected aseptically in either sterile 500 ml or 1000 ml glass bottles, placed on ice in a large chilli-bin, transported to the laboratory within 3 h and processed within 6 h of arrival in the laboratory. The membrane filtration and Enterolert procedures were performed using aliquots from the same thoroughly mixed sample bottle.

Membrane filtration procedure

he standard MF procedure (Anon. 1986, 1993, 1995b) was performed as detailed in "A practical guide to monitoring bacterial indicators in New Zealand waters and effluents" (Donnison 1992). Selection of sample volumes to be filtered varied with sample site, and were based on prior history of the site. At least two different duplicate volumes from each sample were filtered. Depending on the sample source, this involved filtering either 1, 5, 10, 25, 50, 75, 100, or 200 ml volumes of undiluted sample, or 50 and 100 ml volumes of a 1 in 10 dilution (in phosphate buffered saline, pH 7.4) for samples expected to yield high levels of enterococci, such as those from the sewage outfalls and stormwater sites.

Each filtration series was commenced with steam-sterilised filtration units. A sterile 47 mm diameter, 0.45 µm pore size, gridded cellulose acetate membrane filter (Gelman Sciences, Ann Arbor, Michigan, United States) was placed aseptically onto the filter support base and the funnel attached. Before filtration of the sample and in the absence of a vacuum, 20 ml of sterile phosphate buffered saline was poured onto the filter. Following the filtration of each volume of the sample, the funnel was rinsed twice with 20 ml of buffered saline and the filter transferred to the surface of a well dried mE agar plate (Difco Laboratories, Detroit, Michigan, United States) and incubated for 48 h at 41 ±0.5°C.

Membrane filters with any pink-red colonies on the surface were transferred to EIA agar plates (Difco Laboratories) for 20 min of incubation at 41 ±0.5°C to confirm esculin hydrolysis. Pink-red colonies that developed a black or reddish-brown precipitate on the underside of the filter were considered positive for enterococci. These colonies were counted with a Chiltern colony counter (Biolab Scientific, Auckland, New Zealand) and the number of enterococci calculated per 100 ml of sample volume. Pink-red colonies that did not develop a black or reddish-brown precipitate on the underside of the filter were considered negative for enterococci.

Enterolert procedure

The Enterolert procedure was performed according to the manufacturer's instructions (IDEXX Laboratories, Westbrook, Maine, United States). A 1 in 10 dilution of the water sample was made (10 ml of sample plus 90 ml of sterile distilled water) in a sterile 100 ml bottle. One package of the powdered Enterolert reagent was added to the bottle and after shaking to dissolve the powder, the mixture was poured aseptically into a sterile 51-well Quanti-Tray. The tray was then mechanically sealed in a Quanti-Tray sealer, which simultaneously distributed the mixture into the wells, and incubated for 24 h at 41 ±0.5°C. After incubation the tray was viewed in a darkened room by placing it under and within 12 cm of a 365 nm wavelength ultraviolet light with a 6 Watt bulb (De Saga, Heidelberg, Germany). Blue fluorescence in a well was considered a positive reaction for that well and indicated the presence of enterococci. The number of enterococci per 100 ml was determined, based on the number of positive wells counted, by referring to a 51-well MPN table and the result multiplied by the dilution factor of 10. Wells showing no fluorescence were considered negative for enterococci.

Enterolert well-confirmation procedure

The optimum growth of enterococci occurs at 35°C. However, most strains grow at 45°C, tolerate 6.5% sodium chloride, and can hydrolyse esculin in the presence of 40% bile salts (Mundt 1986; Facklam & Sahm 1995). A procedure using these growth characteristics was used to determine the sensitivity and specificity of Enterolert. A total of 844 positive wells and 656 negative wells were tested for the presence of enterococci by steps of selective isolation and biochemical identification. An Oxford pipette (Sherwood Medical, St. Louis, Missouri, United States) with a sterile, disposable polypropylene tip was used to aseptically pierce the underside of the Quanti-Tray and aspirate 20 µl of the well broth. This inoculum was seeded onto bile-esculin agar (BEA) and incubated at 35°C for 24–48 h. No growth after 48 h was considered a negative test for enterococci; the presence of small black colonies was considered as a presumptive positive test for enterococci. Further confirmatory steps included the use of brain-heart infusion/NaCl broth (BHI/NaCl) incubated at 45°C and/or 35°C for 24–48 h, and culture-purity testing using plates and slants of nutrient agar, brain-heart infusion agar, brain-heart infusion/NaCl agar, and sheep blood agar. Gram positive cocci from catalase negative colonies that were found to be BEA positive but negative for growth in BHI/NaCl at 35 and 45°C were further identified using API STREP 20 Kits (bioMerieux Sa, Lyon, France). Full details of this well confirmation procedure are available on request from the authors.

Activity-costing analyses

Activity costing analysis, also called micro-costing (Luce et al. 1996; Drummond et al. 1997), was undertaken in order to measure the variable costs for each test procedure. Each procedure was broken down into a series of activities. An observer measured labour time and recorded consumables used for each activity. The analytic perspective taken was that of an established laboratory and it was assumed that overhead costs would not be altered by either test. Prices were obtained from New Zealand price lists as at 1 October 1997 and were exclusive of goods and services tax (GST) as this is a transfer payment. Labour rates were those paid by testing laboratories in New Zealand at 1 October 1997.

Enterolert costing was broken down into the following activities: (1) preparation and dispensing of sterile distilled water; (2) dilution of water samples, adding reagents, filling, and sealing Quanti-Trays; (3) 24-h incubation; (4) counting fluorescent wells in Quanti-Trays; (5) calculating results; and (6) cleanup and autoclaving of used trays, pipettes, and reagent bottles. These activities were observed for 20 routine Enterolert sample runs.

Membrane filtration costing was divided into the following activities: (1) preparation of sterile phosphate buffered saline, mE and EIA agar plates, and filtration units; (2) filtering of water samples and placement of membrane filters onto mE agar plates; (3) 48-h incubation; (4) transfer of filters to EIA agar plates; (5) 20 min incubation; (6) counting colonies; (7) calculating results; and (8) cleanup and autoclaving of used equipment and materials. These activities were observed for 20 routine membrane filtration sample runs.

Statistics

A comparison of the Enterolert and MF methods for statistical purposes was performed using Windows Excel software programme (Microsoft Corporation, United States). The standard statistical methods used were the correlation coefficient (r) and paired t-test (P). Enterolert's sensitivity, specificity, predictive value positive and predictive value negative were calculated using the false positive and false negative rates obtained from the well confirmation data.

RESULTS

No statistically significant difference between the Enterolert MPN values and the MF counts was found by paired t-test analysis ($P = 0.39$). Analysis of the results showed a strong positive correlation (correlation coefficient, $r = 0.927$) between both methods for 312 samples within the range 0–2400 (Fig. 1). By excluding one outlier (MF = 2400, Enterolert = 560) from the calculation a correlation coefficient of 0.961 was obtained.

The results of 21 samples could not be included in the correlation coefficient or t-test calculations because Enterolert values were obtained that were greater than 2005. The MF counts for these >2005 Enterolert values ranged from 1300 to too numerous to count (TNTC).

During the course of this study a 97-well Quanti-Tray (Quanti-Tray 2000) was released by IDEXX Laboratories and this was evaluated by testing 10 additional water samples. A correlation coefficient of $r = 0.9998$ was obtained for these 10 samples (Enterolert range = 10–15 238 and MF range = 3–14 300).

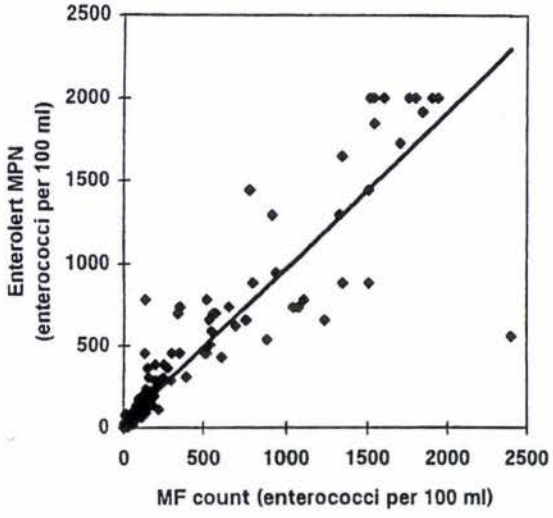

Fig. 1 Correlation between Enterolert® Quanti-Tray most probable number (MPN) values and membrane filtration (MF) counts for 312 samples within the counting range 0–2400. Correlation coefficient between the two methods was 0.927 (Y=10.4 + 0.90X).

The disagreement in the comparative results of the majority of samples made no difference to the water quality acceptability status. However by classifying 48 samples (15%) with variation in single sample maxima results as acceptable water quality by one method but not the other, more samples were found to be acceptable by MF than Enterolert (Table 1).

The results of the positive and negative well confirmation tests are summarised as follows: of the 844 positive wells, 824 (97.6%) were true positive and 20 (2.4%) were false positive; the 20 false positives were identified as *Bacillus* spp. (3 isolates), *Aerococcus viridans* (9 isolates), *Leuconostoc* spp. (6 isolates), *Streptococcus bovis* (1 isolate), and *Streptococcus mitis* (1 isolate). Of the 656 negative wells 654 (99.7%) were true negative and 2 (0.3%) false negative; these 2 false negatives were identified as *Enterococcus faecium* and *E. gallinarum/E. casseliflavus* (good identification to the genus level). Based on these results, Enterolert has a sensitivity of 99.8% and a specificity of 97.0% and its predictive value positive and predictive value negative is 98.0 and 99.7% respectively.

The total variable cost per sample was found to be NZ$18.33 for testing by Enterolert and NZ$22.79 for testing by MF. Details of the two costings and other procedure comparisons are shown Table 2.

DISCUSSION

The results of this study show that Enterolert is a simple and rapid 24-h system for detecting and enumerating enterococci in marine water and has a sensitivity of 99.8% and specificity of 97.0%. In this evaluation Enterolert was found to have a false positive rate of 2.4% and a false negative rate of 0.3%. These results are consistent with the results of studies undertaken in the United States where Enterolert's false positive rate was found to be ~5.0% and the false negative rate ~0.6% (Budnick et al. 1996; Chen et al. 1996). The standard MF method evaluated by Levin et al. (1975) was found to have noticeably higher rates (false positive, 10%; false negative, 11.7%). Based on routine testing of enterococci in recreational waters, several public health laboratories have reported similar rates for the MF method (Budnick et al. 1996).

Rhodes & Kator (1997) evaluated a modified mE medium (mEI), containing the chromogenic substrate indoxyl-ß-D-glucoside, for the enumeration of enterococci in 24 h. Based on the recovery of enterococci from environmental samples, mEI

Table 1 Effect of test methodology on water quality determination of sample.

Recreational use (enterococci per 100 ml)*	No. of samples that pass by membrane filtration but fail by Enterolert	No. of samples that fail by membrane filtration but pass by Enterolert
Bathing beach (104)	11	9
Moderate use (153)	10	3
Light use (275)	9	0
Infrequent use (500)	3	3

*1992 New Zealand recreational water quality guidelines (upper limits).

was found to be ineffective for 24-h membrane filtration enumeration. A study by Messer & Dufour in 1998, suggests that reducing the triphenyltetrazolium chloride (TTC) concentration in another modification of mEI yields an improved 24-h enumeration procedure for enterococci that shows analytical performance (false positive rate 6.0%; false negative rate 6.5%) characteristics similar to those of the standard 48-h MF method.

The higher reported false positive and false negative rates of the standard MF method may be related to the performance variability of membrane filters in the recovery and retention of cells (Geldreich 1977). Toxic and hydrophobic inks causing grid line growth inhibition, toxic and growth promoting substances in the chemical composition of the filter itself, and different pore sizes between batches of filters are examples of the problems that may be encountered. Because of these factors laboratories using MF methods need to implement quality control procedures for each batch of membrane filters (Brenner & Rankin 1990).

Hernandez et al. (1993), showed that fluorogenic substrates incorporating 4-methylumbelliferyl-ß-D-glucoside had effective selectivity for the major faecal enterococci (*E. faecalis*, *E. faecium*, *E. durans*, and *E. hirae*); 100% of strains produced ß-glucosidase and were recovered with good efficiency in the

Table 2 Procedure comparisons: membrane filtration versus Enterolert®.

Component	Membrane filtration	Enterolert
False positive rate	10.0%	2.4%
False negative rate	11.7%	0.3%
Media required	mE agar plates EIA agar plates	Enterolert reagent
Media storage	Prepared agar plates: 1 month at 4°C	Enterolert reagent: 18 months at 4–30°C
Media quality control	Test each batch of plates for sterility and growth with *E. faecalis*, *S. epidermidis*, and *S. bovis* controls	Test each lot once with target levels of *E. faecalis*, *S. marcescens*, and *A. viridans* controls
Equipment and materials required	Rinsing buffers Dilution buffers Glassware and pipettes Membrane filters Filtration units Colony counter Incubator	Dilution water blanks Glassware and pipettes Quanti-Trays Quanti-Tray sealer UV lamp Incubator
Incubator space required	Agar plate dimensions: 50 mm × 9 mm	Quanti-Tray dimensions: 260 mm × 120 mm × 9 mm
Reading of results	Zone size and intensity on EIA agar plate variable depending on colony size and density	Clear positive or negative fluorescence
Sample turbidity	Particulate matter can affect recovery and counting of target colonies on membranes	Reading of fluorescence not affected by turbidity
Suitable counting range (20–60 colonies)	Requires prior knowledge of site or may need repeat filtrations of sample	Not a factor as Quanti-Trays cater for very wide range of counts
Skill level required	Moderate	Minimal
Procedure manipulations	Several	Few
Time per sample set up	8–12 m	3 m
Incubation time	48 h	24 h
Cost per sample:		
Labour	NZ$13.56	NZ$5.93
Consumables	NZ$9.23	NZ$12.40
Total	NZ$22.79	NZ$18.33

fluorogenic medium. Enterolert's accuracy has been demonstrated by Chen et al. (1996), who challenged Enterolert with a variety of non-enterococcal bacteria as well as variety of enterococcus species. The sensitivity of the Enterolert presence/absence test was evaluated by these authors using 25 enterococcus strains; ~96% (24/25) at 1–3 colony forming units (cfu)/100 ml developed fluorescence within 24 h of incubation at 41°C. Only *E. hirae* was not detected in 24 h. In the Quanti-Tray format, Enterolert detected ~95% (121/127) of environmental enterococcal isolates within 24 h; the identities of these isolates included *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. durans*, *E. gallinarum*, and *E. hirae*. Using 17 non-enterococcal bacteria, Enterolert showed no fluorescence at levels up to 2×10^5 cfu/100 ml within 48 h.

In the MF method, confirmed enterococcal colonies develop a black or reddish brown precipitate after incubation on the underside of the filter on the EIA plate. This reaction may vary in intensity, especially when large numbers of different sized colonies are present, resulting in counting errors because of difficulties in the interpretation of the reaction. Reading an Enterolert well is more objective since any degree of fluorescence is considered a positive reaction.

Turbid samples, commonly found in recreational waters, contain high concentrations of particulates that are captured on the surface of the membrane filter during filtration. Contact of the target organisms with the agar surface may be obstructed resulting in the prevention or inhibition of colony formation. Furthermore, counting colonies is more difficult when extraneous matter is present thus necessitating sample dilution and/or repeating the filtration procedure; colony numbers above 60 leads to counting errors and may also necessitate sample dilution and repeat filtrations. However, testing turbid samples or samples from heavily polluted sites with Enterolert is not a problem since turbidity does not affect the reading of fluorescence. Furthermore, the 51-well Quanti-Tray can be used to obtain marine enterococci counts up to 2005 and the 97-well Quanti-Tray allows for quantifying counts of up to 24 000.

The Enterolert system requires very little skill to set up, is easy to read, and less training is required than that necessary for the MF method. Quality control testing is more time consuming for MF because each batch of prepared mE and EIA plates, which have a shelf life of 1 month at 4°C, must be checked for sterility and growth performance characteristics with positive and negative control organisms. The Enterolert powdered reagent, which can be stored for up to 18 months from date of manufacture at room temperature, only requires presence/absence testing of the single reagent lot with defined target levels of positive and negative control organisms. The Enterolert Quanti-Tray does however, require a greater amount of incubator space because of its size (260 mm × 120 mm × 9 mm) compared to the MF petri dish (50 mm × 9 mm). Each Quanti-Tray requires the space of about eight MF plates and this needs to be considered if incubator space is limited and large numbers of samples are to be tested routinely.

Although the cost of consumables per test was found to be more for Enterolert (NZ$12.40) than MF (NZ$9.23), the labour cost per test is considerably less for Enterolert (NZ$5.93) than MF (NZ$13.56). As fixed costs per test for Enterolert and MF were dependent upon the number of samples tested and made up a small proportion of total costs, even at low throughput, they have not been presented in this paper. A laboratory considering a change to the Enterolert system would need to purchase a Quanti-Tray sealer which at 1 October 1997 cost NZ$4500.

Using Enterolert requires much less time per sample to perform (3 min versus 8–12 min) compared to the MF method which requires a battery of manipulations. These time savings as well as the reduction in the incubation time required (24 h for Enterolert; 48 h for MF), decrease turnaround time significantly. In this study, the number of samples in which the water quality was judged acceptable by one method and not acceptable by the other, is relatively small. However, the fact that more of these samples were judged acceptable by MF is of concern in view of Enterolert's greater accuracy. If Enterolert had been used to routinely monitor the recreational waters used in the study, more samples would have failed the single sample upper limit, therefore allowing health authorities to respond more rapidly to potentially hazardous public health situations.

CONCLUSION

Enterolert has been found to be suitable and reliable for detecting and enumerating enterococci in marine waters and Enterolert's sensitivity, specificity, simplicity, and rapidity, indicate that it should be seriously considered as a practical choice in testing the microbiological quality of recreational water.

Currently the frequency and adequacy of recreational water quality monitoring in New Zealand varies considerably from region to region making proper nation-wide comparisons difficult. The results from this study suggest that more widespread use of this new technology in water quality monitoring is warranted so that national enterococcal standards can be effectively implemented. Furthermore, because Enterolert requires considerably less time to perform than the membrane filtration method, monitoring agencies can respond to sudden bacterial threats more quickly and take immediate corrective action which would mean safer recreational waters.

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Indicator Organisms And Water Quality

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This paper takes the form of a review of indicator organisms and marine recreational water quality monitoring. The intent is to encourage clear thought and discussion with respect to the application of indicator organism analyses and the subsequent interpretation and use of results.

Purpose

The presentation will focus on:

- Major waterborne pathogens that can be transmitted in recreational water.
- The New Zealand Recreational Water Quality Guidelines.
- The rationale for using indicator organisms to monitor water quality.
- Some important characteristics of faecal *streptococci* and *enterococci*.
- Methods for detecting and enumerating *enterococci*.
- Distinguishing human from animal sources of faecal contamination.
- Alternative approaches to water quality monitoring and assessment programmes.

Introduction

Marine waters are subject to important changes in their microbiological quality that arise from agricultural use, discharges of sewage or wastewater resulting from human activity or storm water runoff. Sewage effluents contain a wide variety of pathogenic microorganisms such as viruses, bacteria and protozoa that may pose a health risk to humans when effluents are discharged into recreational waters. The density and variety of these pathogens are related to the size of the human population, the seasonal incidence of the illness, and the dissemination of pathogens within the community (Figueras *et al.*, 2000). The organisms are potentially hazardous when contaminated water is used for recreational activities such as swimming and other "high contact" water sports. In these activities there is reasonable risk that water could be swallowed, inhaled, or come in contact with ears, nasal passages, mucous membranes and cuts in the skin allowing pathogens to enter the body (Harrington et al. 1993). In most cases, the ill-health effects from exposure to polluted water are minor and short-lived.. However, the potential for more serious diseases such as Hepatitis A, Giardiasis, Cryptosporidiosis, Campylobacteriosis, and Salmonellosis cannot be discounted (Philip 1991). Until recently scientists believed that gastroenteritis was the main health effect from contact with polluted water, but now it is becoming clear that respiratory health effects also occur and may in fact be more prevalent than gastroenteritis.

Many waterborne pathogens are difficult to detect and / or quantify and the specific methodology to detect some of them in environmental water samples has still to be developed. Therefore faecal bacteria are commonly used today to signal the presence in water of pathogens that may cause gastrointestinal disease in humans.

New Zealand Recreational Water Quality Guidelines

In New Zealand, *Enterococci* are used as indicators of faecal pollution of marine waters and for compliance monitoring the *Recreational Water Quality Guidelines* (Ministry for the Environment, 1999) propose a four tier management framework based on *Enterococci* levels in water samples, namely:

- **Surveillance/Green mode** - Running median less than 35 *Enterococci* per 100ml - which involves routine (eg. weekly) sampling.
- **Alert/Amber mode I** - Running median greater than 35 *Enterococci* per 100ml and no single sample greater than 136 *Enterococci* per 100ml - exceedences require investigations into the causes of the elevated levels and increased sampling (twice weekly) to enable the risks to bathers to be more accurately assessed.
- **Alert/Amber mode II** - Single sample greater than 136 *Enterococci* per 100ml (irrespective of running median) - again, exceedences require investigations into the causes of the

elevated levels and increased sampling (daily), and sanitary surveys to identify sources of contamination.

- **Action/Red mode** - Two consecutive single samples (within 24 hours) greater than 277 *Enterococci* per 100ml (irrespective of running median) - exceedences require the local authority and health authorities to warn the public that the beach is considered to be unsafe, erect warning signs, conduct sanitary surveys and daily sampling.

As shown above, the guidelines use a combination of annual (seasonal) median values and single sample maxima s to assess the safety of the marine waters and assist water managers to implement the Resource Management Act (RMA) 1991 and the Health Act 1956. The system is analogous to traffic lights:

- **Green** - "Safe" for recreational use i.e. clean waters.
- **Amber** - "Potentially unsafe" for use i.e. potentially contaminated waters.
- **Red** - "Highly likely to be unsafe" for use i.e. highly likely to be contaminated.

In marine waters, adherence to the guideline values and use of the management framework should ensure that people using the water for recreational are not exposed to significant health risks. However, because these guidelines were developed from studies relating to bacteriological indicators to illness in the general public, water conforming to the guideline values may still pose a potential health risk to high-risk user groups such as the very young, the elderly and those with impaired immunity.

These guideline values are based on the *1994/1995 New Zealand Marine Bathing Health Effects Study* (McBride *et al.*, 1998). The key findings of this study are:

- The relationships in New Zealand between indicator bacteria and health effects are consistent with those found overseas (Cabelli et al. 1982).
- There is no noteworthy difference between the health risks associated with animal and human sources of contamination. Therefore, the guideline values should apply irrespective of location and time. For example, there is no justification, from a health perspective, for not sampling after heavy rainfall if people are swimming or likely to be swimming. That is, sampling programmes should be based on the number of people (likely to be) swimming, whether or not there has been a heavy rainfall event.
- Of the easily measured bacteriological indicators, *Enterococci* are the preferred indicator to show a relationship to health risks in marine waters.
- *Enterococci* levels in shallow water correlate well with health risks. Therefore, sampling can take place at 0.5m depth rather than at chest depth, as proposed in the previous guidelines (McBride et al., 1992). Sampling at chest depth can be hazardous to those collecting samples.

The 1999 guidelines use "maximum acceptable" swimming-associated illness risks of 19 per 1,000 bathers for marine water (8 per 1,000 for fresh water) that are consistent with international practice (Cabelli, 1983).

Rationale for using indicator organisms to monitor water quality

To control water contamination from animal and human faecal pollution and to protect the public from acquiring waterborne diseases, we must have reliable methods for detecting and measuring pollution. The quality of recreational water is determined largely by bacteriological analyses in order to establish whether the water contains faecal organisms (not necessarily pathogens) whose presence is indicative of pollution by animal or human wastes. Analysis of water samples on a routine basis for each individual type of pathogen is not practical and pathogen isolation and identification is usually only attempted following a waterborne disease outbreak.

There are a number of reasons why pathogen identification is rarely attempted during routine assessment of water quality:

1. Pathogens may be emitted intermittently from the diseased host so that the testing programme may miss them
2. Pathogen numbers may be diluted in the receiving waters making detection more difficult.
3. The particular pathogen being tested may not be easily culturable.
4. The test methodology may not be available for pathogen testing.
5. Multiple strains of the pathogen may have to be targeted for the satisfactory detection.
6. Isolation and identification of the pathogen may involve many tests and can be expensive and time-consuming.

By implication, indicator organisms should signal the potential presence of organisms that cause gastrointestinal disease. Commonly used indicator organisms of water quality are organisms originating in the human and animal gastrointestinal tract and includes aerobic and facultative anaerobic organisms such as *Escherichia coli* (faecal coliforms) and organisms belonging to the genus Enterococcus. The purpose of a bacterial indicator is to allow prediction of the health risk associated with the water under consideration. To ensure the predictive value of indicator tests, the indicator organism must meet certain criteria:

1. Must always be present in the presence of pathogens.
2. Must be derived exclusively from the same source as the pathogen.
3. Must occur in high numbers to allow for a safety margin.
4. Must behave and survive like the pathogen in the environment.
5. Must resist treatment and disinfection to a similar or slightly greater extent than the pathogen.
6. Must be easily detected by simple, reliable and rapid methods.
7. Must be easily detected, even in the presence of other organisms.

The search for the ideal indicator for microbiological water quality has been elusive. In no case is any indicator entirely suitable for all pathogens and no single indicator or approach is likely to represent all the facets and issues associated with contamination of water with faecal matter. For example, protozoa and viruses have different survival properties; enteric viruses and protozoan cysts are relatively stable in moist conditions. Generally, if indicator organism levels are high, then there is good prediction that virus and protozoan levels are high also; but low levels of indicator organisms do not necessarily indicate an absence of viruses and protozoa (Loutit, 1990). While the indicator organism concept has been used successfully for a long time, there are still many questions concerning the effectiveness of the way in which water quality is measured and monitored because a number of environmental and physical factors may influence the usefulness of faecal bacteria as indicators. Other non-bacterial indicators (e.g. coliphages, F-specfic RNA phages, *Cryptosporidium*, faecal sterols, caffeine, detergents, sanitary plastics etc. can be appropriate "baskets" of microbial and chemical determinands

for identifying and apportioning human and animal sources of faecal pollution (Sinton et al., 1998).

Major characteristics of Faecal streptococci and Enterococci

Faecal *streptococci* have received widespread acceptance as useful indicators of faecal pollution in natural aquatic ecosystems. These organisms show a close relationship with health hazards (mainly for gastrointestinal symptoms) associated with bathing in marine and freshwater environments (Cabelli *et al.* 1982, 1983; Dufour 1984; Kay et al 1994; McBride *et al.*, 1998; WHO 1998). They are not as ubiquitous as coliforms, they are always present in the faeces of warm-blooded animals, and it is believed they do not multiply appreciably in sewage contaminated waters. Their die-off rate is slower than the decline of coliforms in seawater and persistence patterns are similar to those of potential waterborne pathogneic bacteria (Sinton *et al.*, 1993a, 1993b).

The group of organisms called faecal *streptococci* includes species of different sanitary significance and survival characteristics (Sinton & Donnison 1994). The proportion of the species of this group is not the same in animal and human faeces (Rutkowsky & Sjogren 1987; Poucher *et al.*, 1991). The taxonomy of this group, comprising species of two genera *Enterococcus* and *Streptococcus*, has been subject to extensive revision in recent years (Facklam *et al.*, 1995). Although several species of both genera are included under the term *enterococci* (Leclerc *et al.*, 1996), the species most predominant in polluted aquatic environments are *Enterococcus faecalis*, *E. faecium*, *and E.durans.*

Enterococci includes all the species described as members of the genus *Enterococcus* that fulfil the following criteria:

- Growth at 10°C and 45°C.
- Resistance to 60°C for 30 minutes.
- Growth at pH 9.6 and at 6.5% NaCl.
- Ability to reduce 0.1% Methylene Blue.

The most common environmental species fulfil the above criteria and therefore in practice the terms faecal streptococci, *enterococci*, intestinal *enterococci* and *enterococcus* group can be considered synonymous (Figueras *et al.*, 2000).

Two case studies will be presented in order to demonstrate the effects of meteorological events on *enterococci* levels as well as some of the problems associated with distinguishing human from animal sources of contamination. The first study, on the impact of duck droppings on water quality at Hataitai Beach (Abbott *et al.*, 2000; Robertson 2000), highlights some of the problems associated with the number of samples necessary and from which sampling sites for adequate water quality monitoring; the second study, on the water quality of Evans Bay (Svagzdiene 2001), highlights some of the problems of stormwater runoff and the influence of rainfall on *enterococci* counts.

Methods for detecting and enumerating Enterococci:

There are a number of methods that can be used for the analyses of enterococcal densities in marine water samples. These include the membrane filtration (MF) techniques and multiple tube fermentation techniques for the most probable number (MPN).

For the previous New Zealand Recreational Water Quality Guidelines (McBride *et al.*, 1992) the approved MF method for *enterococci* was a two-step procedure; incubation of the membrane filter at 41°C on a selective and differential agar medium (mE) for 48 hours followed by transfer of the membrane to an *in situ* esculin-iron agar (EIA) substrate medium and incubation for 20 minutes at 41°C. Pink - red colonies on the membrane producing a brownish black precipitate on EIA were identified as *enterococci*. The brownish black precipitate was the result of the hydrolysis of esculin to glucose and coumarin by the enzyme β-glucosidase. Coumarin forms a black precipitate in the presence of ferric citrate. Although this MF method has been shown to efficiently recover *enterococci* from marine and estuarine waters, the false positive and negative rates are reported to be 10.0 and 11.7% respectively (Levin *et al.*,1975; Budnick *et al.*, 1996). The MF method requires the dilution of water samples which contain high bacterial numbers in order to obtain counts in the acceptable counting range of 20 - 60 colonies (Donnison 1992). Turbid samples can block the membranes, and the growth of competing bacteria can often cause inhibition of growth of target organisms or mask the colour changes associated with carbohydrate fermentation (Fricker & Fricker 1996).

The MPN test has been used in the microbiological monitoring of water quality for many years (McGrady 1915) and its validity and suitability for estimating bacterial numbers is explained in Cochran's classic paper (1950) and by Woodward (1957). The MPN method for *enterococci* is a three-stage test which takes 4 days to complete. The first (presumptive) stage uses liquid medium containing azide dextrose to inhibit non-target bacteria. All azide dextrose broth tubes showing turbidity after 24 or 48 hours incubation are subjected to confirmation on the same media used for the membrane filtration method (Donnison 1992). The MPN method has been found to give statistically equivalent results to the membrane filtration method even though the techniques are significantly different (Fullerton 1995). Although the MPN method is particularly useful for turbid water samples, sediments and effluents, traditional MPN methods are time consuming because of the number of tubes and sample manipulations required and are therefore unsuitable when it is necessary to process large numbers of water samples.

Both of the methods described above are labour intensive, tedious, and because they require a minimum of 48 - 72 hours before results can be obtained, they cannot be used for current New Zealand compliance monitoring programmes since the 'Action/Red' mode management framework requires two consecutive single samples analyses within 24 hours. The *New Zealand Recreational Water Quality Guidelines* states, that unless an alternative method is validated to give equivalent results for marine waters, the 24 hour EPA 1600 MF Method or the Enterolert® must be used to enumerate *enterococci*.

The EPA1600 MF Method - also called the mEI method - was developed for the United States Environmental Protection Agency (Messer & Dufour 1998). This mEI membrane filtration medium contains the chromogenic substrate indoxyl-β-D-glucoside for use in a single step procedure for enumeration of *enterococci* in 24 hours. Like the mE medium, the mEI medium also contains nalidixic acid, cycloheximide, but a reduced amount of triphenyltetrazolium chloride. *Enterococci* colonies produce an insoluble indigo blue complex, which diffuses into the surrounding medium forming a blue halo around the colony. Based on the recovery of *enterococci* from environmental samples the mEI medium shows analytical performance (false positive rate 6.0%; false negative, rate 6.5%) characteristics similar to those of the standard 48 hour mE MF method.

The MPN principle, as used Enterolert® Quanti-Tray™ system (IDEXX Laboratories, United States), allows for the

inoculation, with minimum sample manipulation, of a greater number of wells than the standard MPN method. The Quanti-Tray is a sterile, disposable tray containing 51 or 97 separate wells. Sealing of the sample-filled tray is accomplished by the use of a specially designed Quanti-Tray sealer which automatically distributes and seals a 100 ml sample. The Enterolert system, designed to detect *enterococci* in water in 24 hours, is based on Defined Substrate Technology (Edberg *et al.*, 1988; Fiksdal *et al.*, 1994) that has been used successfully to monitor faecal pollution of drinking water and recreational waters in significantly less time than membrane filtration procedures. Enterolert utilises 4-methylumbelliferyl-β–D- glucoside as the defined substrate nutrient indicator. This compound, when hydrolysed by enterococcal-β–glucosidase, releases 4-methylumbelliferone which exhibits fluorescence under a UV_{365} lamp. The number of *Enterococci* per 100 ml is determined, based on the number of positive (fluorescent) wells counted, by referring to a 51 or 97 - well IDEXX MPN table or using an IDEXX version 3.0 MPN software programme. Wells showing no fluorescence are considered negative for *enterococci*.

Abbott *et al.* (1998) evaluated Enterolert® for the enumeration of *enterococci* in a large study involving 343 marine water samples from selected sites in the Wellington area of New Zealand. Statistical analysis of parallel test results showed a strong linear correlation (r = 0.927) and no significant difference between Enterolert® and the 48 hour mE membrane filtration method by paired t-test analysis (P = 0.39). Based on the 2.4% false positive and 0.3% false negative rates obtained, Enterolert was found to have a sensitivity of 99.8% and a specificity of 97.0%. These results are consistent with the results of studies undertaken in the United States, Britain and Sweden where Enterolert's false positive rate was found to be ~5.0% and the false negative rate ~0.6% (Budnick *et al.*,1996; Chen *et al.*, 1996; Fricker & Fricker 1996; Eckner, 1998).

As mentioned earlier, the New Zealand Recreational Water Quality Guidelines states, that although the 24 hour mEI method or the Enterolert system must be used to enumerate *enterococci* in marine waters, other methods can be used if they are validated to give equivalent results. The use of chromogenic and fluorogenic substrates for the detection of β-glucosidase activity to differentiate *enterococci* in new culture media has recently been described (Manafi 2000). The chromogenic substrate 5-bromo-4-chloro-3-indolyl-βd-glucopyranoside (XGLU), in Chromocult *Enterococci* broth and Readycult *Enterococci* media (Merck, Germany) utilizes the β-d-glucosidase reaction as an indicator for the presence of *enterococci*. XGLU is liberated and rapidly oxidized to bromochloroindigo which produces a blue colour in Chromocult as well as blue colored *enterococci* colonies in Chromocult *Enterococci* agar.

The results obtained with pure cultures showed that 3% of enterocccci strains gave false positive results and 0% were false negative. Using XGLU in a presence / absence procedure to detect *enterococci* in water resulted in positive results after 24 hours incubation with 10 cfu/100 of water sample (Manafi & Windhager 1997). RAPID enterococcus agar (Sanofi, France) is a new medium using chromogenic XGLU for the detection of enteroccoci and also contains a selective mixture for the inhibition of gram negative and β-glucosidase positive bacteria other than *enterococci*.

New approaches to water quality monitoring programmes: Despite the evident successes in the protection of public health, present approaches to recreational water quality monitoring suffer several limitations. While current water quality guidelines and associated regulations have had some success in driving cleanups, increasing public awareness, contributing to informed personal choice and contributing to a public health benefit, these successes are however, difficult to quantify. The need to control and minimize adverse health effects has obviously been the principal concern of water quality monitoring programmes. Present monitoring programmes for the microbiological quality of recreational water are primarily or exclusively based on compliance with indicator organism counts. A number of constraints are evident in current programmes:

- Management actions are largely retrospective.
- While the risk to health is primarily from human excreta, the traditional indicator organisms may also originate from other sources.
- There is poor inter-laboratory and international comparability of microbiological analytical data.
- While beaches are classified as safe or unsafe, there is a gradient of increasing severity, variety and frequency of health effects with increasing sewage pollution and it is desirable to promote incremental improvements prioritising "worst failures"

In view of the above constraints, a modified approach to the regulation of recreational water quality has recently been proposed by the World Health Organization (WHO 1999) for improved protection of public health with minimum necessary monitoring effort. This approach could provide greater scope for interventions, especially for those within the resources of local authorities. The approach has become known as the "Annapolis Protocol" which concludes that the proposed alternative strategies are possible and should be tested and promoted. Some to the main issues addressed in the Annapolis Protocol are:

- Studying relationships between factors that affect beach water quality and the ability of monitoring schemes to detect these changes.
- A classification scheme through which a beach would be assigned a class (very poor, poor, fair, good, or excellent), based upon health risk.
- Enabling local management to respond to sporadic or limited areas of pollution and thereby upgrade a beach classification.
- Scheme that provides a generic statement of the level of risk and that indicates the principal management and monitoring actions likely to be appropriate.
- Major advantage of classification scheme, as opposed to a pass/fail approach, lies in its flexibility since a large number of factors can influence the condition of a given beach.
- Allows regulators to invoke mitigating approaches for beach management. • Any microbiological analytical result provides information on only a moment in time, while microbiological quality may vary widely and rapidly even within a small area.
- It is possible to effectively discourage use of areas of poor quality, or discourage use at times of increased risk.
- Level of microbiological monitoring required for a given beach may largely depend upon its classification. Beaches classified as very high quality or very low quality ("excellent or "very poor"), for example may only need a

microbiological quality assessment every few years to verify that their status has not changed.

- Mid-level ("good, "fair" and "poor") beaches may require an annual, low level-level microbiological quality assessment, with 20 samples being taken at a minimum of four sites on five occasions evenly spaced throughout the bathing season. Further samplings may be necessary if abnormally high sample results are found.

A classification scheme of the type proposed would be of value if it accomplishes one or more of the following:

1. Contributes to informed personal choice. In other words individuals, by using the information provided, can modify their exposure. This implies inter-location comparability and an informed public.
2. Contributes to local risk management. For example, by excluding or discouraging access to areas or at times of increased risk and thereby reduce overall exposure.
3. Assists in making maximum use of the minimum monitoring effort. 4. Assists local decision-making regarding safety management. 5. Encourages incremental improvement and prioritises areas of greatest risk. In order to evaluate the above, both field testing, including extensive sampling of sites in a number of intensive studies, and an evaluation of the scientific validity of the approach, has been proposed by the WHO 'Annapolis Protocol' consultative group.

Conclusion: Today, one can hardly find a popular beach that has not at some time been affected by pollution of one kind or another, with consequent degradation of the suitability of the water for recreational activities. Diseases acquired from recreational use of contaminated waters are of great concern to public health agencies and the tourism industry. Therefore management of coastal water quality becomes the key to managing this resource. Water quality is usually maintained by establishing water quality guidelines and/ or standards and implementing monitoring programmes to ensure that the quality of the water is safe for recreational use. There is worldwide consensus among scientists that the bacterial indicators used in establishing marine water quality have been useful in controlling water contamination from faecal pollution and thus protecting the public from acquiring waterborne diseases. However, there are still many questions concerning the effectiveness of the way in which water quality is measured and monitored because a number of environmental and physical factors influence the usefulness of faecal bacteria as indicators. The search for the ideal indicator for microbiological water quality has been elusive. In no case is any indicator entirely suitable for all pathogens and no single indicator or approach is likely to represent all the facets and issues associated with contamination of water with faecal matter. Currently, the primary goal of monitoring marine recreational waters is to determine the levels of faecal bacteria that may indicate the presence and/or absence of pathogens rather than indicate the presence or absence of any one specific pathogen. In order to improve the effectiveness of water quality monitoring new approaches and new detection methods are necessary. Recent advances in molecular techniques such as the Polymerase Chain Reaction (PCR) for detecting and quantifying specific pathogens like enteric viruses and protozoa, should overcome some of the limitations associated with using bacterial indicators to monitor the microbiological quality of recreational waters.

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Indicator Organism Sources and Recreational Water Quality: A Study on the Impact of Duck Droppings on the Microbiological Quality of Water at Hataitai Beach.

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PRESENTED AT THE NEW ZEALAND WATER & WASTES ASSOCIATION'S 'WATER 2000' CONFERENCE - CARLTON HOTEL - AUCKLAND - 22/3/2000

ABSTRACT

During 1998 the Wellington City Council carried out remedial work, over several months and at a cost of more than $400,000, on leaking sewers and private drain faults that were suspected as being the primary cause for the high bacterial counts at Hataitai Beach. Accordingly, Hataitai Beach was re-opened for swimming in December 1998 and remained open throughout the 1999 bathing season. In this study 274 marine water samples were collected from various sites at Hataitai Beach from December 1998 to August 1999 for the detection and measurement of indicator organisms. Results were examined for compliance with the 1998 New Zealand Bacteriological Water Quality Guidelines. *Enterococci* and *E.coli* results were also used to determine the correlation between these indicator organism levels and duck numbers at the various sites as well as the influence of meteorological events on the water quality. For samples collected from all sites during the bathing season (10/12/98 to 25/3/99), a median of 41 *Enterococci* per 100ml was obtained and over this period 14 samples were in the Alert/Amber Mode 2 (single sample between 136 and 277 *Enterococci* per 100ml). On 24 occasions the all sites samples yielded results in the Action/Red Mode (single sample greater than 277 *Enterococci* per 100ml). For samples collected from sites frequently populated by ducks (B3) the median was 160 *Enterococci* per 100ml over the bathing season. These B3 site samples yielded results in the Alert/Amber Mode 2 on 8 occasions and in the Action/Red Mode on 11 occasions. On 4 occasions during the bathing season B3 site results of greater than 1000 *Enterococci* per 100ml were obtained. While a combination of waterfowl wastes and meteorological events may be the likely explanation for the high bacterial counts at Hataitai Beach, further investigations into the most probable pollution sources at Hataitai Beach are clearly warranted so that the safety of the beach as a whole can be properly assessed.

KEYWORDS

Marine water quality, Hataitai Beach, ducks, indicator organisms, Enterococci, Enterolert, coliforms, E.coli, Colilert, pollution, health.

INTRODUCTION

Between 50 and 100 ducks frequent Hataitai Beach, a small Evans Bay beach that is a popular location for swimming, canoeing, windsurfing and fishing. Since 1994 the water quality at Hataitai beach had deteriorated to the extent where the compliance values for indicator organisms were regularly exceeded (Berry 1996, 1997, & 1998). The high frequency of exceedences resulted in Hataitai Beach being closed for bathing between February 1996 and January 1997, and again in January 1998 (Murphy 1996; Samson 1998). Results of routine laboratory tests performed on marine water samples by the Wellington City and Regional Councils' laboratories have been unable to determine if the raised bacterial levels were of animal or human origin. Consequently it has not been established if the ducks at Hataitai beach are the main reason for the high counts or whether they contribute to the pollution problem (Dominion report 1996; Lucas 1998; Samson 1998). Since leaking sewers and private drain faults were suspected as being the primary cause for the high bacterial counts at Hataitai Beach, the Wellington City Council carried out remedial work at a cost of more than $400,000 over several months in 1998. Hataitai Beach was re-opened for swimming in December 1998 and remained open throughout the 1999 bathing season (Baker 1998; Berry 1999).

A study on the Island of Jersey (Wyer et. al., 1995) suggests that water fowl, such as ducks and swans, can influence water quality compliance and a Canadian study (Levesque et al., 1993) on ringed - billed gull droppings, found that these birds contribute to the microbiological degradation of recreational water. Feare et al., (1999) demonstrated that bacteria present in waterfowl droppings constitute a potential health risk to humans exposed to the droppings during recreational activities. In a

study on the effects of waterfowl on indicator organism levels in water, Standridge et al., (1978) showed that faecal coliforms that were deposited by mallard ducks multiplied in the beach sand and that these bacteria were subsequently transported to the water resulting in high faecal coliform counts in the swimming areas. The authors report that ducks have been implicated as carriers and disseminators of *Salmonella* and that beach closures based on contamination from ducks is warranted. A number of studies have shown that bacteria isolated from waterfowl droppings, such as *Campylobacter, Salmonella, Escherichia* and *Aeromonas*, have the potential for human pathogenicity which can lead to infection and disease, notably diarrhoea, gastroenteritis and marine acquired wound infections. (Gould et al., 1978; Jones et al., 1978; Hussong et al., 1979; Levesque et al., 1993; Hatch, 1996; Clarke et al., 1998). With regard to protozoal pathogens, studies by Graczyk et al., (1996 & 1998) provide clear evidence that waterfowl can disseminate *Giardia* cysts and *Cryptosporidium* oocysts in the environment and that these protozoa in water may have epidemiological implications. Recent publications on cercarial dermatitis (swimmers itch or duck itch) indicate that this condition, which is associated with ducks and human recreational water activities, should be considered as another emerging global parasitic disease (De Gentile et al., 1996; Chamot et al., 1998).

In this paper we report the findings of phase two of the study which details the microbiological quality of water at Hataitai Beach from December 1998 to August 1999. The objectives of this part of the study were to:

1. Determine if there was a significant improvement in water quality since the 1998 stormwater and sewer upgrades.
2. Identify the potential sources of faecal pollution if compliance monitoring revealed no significant improvement in water quality.
3. Establish if ducks at Hataitai beach have an impact on the water quality.

METHODS

A total of 274 marine water samples were collected over a period of 9 months from 10/12/98 to 9/8/99. The samples were collected from sites along Hataitai Beach which is approximately 90 meters long. The majority of samplings were at beach sites designated B1, the southern end adjacent to the public toilets and changing sheds; B2, the middle of the beach; B3, the northern corner where ducks are frequently seen either loafing or feeding on the beach, or swimming and feeding on the water. Samplings were mostly in dry weather conditions when indicator organism counts would not be influenced by stormwater run-off. Samples were taken at approximately adult knee depth (about 0.5m) from below the surface using the Mighty Gripper sampling device, between 0800 and 1800 hours, and mostly within 1 hour of high tide. Samples were collected aseptically in sterile 500 ml glass bottles, placed on ice in a large chilli-bin, transported to the laboratory within 30 minutes and processed within 1 hour of arrival in the laboratory. Data on weather conditions, tides, presence of debris, seaweed, and duck numbers were recorded at each sampling event. Daily Wellington Airport climatological data for the months December 1998 to August 1999 was obtained from MetService in order to determine if rain or stormwater run-offs preceding the sampling days had any effect on indicator organism levels.

Samples were analysed in the Microbiological Water Research Laboratory at Massey University Wellington for the detection and enumeration of *Enterococci, Total coliforms*, and *Escherichia coli*. The Enterolert system (IDEXX Laboratories, Westbrook, Maine, United States) was used for *Enterococci* enumeration. Enterolert is the recommended method in the 1998 bacteriological water quality guidelines for monitoring marine water (Ministry for the Environment, 1998) and the system has been shown to have advantages over membrane filtration methods for rapid water quality testing. (Abbott et al., 1998).

The Enterolert procedure was performed according to the manufacturer's instructions. A 1 in 10 dilution of the well-mixed water sample was made (10 ml of sample plus 90 ml of sterile distilled water) in a sterile 100 ml bottle. One package of the powdered Enterolert reagent was added to the bottle and after shaking to dissolve the powder, the mixture was poured aseptically into a sterile 97-well Quanti-Tray. The tray was then mechanically sealed in a Quanti-Tray sealer, which simultaneously distributed the mixture into the wells, and incubated for 24 hours at 41 ±0.5°C. After incubation the tray was viewed in a darkened room by placing it under and within 12 cm of a 365 nm wavelength ultraviolet light with a 6 Watt bulb. Blue fluorescence in a well was considered a positive reaction for that well and indicated the presence of *Enterococci*. The number of *Enterococci* per 100 ml was determined, based on the number of positive wells counted, by referring to a 97 - well MPN table (IDEXX version 3.0 MPN software programme) and the result multiplied by the

dilution factor of 10. Wells showing no fluorescence were considered negative for *Enterococci*.

For *Total coliforms* and *Escherichia coli* detection and enumeration the Colilert–18 system (IDEXX Laboratories) was used. Colilert–18 is a rapid enzymatic technique that is particularly useful for monitoring faecal pollution of coastal beach water (Palmer et al., 1993; Fiksdal et al., 1994).

The Colilert-18 procedure was performed according to the manufacturer's instructions. A 1 in 10 dilution of the well-mixed water sample was made (10 ml of sample plus 90 ml of sterile distilled water) in a sterile 100 ml bottle. One package of the powdered Colilert-18 reagent was added to the bottle and after shaking to dissolve the powder, the mixture was poured aseptically into a sterile 97-well Quanti-Tray. The tray was then mechanically sealed in a Quanti-Tray sealer, which simultaneously distributed the mixture into the wells, and incubated for 18 hours at 35 ±0.5°C. After incubation the tray was viewed for yellow wells which confirmed the presence of *Total coliforms*; the tray was then viewed in a darkened room by placing it under and within 12 cm of a 365 nm wavelength ultraviolet light and blue fluorescence was considered a positive for *Escherichia coli*. The number of *Total coliforms* and *Escherichia coli* per 100 ml was determined as mentioned above by referring to a 97 - well MPN table (IDEXX version 3.0 MPN software programme) and the result multiplied by the dilution factor of 10. Wells showing no yellow colour were considered negative for *Total coliforms* and wells showing no fluorescence were considered negative for *Escherichia coli*.

Enterococci counts were used for compliance monitoring as per the 1998 bacteriological water quality guidelines which proposed a three tier management framework:

Surveillance/Green mode – Running median less than 35 *Enterococci* per 100ml - which involves routine (eg. weekly) sampling.

Alert/Amber mode I – Running median greater than 35 *Enterococci* per 100ml and no single sample greater than 136 *Enterococci* per 100ml – exceedences require investigations into the causes of the elevated levels and increased sampling (twice weekly) to enable the risks to bathers to be more accurately assessed.

Alert/Amber mode II – Single sample between 136 and 277 *Enterococci* per 100ml (irrespective of running median) – again, exceedences require investigations into the causes of the elevated levels and increased sampling (daily), and sanitary surveys to identify sources of contamination.

Action/Red mode – Single sample greater than 277 *Enterococci* per 100ml (irrespective of running median) - exceedences require the local authority and health authorities to warn the public that the beach is considered to be unsafe, erect warning signs, conduct sanitary surveys and daily sampling.

The *Total coliforms* and *Escherichia coli* counts were used to determine the most likely sources of pollution when this occurred.

Statistical calculations on the data were undertaken using Microsoft Excel 97. Medians, correlation coefficients, ratios and histograms were calculated or determined using this program.

RESULTS

For samples collected from all sites from December 1998 to August 1999 a median of 31 *Enterococci* per 100ml was obtained. For all site samplings during the bathing season (10/12/98 to 25/3/99) the median was 41 *Enterococci* per 100ml.

For samples collected from the B3 sites from December 1998 to August 1999 the median was 70 *Enterococci* per 100ml and during the bathing season the median for these B3 sites samples was 160 *Enterococci* per 100ml. These median values and those of the B1 and B2 sites are shown in Table1.

Sites	Dec-Aug 10/12/98 to 9/8/99	Bathing Season 10/12/98 to 25/3/99	February 2/2/99 to 27/2/99	March 4/3/99 to 25/3/99
All	31 (274 Samples)	41 (156 Samples)	31 (85 Samples)	158 (33 Samples)
B1	20 (73 Samples)	20 (43 Samples)	20 (19 Samples)	97 (11 Samples)
B2	31 (72 Samples)	41 (42 Samples)	36 (19 Samples)	265 (11 Samples)
B3	70 (79 Samples)	160 (46 Samples)	112 (22 Samples)	301 (11 Samples)

Table 1. Hataitai Beach sampling site medians (Enterococci per 100ml)

During the bathing season samples from all sites yielded results in the Alert/Amber Mode II on 14

occasions and in the Action/Red Mode on 24 occasions. The B3 site samples yielded results in the Alert/Amber Mode II on 8 occasions and in the Action/Red Mode on 11 occasions during the bathing season. These exceedences and those of sites B1 and B2 are shown in Table 2.

Sites	Alert Amber Mode II (Single sample between 136-277 Enterococci per 100ml)	Action Red Mode (Single sample greater than 277 Enterococci per 100ml)
ALL (156 Samples)	14	24
B1 (43 Samples)	2	5
B2 (35 Samples)	4	8
B3 (36 Samples)	8	11

Table 2. Number of exceedences of the water quality guideline limits at Hataitai Beach during the bathing season (10/12/98 – 25/3/99)

On four occasions during the bathing season B3 sample results of greater than 1000 *Enterococci* per 100ml were obtained. The *Enterococci* counts for all site samples and the B3 sites samples during the bathing season and the period 10/12/98 to 9/8/99 are shown in Figures 1, 2, 3 and 4.

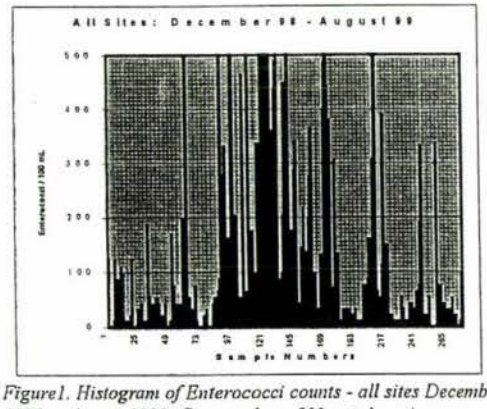

Figure1. Histogram of Enterococci counts - all sites December 1998 to August 1999.(Counts above 500 not shown)

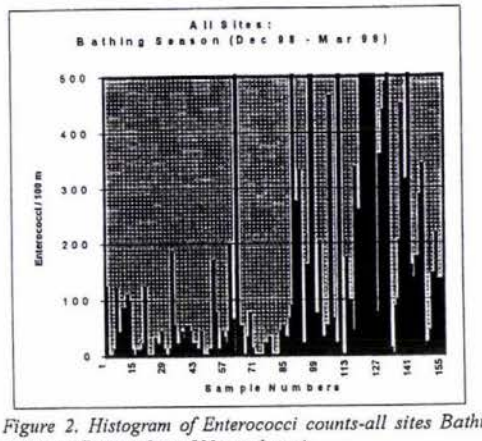

Figure 2. Histogram of Enterococci counts-all sites Bathing Season. (Counts above 500 not shown)

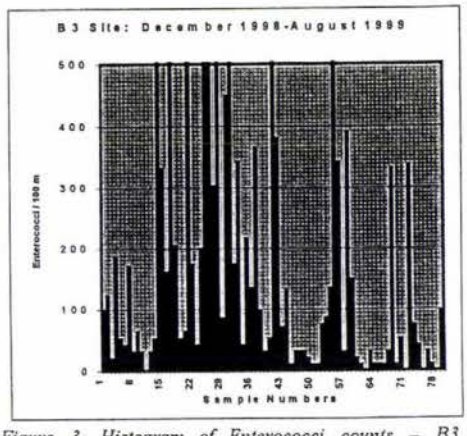

Figure 3. Histogram of Enterococci counts – B3 site December 1998 to August 1999. (Counts above 500 not shown)

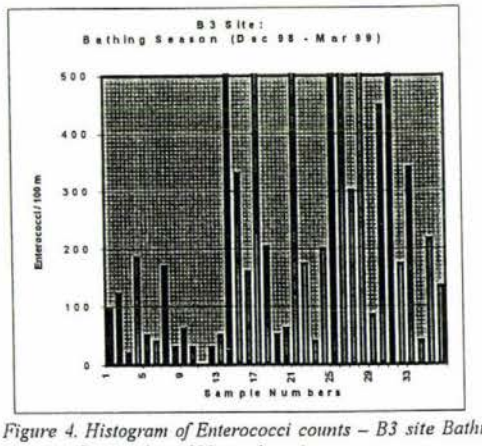

Figure 4. Histogram of Enterococci counts – B3 site Bathing season. (Counts above 500 not shown)

No correlation was found between rainfall in the preceding 24, 48 or 72 hours of sampling and the *Enterococci* counts (Table 3).

Rainfall (mm)	24 hours (Enterococci)	48 hours (Enterococci)	72 hours (Enterococci)
0	0-1334	0-1334	0-1334
10-20	0-3448	0-3448	0-3448
20-55	20-216	10-1182	0-1182

Table 3. Effects of rainfall preceding sampling on Enterococci ranges per 100ml.

No relationship was found between rainfall and *Enterococci* counts over the bathing season and neither was any relationship found between rainfall and the *Enterococci* counts at individual sites. *r* values of between 0.08 and 0.17 were found in each of the above cases.

No correlation was found between the number of ducks and either the *Total coliform*, *E.coli*, or *Enterococci* counts. The duck numbers on the beach or in the water at the time of sampling during this study ranged from 0 to 96. While several very high counts of the indicator organisms were obtained from water samples that were collected when large numbers of ducks were present, high counts were also obtained from samples when few or no ducks were present at the sampling sites.

In order to determine the sources (animal or human) of the indicator organisms the ratios of *E.coli* to *Enterococci* were calculated and plotted as shown in Figure 5

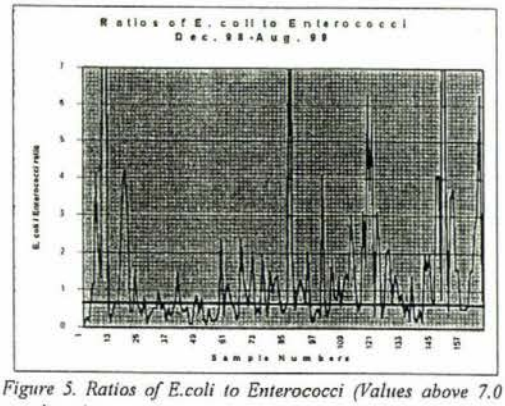

Figure 5. Ratios of E.coli to Enterococci (Values above 7.0 not shown)

The ratios for samples from all sites varied between 0.08 and 19.5. Ratios for samples from the B3 site varied between 0.08 and 4.1 with a median of 0.8. Because ratios were above and below 0.7 (<0.7 indicates animal faecal pollution) a specific faecal pollution source could not be categorically established.

DISCUSSI0N

Although we have been unable to categorically establish the reasons for the high indicator organism levels at Hataitai Beach during the course of this study, the results clearly indicate that despite the stormwater and sewer upgrades there is still a pollution problem at the beach. These results are in direct contrast to those that were published in June 1999 by the Wellington Regional Council which showed 4 exceedences in 17 samples from the B1 site over a 12 month period. The WRC report states "Water Quality has improved significantly at Hataitai Beach as a result of sewer and stormwater upgrades by Wellington City Council" (Berry 1999). Wellington City Council results of 26 samples taken during January and February 1999 shows a median of 56 *Enterococci* per 100ml and includes site B3 *Enterococci* values of 150, 380, 1000 and 1000, and site B1 values of 270 and 540 *Enterococci* per 100ml.

We have in this study found no significant relationship between rainfall and high indicator organism counts thus ruling out the likelihood of the influence of stormwater run-off. Most samplings occurred in dry weather and from the Metservice data no association was found between increased counts and rain on days preceding sampling. Further more, in a preliminary study in July and August 1998 we could find no correlation between the marine water quality near a stormwater outlet at Greta Point (about 150 meters north of the B3 site) and the water quality at Hataitai Beach.

Using the ratios of *E.coli* to *Enterococci* to determine the likely sources of pollution has been of limited value since ratios of above and below 0.7 were obtained (Faecal coliform : Faecal streptococci ratios of >4 0 indicate human faecal pollution and ratios <0.7 indicate animal pollution). The application of ratios to environmental samples needs many qualifications and ratios have been reported to be too unreliable to be useful in characterising pollution sources (Sinton et al 1993; 1994; 1995; 1998). However, the *E.coli* to *Enterococci* ratio shift approach, based on survival of organisms in stored

samples could be a better option for determining faecal pollution sources.

While the results from site B3 samples indicate that ducks could be responsible for the high counts that were frequently obtained at this site, we found no significant correlation between the number of ducks and either the *Total coliform*, *E.coli*, or *Enterococci* counts. However despite these findings a combination of waterfowl wastes and meteorological events may still be the most likely cause of the high bacterial counts at Hataitai Beach.

The work by Standrige et al., (1978) could explain the reason why in this study we obtained several high counts of the indicator organisms when large numbers of ducks were present at the sampling sites and also when few or no ducks were present. These authors found that resident mallard ducks frequently defaecate onto the beach sands and that faecal organisms in the droppings not only survived in the sand but multiplied rapidly during the first week. The authors demonstrated the presence of sufficient nutrients in the beach sand and near shore sediments to support microbial growth and leach into the water. Furthermore, the authors showed that faecal bacterial organisms introduced into the sands can be carried into the water by tides, rain, wind erosion and also the action of swimmers.

Levesque et al (1993) has shown that gulls contribute to the bacteriological degradation of recreational water. They measured the number of faecal coliforms in the water prior to and after attracting these birds to the beach with food. Not only was there a rapid increase in the concentration of faecal coliforms but also an increase in *Salmonella* and *Aeromonas* organisms.

Feare et al (1999) demonstrated that the droppings of waterfowl contain bacteria and that these bacteria can survive in droppings for up to four weeks and possibly longer under conditions prevailing in summer. Many of the bacteria isolated by these authors do have the potential to cause infections in humans. Bacterial infections could occur after exposure to a contaminated environment for example through swimming in polluted water or the ingestion of food inadvertently contaminated by waterfowl faeces.

The risk of humans acquiring infections from ducks at Hataitai Beach depends on the following:

- Presence of pathogenic organisms in the duck droppings.
- Survival of pathogenic organisms in the droppings after deposition on the beach sand and in the water.
- Frequency with which ducks deposit their droppings.
- Distribution of the droppings in areas where humans are likely to come into contact with the droppings.
- Types of recreational activities that expose humans to the droppings.
- Human susceptibility to infection from the pathogens in the droppings.

CONCLUSION

The results of this study clearly indicate that further investigations into the sources of contamination at Hataitai Beach are warranted so that the safety of the beach as a whole can be properly assessed and lead to appropriate management decisions for protecting beach users from waterborne infectious diseases. If the ducks are shown to be a major source of pollution at Hataitai Beach and it can be established that there is a risk of humans contracting infections from being exposed to the duck droppings, then common sense dictates that we should limit their food sources in the areas surrounding the beach and officially prohibit people from feeding the ducks.

Phase three of this study is continuing throughout 2000 and should include:

- Routine monitoring of indicator organism levels.
- Attempting more reliable methods to distinguish animal from human sources of pollution such as:
  - Speciation of *Enterococci*
  - Assemblages and ratio shifts
  - Antibiotic resistance patterns
  - DNA based techniques
    - (eg PCR of gene sequences)
- Determine the risk of humans contracting infections from being exposed to the duck droppings. The presence and survival of pathogens part of this risk assessment should include the detection of pathogenic organisms in the duck droppings and the water including bacteria like *Salmonella, Campylobacter, Aeromonas* and protozoa like *Giardia and Cryptosporidium*.

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