HEALTH SIGNIFICANCE OF HETEROTROPHIC BACTERIA IN DRINKING WATER

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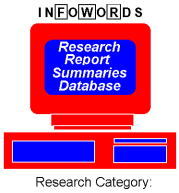

Waste Quality & Health:
Toxicity & Health

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HEALTH SIGNIFICANCE OF HETEROTROPHIC BACTERIA IN DRINKING WATER

REPORT - JUNE 1996

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EXECUTIVE SUMMARY

Objectives:

1 To determine whether HPC organisms may be of significance for health

2 to determine whether it might be appropriate to develop standards for individual species or genera.

Methods

The techniques used for the detection of heterotrophic bacteria (HPC) in water were reviewed to establish if they are appropriate for the study of the health significance of heterotrophic bacteria. After reviewing the possible ways of assessing the potential virulence of HPC that commonly occur in water a battery of tests was selected and set up in order to apply it to the virulence assessment of fresh isolates. The tests selected included assays for: Verotoxins using Vero cells: LT or CT type toxins using Y1 cells; adhesive and invasive factors in Hep-2 cells; and haemolysins using horse, human and sheep erythrocytes.

After a preliminary investigation the method chosen for the isolation of HPC for further study was the inoculation of volumes (0.1 and 0.5ml) of water onto R2A medium and incubation at 30°C and 37°C for seven days to maximise the recovery of as wide a range of heterotrophs a possible. HPC were isolated from 18 drinking water distribution systems in England and Wales and tested for the presence of potential virulence factors

Over 1000 isolates were collected and attempts were made to identify them by basic biochemical tests in combination with commercial identification kits. A preliminary trial was performed of a PCR based method for the detection of specific genetic markers in water using *Campylobacter* as a model.

Conclusions

The population of HPC organisms in normal tap water includes small numbers of organisms that possess activities on tissue cultures (markers) similar to those associated with the virulence markers of some recognised enteropathogenic species of bacteria.

These activities are not associated with any particular species or phenotypic group of bacteria or recognised enteropathogenic species

If these organisms can produce disease they would probably need to be present in water in high numbers in excess of the guideline values for the HPC at 37°C after 48 hours incubation.

More extensive trials should be undertaken to compare the use of R2A medium and YEA and spread versus pour plates for the determination of the HPC count at 37°C and the effect of using chelating agents to improve recovery of HPC bacteria.

There is insufficient evidence at present to indicate a need for the development of standards for individual species but this should be continually reviewed in the light of new epidemiological information.

There is an urgent need for taxonomic studies on HPC bacteria in order to improve the ability to identify them and further our understanding of any role, if any, they may have in the epidemiology of gastrointestinal disease. The strains collected in this study could form the basis of such studies.

Although appropriate for research projects the tissue culture based assays are too laborious for the application as routine test methods in water laboratories

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INTRODUCTION

A wide variety of heterotrophic bacteria can be found in drinking water. These bacteria include those that have survived the water treatment processes used to supply potable water, and others which are able to grow within the distribution system either in biofilms, or within the water under favourable conditions (relatively high biodegradable dissolved organic carbon and low residual chlorine). The counts of these organisms vary throughout the year, and in warmer months may multiply considerably (Report 71 1994). Sudden changes in these counts are thought to be more significant than the absolute value, although consistently high counts should be investigated (EC Council Directive 80/778). Changes in the count at 37^{o}C are thought to give an early sign of pollution potentially of human or animal origin. In contrast counts at 22^{o}C reflect seasonal, environmental and other changes (Report 71 1983), but these differences are not clear cut. The counts are thought to have little health significance in themselves, but provide a general indication of the hygienic quality of water supplies, and provide a useful means of assessing the performance of water treatment processes (Report 71 1994).

A randomised intervention trial in Canada showed that, over a 15 month period, people in 299 households that had domestic reverse osmosis water filters installed had significantly fewer gastrointestinal symptoms than people in 307 households without such filtration. Furthermore in households with filtered water the incidence of symptoms of diarrhoeal disease correlated with higher counts of heterotrophic bacteria growing at 37°C (HPC). These results implied that some component of the heterotrophic bacterial population may cause significant levels of gastrointestinal infection within a population. (Payment *et al.* 1991a & b). Whilst the validity of these results has yet to be confirmed by further research it has caused others to question the possible role of heterotrophic bacteria as a cause of gastrointestinal disease.

The objectives of the work reported here were to determine:

1 whether HPC organisms may be of significance to health

2 whether it may be appropriate to develop standards for individual species or genera

The work was carried out in two phases

Phase 1 This included: a review of the techniques for measuring HPC; an assessment of whether the culture techniques used for the microbiological examination of water supplies are appropriate for a study of the health significance of HPC; a review of the possible means of assessing the virulence of HPC, and the development of a sequence of tests that might be applied to virulence assessment.

Phase 2 This included the isolation and identification of HPC from within distribution systems and testing them for the presence of potential virulence factors using the range of tests selected in phase 1.

1.1 POSSIBLE LINKS BETWEEN HPC AND DISEASE

The heterotrophs which may be present in a glass of water drunk by the consumer can be derived directly from the incoming mains water itself or from areas of microbial colonisation in the household water system, particularly the tap itself. Organisms colonising a kitchen tap, for example, may have originally been introduced from the supply water. They may also have originated from the tap becoming contaminated from external sources in the kitchen during normal use such as the washing of vegetables or poultry. Thus it is possible for tap water to become contaminated with potential pathogens even though they are absent in the incoming mains water.

There are several possible explanations for the apparent association of a raised heterotrophic count with a higher incidence of gastrointestinal symptoms in the absence of any detected pathogens in the water.

1 The population of HPC may contain species of bacteria that have not previously been recognised to cause gastrointestinal disease.

2 Certain individual potentially pathogenic species may be present as natural components of the HPC flora but not be readily detected by the selective procedures used although they are capable of growth on the media. Examples of these could be *Aeromonas* species or *Yersinia* species both of which can occur in water. The aeromonads and other bacteria could possibly be missed because they are more sensitive than many of the other heterotrophs to copper ions that might have entered the water during its passage through the domestic piping. Under normal circumstances, exposure to these copper ions may only be for very brief periods during passage through the piping or following being washed out of the area of colonisation in the outlet. Exposure during the sampling process and transit to the laboratory could be longer and copper sensitive organisms such as *Aeromonas* species may not survive sufficiently well to enable them to be detected by the normal procedures. In contrast the potentially pathogenic *Yersinia enterocolitica* could possibly be present but not recognised because strains of this species do not normally produce lactose and so would not be detected by the common methods used to determine coliforms in water. In a study which investigated the use of presence / absence techniques for the detection of coliforms in drinking water, methods which utilised the detection of galactosidase activity for the detection of coliforms, rather than the more traditional acid production from lactose, detected more coliforms. One group of coliforms commonly missed by the traditional methods were *Yersinia* species (Lightfoot, Tillett and Lee 1996).

3 Other recognised pathogenic species such as *Campylobacter* which may be present but are not readily detected either because they do not grow on the media used or they exist in a viable but non-culturable state.

4 Colonisation of the intestine by high numbers of certain HPC may cause a disturbance in the normal gut flora.

5 The presence of high numbers of HPC in the water may produce preformed toxins which cause gastrointestinal disturbance. The production of toxins in sufficient quantities to cause intestinal disturbance seems the least likely possibility since this would require considerable microbial growth.

A variety of approaches may be taken to determine if components of the HPC are potentially virulent.

1 Direct testing of freshly isolated strains of heterotrophs for a range of virulence markers.

2 Examining water for the presence of specific recognised pathogens by the appropriate isolation methods.

3 Examination of water for recognised pathogens using the polymerase chain reaction (PCR) to detect the presence of genetic material characteristic of known pathogens. This method may detect culturable, dead or viable but non-culturable forms of bacteria.

4 Direct examination of concentrates (filtrates) of water for the presence of known virulence genes using PCR and molecular biological probing techniques.

The work undertaken in this contract was directed primarily at approach 1.

1.2 SOURCES OF INFORMATION

A water bacteriology database was constructed by searching a range of scientific literature data bases including: Aqualine CD 1964-95; Biosys Previews online 1970-95; Chemical Abstracts online; 1967-95; Medline (CD-Plus) 1990-95; Medline online 1966-95; PHLS Library Bulletin on disk 1988-95; Science Citation Index on-line 1987-95; Waterlit on-line 1977-95; and Water Resources Abstracts on-line 1967-95.

1.3 METHODS FOR MEASURING COUNTS OF HETEROTROPHIC BACTERIA

For the purposes of the literature search and reviews we have used the following definition of **heterotrophic bacteria:**

> prokaryotic organisms that use reduced, preformed organic molecules (usually from other organisms) as carbon sources (Prescott *et al.* 1993).

This definition includes most bacteria of medical significance, but excludes cyanobacteria (commonly known as blue-green algae). A practical definition of heterotrophic bacteria is *bacteria that can grow on non-selective media containing mineral salts and a source of organic nutrients such as yeast extract.* Such organisms are commonly called heterotrophic plate count organisms (HPC).

The majority of the bacteria that occur in water must be heterotrophs. However the cultural methods commonly used in routine water microbiology to determine the numbers of heterotrophs will not detect the fastidious or slow growing bacteria. In addition the various techniques used for determining the numbers of heterotrophic bacteria present in drinking water all give different results because many organisms are not detected by a single method alone. The factors which influence the detection of these organisms are: the degree and the type of stress the organism is under for example heat shock, disinfectant, and its adaptation to the aquatic environment; composition of the culture medium; temperature of incubation; the resuscitation method; the length of incubation; the ability of the technique to detect viability; and the indicator of viability used.

Culture has traditionally been used as the method for detecting heterotrophic bacteria in water. However a variety of other methods for counting organisms in water have been used and these are summarised in Table 1.1 They include direct microscopy methods using vital stains which are good at detecting organisms which may not be recovered by cultural methods (Byrd *et al.* 1991; Rodrigues *et al*. 1992) and impedance (Noble *et al*. 1991).

Table 1.1 Methods for determining the count of heterotrophic bacteria in water samples

Type of method	Method	References
Cultural methods	Pour plate	Greenberg *et al.* 1985; Report 71 1994; Höll 1972; BSI 1989a and 1989b
	Spread plate	Greenberg *et al.* 1985; Report 71 1994; BSI 1989b
	Membrane filtration	Greenberg *et al.* 1985; BSI 1989a
	Spiral plate	Wada 1992
	Most probable number tube method	BSI 1989a
	Presence / absence	BSI 1989a
Direct microscopic counts	Acridine orange direct count	Report 71 1994; Byrd *et al.* 1991
	5-cyano-2,3-ditolyl tetrazolium chloride (CTC) count	Rodriguez *et al.* 1992
	4',6-diamidino-2-phenylindole count (4D2P)	Rodriguez *et al.* 1992
	DAPI count	Coleman 1980
	2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT) count	Tabor & Neihof 1982; Zimmerman *et al.* 1978
	Kogure method	King & Parker 1988
Physical measurement of growth	Impedance	Noble *et al.* 1991

1.3.1 Culture media, resuscitation and temperature

A variety of cultural conditions have been used to isolate heterotrophic bacteria from water, and the temperatures range from 0°C to 37°C. In the UK pour plate or spread plate counts are preformed using yeast extract agar (YEA; also known as plate count agar or tryptone glucose yeast agar) incubated at 22°C ± 1°C for 72 ± 3hr, and 37°C for 24 ± 3 hours, or R2A agar (Reasoner & Geldreich 1985) incubated at 22°C ± 1°C for 5-7 days, and 30°C for 3 days (Report 71 1994). R2A agar is a low nutrient medium which is good at recovering damaged or stressed organisms. In the UK YEA is used for compliance testing. The UK common practice is for the incubation step at 37°C to only last 24 hours although the EC Drinking Water Directive states 48 hours. In America the media used include tryptone glucose extract agar (TGA), YEA, R2A and m-HPC agar (Greenberg *et al*. 1985).

Nutrient gelatin has been used for plate counts for psychrophilic organisms and was reported to give better counts than those using agar containing media but the medium is unsuitable for performing counts at over 25°C and has generally been superseded by agar containing media.

1.3.1.1 Pour plate

The pour plate method has the advantage that colonies will grow discretely within the medium and spreading organisms and wet plates are not usually a problem. The technique can be used to examine sample volumes of 1ml or less. The main disadvantage is that the method can limit recovery of the maximum number of organisms, irrespective of the medium used, due to the heat shock associated with mixing the water with liquid agar at around 45°C. The nutritionally enriched YEA medium is not ideal for recovering starved bacteria (Greenberg *et al.* 1985). The fact that colonies are distributed throughout the medium means that it can sometimes be difficult to distinguish between small colonies and particulate matter and bubbles.

1.3.1.2 Spread plate

This technique will recover more organisms than pour plates because it eliminates the heat shock caused by the use of liquid agar. The technique requires less time and space than the pour plate, and the colonies are all on a single plane, which makes it easier to distinguish between small colonies and particulate matter and bubbles. The limitation of the technique is the small volume which can be tested (between 0.1 and 0.5ml), although in practice this may give a sufficient detection sensitivity.

1.3.2 Membrane filtration

For examining larger volumes of low turbidity waters membrane filtration is useful if low counts are expected. There are some problems associated with the quality of membrane filters used, and with contamination (Greenberg *et al*. 1985). Many aquatic bacteria, particularly from oligotrophic waters, produce very small cells that may pass through the normal 0.45µm pore sized membranes used for most drinking water analyses. These small cells will be trapped by 0.2µm pore sized membranes. It is also sometimes relatively difficult to count colonies on membranes unless a low powered binocular dissection microscope is used to examine the membranes. The method that Payment *et al.* 1991b used to determine the heterotrophic population was filtration of the water through a membrane which was then placed on R2A.

1.3.3 Acridine orange direct count (AODC)

The AODC is a count of all viable and non-viable bacteria, yeasts and moulds in a water sample (Hobbie *et al*. 1977; APHA 1989; Report 71 1994), and gives higher counts than cultural methods. It is a quick way of determining the total microbial content of water.

1.3.4 EC guidelines

The EC Drinking Water Directive states that the guide levels (GL) of total bacterial counts for water supplied for human consumption are 10/ml for the plate count at 37^{o}C after 48 hours, and 100/ml for the plate count at 22^{o}C after 5 days incubation. There is no maximum admissible concentration (MAC). For disinfected water the GL value should be considerably lower at the point where it leaves the processing plant. If during successive sampling any of these values are consistently exceeded a check should be carried out. For water in closed containers (bottled water) the GL is 5/ml for the plate count at 37^{o}C, and 20/ml for the plate count at 22^{o}C. The MAC is 20/ml for the plate count at 37^{o}C, and 100/ml for the plate count at 22^{o}C (EC Council Directive 80/778). These guidelines appear to derive from German regulations (Höll 1972).

1.3.5 Bactericidal compounds in water samples

Most potable water supplies are treated with chlorine and any residual chlorine in the water will continue to act during the transport of the sample to the laboratory. Consequently sodium thiosulphate is normally added at the time the sample is collected to neutralise any residual chlorine remaining in the water. Apart from the ions present initially, water may pick up certain metal ions, particularly copper, during distribution as a result of solution of the metal ions from pipes. Copper, in particular, is toxic to a range of bacteria notably species of *Aeromonas* (Versteeg *et al.* 1989, Domek *et al.* 1984). In the Netherlands it has been recommended that ethylene diamine tetraacetate (EDTA, final concentration 50 mg/l) should be added with the thiosulphate to neutralise the effect of copper on the recovery of *Aeromonas* species in particular (Versteeg *et al*. 1989).

Sodium thioglycollate (1 gram/litre) has also been suggested as a neutralising agent for metal ions such as copper and silver in water samples (Pyle *et al.* 1992).

1.4 SUITABILITY OF METHODS FOR DETECTING POTENTIALLY VIRULENT HETEROTROPHIC BACTERIA IN WATER

The investigation of the role of unidentified and previously unrecognised pathogens HPC in gastrointestinal disease by any of the above mechanisms will require the ability to detect their presence, preferably by isolation. For these reasons the direct microscopic and physical impedance methods are not appropriate. Preliminary studies have been carried out at the Public Health Laboratory Service (PHLS) Water and Environmental Microbiology Research Unit (WEMRU) of the effect of EDTA on the recovery of heterotrophs from tap water artificially contaminated with pure cultures of specific organisms and lowland river water. These experiments confirmed that EDTA can enhance the recovery of certain species, particularly *Aeromonas*, and coliforms and other naturally occurring aquatic heterotrophs present in the river water. However there was considerable variation between species and there appeared to be little, if any, evidence of higher recoveries of the heterotrophs occurring naturally in the unspiked tap water. The results of these pilot studies and the work of Versteeg *et al.* 1989 suggested that the use of EDTA warranted further investigation.

The most widely used media for the determination of HPC in Europe is the yeast extract agar (YEA) which is also used in the UK for regulatory testing. Of the other media that have been used for detection of HPC, the R2A agar of Reasoner & Geldrich 1985 has consistently been shown to yield the highest counts. Prior to isolating heterotrophs for identification and virulence testing and while virulence testing procedures were being set up it was decided to carry out a pilot study with the following aims:

1. To establish the levels of HPC likely to be encountered in drinking water as normally drawn from the tap in comparison to the numbers present in samples collected in the normal manner for compliance testing.
2. To determine the effect of EDTA on the recovery of heterotrophs.
3. To compare the use of R2A and YEA agars for the recovery of heterotrophs.
4. To determine the effect of incubation time and temperature on the HPC.

1.5 PILOT STUDY: PRACTICAL COMPARISON OF METHODS FOR DETERMINING THE HETEROTROPHIC COLONY COUNT

Full details of the methods used, the results and the analysis are described in Appendix 1. In order to obtain samples representing a range of types of domestic cold water and different geographical regions samples were collected and processed by nine Public Health Laboratories throughout England and Wales.

1.5.1 The effect of incubation time

For all methods the count continued to increase throughout the 14 day incubation period at all temperatures but this effect was most marked at 20°C. At 30°C and 37°C the largest change in count occurred between the 24 and 48 hour readings. By 7 days the mean count at 30°C was 87% of the final mean count and at 37°C it was 94% of the final mean count. In contrast, the mean count at 20°C had only reached 62% of the final mean count by 7 days.

The standard method in the UK is to use pour plates with YEA and incubate the plates for only 24 hours rather than the 48 which is specified in the EC Drinking Water Directive. The counts using the pour plate method at 37°C after 24 and 48 hours incubation were compared using a simple two factor analysis of variance. The counts after 48 hours were significantly higher than the counts after 24 hours ($p=0.02$)

1.5.2 Comparison of counts by spread plates (R2A), pour plates (YEA) and filtration (YEA)

The spread plate technique using R2A medium consistently gave the highest counts at all temperatures in the presence or absence of EDTA. The pour plate method also gave higher counts than the filtration method. The spread plate method on R2A often gave counts that were one or even two orders of magnitude higher than the counts obtained by the filtration method. The counts on R2A by the spread plate method were generally higher than on YEA by the standard pour plate method.

1.5.3 The effect of incubation temperature

As might have been expected, there was a considerable difference between the counts recorded at the different temperatures and this was significantly different. The counts at 20°C exceeded those at 30°C and in turn these were greater than the counts at 37°C

1.5.4 The effect of EDTA

A comparison of the two sets of counts in the presence and absence of EDTA by a two factor analysis of variance confirmed that there was no significant difference in the means of the two sets of results and that this was true for both immediate and post-flush samples

1.5.5 Comparison of immediate and post flushed samples

The counts of the pre flushed samples, that is the samples of water withdrawn from the taps without previously disinfecting or running them, were consistently higher than the counts on samples collected after the taps had been disinfected and flushed.

1.5.6 Discussion

It was to have been expected that the counts on samples taken without flushing and disinfection of the taps would have been higher than those after disinfection since it would be expected that a significant amount of contamination is derived from the colonisation of the tap. Similarly it was expected that the counts at 22°C would be higher than at 30°C or 37°C since this is the normal pattern in water samples from distribution systems. Workers using R2A medium have consistently found it to give higher counts than with other media and the results described here are yet another confirmation of this effect.

The membrane filtration method using YEA gave appreciably lower counts in comparison to both the spread plate method using R2A and the standard UK pour plate method using YEA. The inefficiency of the membrane method is probably a result of a combination of factors. The surface area of the membranes is much less than that of the plates used for the other two methods, leading to crowding of bacterial colonies on the membranes. This crowding caused reduced counts due to competition and coincidence of colonies (colonies growing on top of one another) making it difficult to count them accurately. Several laboratories noted that the colonies on the membranes were extremely small and could only be counted with a lens or dissecting microscope. In their studies Payment *et al.* 1991a & b filtered water and incubated the membrane on the surface of R2A medium in order to determine the heterotrophic count. Although the use of R2A may have encouraged higher counts than YEA, the membrane filtration method is likely to have considerably underestimated the count.

In contrast to the preliminary studies using spiked samples the addition of EDTA to the sample containers appeared to have no effect on the actual numbers of bacterial colonies grown from tap water samples. All of the samples were collected from taps connected to copper piping and there did not appear to be any obvious difference in the colony types of the bacteria growing with and without EDTA treatment. These results suggest that the majority of bacteria growing from water samples are resistant to the copper levels commonly experienced in drinking water distribution systems in domestic households.

The results of this preliminary trial indicated that the best method for detecting the largest number of bacteria in domestic tap water samples is to spread subsamples onto the surface of R2A medium. Higher counts were obtained at 20°C than at 30°C and 37° but the counts at 30° were intermediate to those at 20°C and 37°C. To be capable of growth in the human intestine bacteria must be able to grow at body temperature (37°C). Many bacteria growing at 20°C will not be

capable of growth at 37°C whereas the majority detected at 30°C and, *a priori*, those detected at 37°C, will be capable of growth within the human body. For some of the virulence assays growth at 37°C is required. Accordingly the methods chosen to isolate bacteria from drinking water for the second phase of the study were to collect water without adding EDTA but adding sodium thiosuphate to neutralise any residual chlorine followed by inoculating portions of the water onto the surface of plates of R2A medium which would then be incubated at 30°C and 37°C for up to seven days prior to the selection of the bacterial colonies for further testing

1.6 VIRULENCE FACTORS OF ORGANISMS THAT CAN BE WATER BORNE AND THEIR DETECTION

Methods to be used for detecting potential virulence markers in bacteria present in water must be: capable of detecting as wide a range of markers as possible; readily applicable to a large number of samples at a time; relatively rapid and reliable. The virulence factors produced by the genera of bacteria that can be waterborne can be broadly divided into adhesins, haemolysins, cytotoxins, enterotoxins and invasiveness, that is the ability to invade and grow in cells (Nichols *et al.* 1995a). The specific virulence factors that have been described for the enteropathogens or putative enteropathogens that can be found in water are summarised in table 1.2. The expression of most of these virulence determinants can be determined by biological assays in a range of animals. However these are not practical for routinely screening the pathogenicity of large numbers of isolates by water undertakings as it would require the extensive use of animals. Apart from the expense involved in keeping animals and the relative slowness of some of the assays it is doubtful that this approach could be considered ethical for routine use in view of the low incidence of disease of unknown cause associated with drinking water in Britain. The use of animal models was therefore discounted when selecting tests for use in the screening of isolates in phase 2.

Tissue cultures , like animal models, can give a response to a relatively wide range of toxins. Many ELISA or gene probe techniques have been developed for many of the toxins and these are summarised in tables 1.1 and 1.2. However, although these can sometimes detect closely related toxins from different bacterial strains or species they are inherently more specific and therefore less suitable than tissue cultures for screening many isolates of unknown identity and activity. Thus a selection of tissue cultures is likely to offer the most sensitive method of screening large numbers of isolates for biological activity.

Although the different groups of virulence determinants can be found in a range of bacterial genera many of them are related in terms of their biological actions and sometimes genetically. Thus the action of several of the determinants listed in table 1.2 can be detected by a relatively small range of biological assays based on the action of bacterial cells or their culture supernatants on tissue cultures. Cholera toxin (CT) is similar in action and closely related genetically to the heat labile enterotoxin (LT) of *E. coli* . These two toxins are said to be cytotonic in their action and other cytotonic enterotoxins have been described in *Aeromonas* species, and *Vibrio* species. The

cytotonic enterotoxins all have similar action on certain tissue cultures but the adrenal Y1 cells which were originally used to assay CT and LT have been used most widely and so this cell line was considered to be a suitable candidate for screening purposes.

Invasiveness is a virulence determinant in *Shigella* species and enteroinvasive strains of *E. coli* (EIEC) which can be determined in Hep2 cells. The same cell line can also be used to assay adhesiveness which is a determinant in some pathogens such as enteropathogenic *E. coli* (EPEC).

Vero cells are used primarily to detect the Vero cytotoxins found in enterohaemorrhagic *E coli* but similar activities have been observed in a range of other organisms including *Aeromonas, Shigella,* and *Vibrio* species. In addition some cytotonic, LT or CT-like, toxins can also produce a reaction in this cell line.

The same type of enterotoxin can be expressed differently in different species. In some cases the toxin is released from the cell into the culture medium and in others it may remain cell bound. The expression of the toxin may also depend on the cultural conditions. Thus it is important when screening isolates of unknown activity to use both culture supernatants and whole cells in the assays. Ideally a range of cultural conditions would also be screened but this is not practical when screening a large number of unknowns.

Haemolysins against erythrocytes from a range of different species have been proposed as virulence markers in some species of *Aeromonas* and *Vibrio*. In particular the virulence of *V. parahaemolyticus* is associated with a haemolysin active on human erythrocytes. Although the importance of haemolysins is unclear in many other species they can be screened for very easily at little cost.

The heat stable ST-type enterotoxins of *E.* coli may not readily be detected by tissue cultures although they can be detected by their action in animal models such as suckling mice and ligated ileal loops of pigs or other animals. Apart from animals the only way of detecting these activities is the use of molecular biological techniques. Genetic probes can be used relatively easily to detect the presence of genes or closely related sequences in a large number of isolates at a time. The technique of polymerase chain reaction (PCR) can also be used to amplify and detect specific genetic sequences in a specimen. The application of the technique to water is, however, still in its infancy. The technique could be particularly useful for detecting organisms which are not readily cultured in the laboratory. This could be because they cannot grow on artificial media, e.g. viruses, or they are present as such a small proportion of the total bacterial population that they cannot be readily seen because of overgrowth by the competing flora, e.g. legionellae or because they have entered a viable but non-culturable state.

Campylobacter species are enteropathogens that are both relatively difficult to culture because they have special growth requirements and are also reported to enter a viable but not culturable state in the environment including water. In the viable but non-culturable state they are believed to remain infective but cannot be cultured on artificial media. Their presence can, however, be detected by PCR. A PCR assay has been developed recently at the Manchester Public Health Laboratory specific for the enteropathogens *Campylobacter jejuni, C. coli* and *C. upsaliensis* and is subject to an international patent application number PCT/GB94/01967. Water has been incriminated as a potential source of infection by campylobacters and thus the detection of campylobacters by PCR could be used as a model for the detection of specific enteropathogens in water.

1.7 PLAN OF TESTING FOR VIRULENCE DETERMINANTS IN PHASE 2

In order to examine as wide a range of organisms as possible from different types of water, strains would be isolated from samples collected by different Public Health Laboratories throughout England and Wales. As many of the strains as practicable in the time available would be tested for the following:

Haemolysins using a range of blood cells from different animals including horse, human and sheep blood.

Cytotoxins / enterotoxins using Vero cells and Y1 adrenal cells

Invasiveness and adhesiveness using Hep2 cells.

In addition if possible a PCR assay for *Campylobacter* would be used to attempt to determine the feasibility of using this method to detect unculturable pathogens in drinking water samples

Table 1.2 Virulence factors of organisms which can be waterborne

Description	Virulence Factors	DNA probes	PCR primers
Aeromonas hydrophila/sobria	cytolytic toxin		
	cytotonic toxin (cytotonin)		
	enterotoxin		
	aerolysin asoA		
	protease		
	haemolysin		
	haemagglutin		
	acetylcholine esterase		
Campylobacter jejuni/coli/lari	cytolethal distending toxin		
Citrobacter freundii	SLT	SLT2	+
Clostridium difficile	Toxin A		
	Toxin B		
Clostridium perfringens	cytotoxic enterotoxin		
Diffusely adherent *E.coli* DEAC	DA	daa	-
	AIDA-1	-	
Enteroaggregative *E.coli* EAggEC	EAST1	astA	-
	AggA	EAggEc	-
Enterohaemorrhagic *E coli*	Vero cytotoxin (Shiga like) VT1	VT1	+
(Vero cytotoxin producing)	Vero cytotoxin (Shiga like)	VT2 VT2	+
	Vero cytotoxin VT2 variants	VT2 variants	+
	AE lesions	eae	+
Enteroinvasive *E.coli* EIEC	Invasion	ial	+
		paB	+
Enteropathogenic *E.coli* EPEC	Bundle forming pili	EAF	+
	AE lesions	bpf	+
	94 Kda OMP	eae	+
Enterotoxigenic *E.coli* ETEC	heat-stable enterotoxin STa	STa1	+
	heat-stable enterotoxin Stb	STa2	+
		STb	+
	heat-labile enterotoxin LT	LT1	+
		LT2	+
	Colonisation factors		
Klebsiella pneumoniae	heat-stable enterotoxin ST	ST	+
Plesiomonas shigelloides			
Pseudomonas aeruginosa	exotoxin A		
Salmonella spp		spvABC	
Shigella spp	Shiga toxin gene stx		
	aerobactin		
	group specific O antigen		
	superoxide dismutase sodB		
	invasion gene virB		
	invasion gene ipaABCD		
	invasion gene ippI		
	invasion gene invGF		
	invasion gene invAJKH		
	intracellular spread gene virG		
	plasmid antigen gene ipaH		
	plasmid expression gene malA		
	plasmid expression gene galU		
	plasmid expression gene glpK		
	keratoconjunctivitis gene kcpA		
Vibrio cholerae	Cholera toxin		+
Vibrio parahaemolyticus	haemolysin		
Yersinia enterocolitica	heat-stable enterotoxin	yst	
	lipopolysaccharide O side chain		

Table 1.3 Methods for detecting toxins and other virulence factors involved in gastrointestinal disease

Toxin	Organism	Test	Reference
Shiga toxin	*E. coli*	ELISA	Acheson *et al*. 1990
Shiga like toxin II	*E. coli*	ELISA	Acheson *et al*. 1990
Virulence	*Y. enterocolitica*	desferal-attenuated murine model	al-Hendy *et al*. 1992
Vero-cytotoxin	*E.coli*	Mitomycin C enhanced vero cell assay	al-Jumaili *et al*. 1992
Exotoxin A	*P.aeruginosa*	Mouse lethality bioassay	al-Ssum 1992
Enteropathogenicity	*E.coli*	Attaching and effacing cells in rabbit ileal loop	Albert *et al*. 1992
Enteropathogenicity	*E.coli*	Fluorescent actin staining of Hep2 cells	Albert *et al*. 1992
Enteropathogenicity	*E.coli*	AE lesion gene hybridisation	Albert *et al*. 1992
Heat stable enterotoxin (ST)	*E.coli*	Suckling mouse bioassay	Alessio *et al*. 1993
Heat stable enterotoxin (ST)	*E.coli*	ELISA	Alessio *et al*. 1993

Isolation and testing of heterotrophs for virulence markers

2.1 METHODS

The methods will only be described briefly here. Full details are given in appendix 2

2.1.1 Isolation of strains for testing

Water samples were collected from public supplies to single occupancy or multiple occupancy domestic properties by 9 Public Health Laboratories throughout England and Wales. Each laboratory collected a sample with and without disinfection of the outlet from a single outlet in each of two properties. Organisms were cultured by directly inoculating 0.1ml volumes of 1:10 and 1:100 dilutions of the water onto the surface of plates of R2A medium and incubating at 30°C and 37°C for 7 days. Thirty colonies at each temperature were subcultured and sent to the WEMRU for identification and virulence testing.

2.1.2 Identification

On receipt in WEMRU isolates were subcultured and stored on R2A slopes at 4-8°C, at room temperature in the dark and on glass beads at –40°C. Attempts were made to identify the isolates using routine bacterial tests in conjunction with commercial identification kits (BBL Crystal for gram-negative glucose-fermenters and API20NE for gram negative non-fermenters).

2.1.3 Haemolysin

Strains were screened for haemolytic activity against horse, human and sheep erythrocytes using multipoint inoculation techniques and incubation for 24 hours at 37°C.

2.1.4 Verotoxin assays

Heated (100°C for 15 minutes) and unheated filter sterilised supernatants from shaken 18-24h trypticase soy broth cultures of each strain were incubated with Vero cells for 4 days at 37°C. The cells were then fixed, stained and examined microscopically for evidence of cytotoxic activity

2.1.5 Activity on Y1 cells (LT type toxic activity)

Heated and unheated filter sterilised trypticase soy broth supernatants and whole cells were incubated with adrenal Y1 cells for 24h incubation at 37°C. The cells were then fixed, stained and examined microscopically for evidence of cytotonic or cytotoxic activity.

2.1.6 Test for adhesion

The bacteria were grown overnight without shaking at 37°C in peptone water and dilutions of the culture were incubated with Hep2 cells for 3 hours at 37°C before washing fixing staining and microscopic examination for the presence of bacteria adhering to the tissue culture cells..

2.1.7 Test for invasiveness

The bacteria were grown overnight without shaking in nutrient broth at 37°C. Portions of the diluted culture were incubated with Hep2 cells for 2 hours , the cells washed to remove free bacteria and then incubated in the presence of gentamicin, lysozyme and growth medium for 3 hours before fixing, staining and examining the cells microscopically. Any bacterial cells that have not invaded the Hep2 cells are killed and lysed by the gentamicin and lysozyme so that any bacterial cells that are observed inside cells must have invaded the cells.

2.2 RESULTS

1127 isolates were received from 9 Public Health Laboratories in England and Wales. Table 2.1 is a summary of the samples received by the WEMRU for identification and toxin assay. Each laboratory sampled two sites, of these 10 were located in a single dwelling site i.e. family occupied properties which yielded a total of 682 isolates, 421 (62 %) preflush (mean 45.11, median 36) and 261 (38%) postflush (mean 29.8, median 30). Eight sites were located within a multioccupancy dwelling e.g. a student hall of residence or a nurses home and yielded a total of 445 isolates of which 265 (60%) pre-flush (mean 40.71, median 40) and 180 (40%) postflush (mean 23.7, median 30).

2.2.1 Identification

Several isolates failed to grow on subculture, 902 of the viable isolates were Gram-stained. Of these, 396 were Gram-negative (Gram-ve) of which 376 were Gram -ve bacilli. Of the remaining isolates 463 were Gram-positive (Gram + ve) of which 442 were Gram +ve cocci. The remaining 43 isolates tested were Gram-variable. Of the preflush isolates 246 were Gram-negative, 303 were Gram-positive and 27 gave a Gram-variable result (total 580 tested). Of the postflush isolates

tested; 150 were Gram-negative; 157 were Gram-positive and 16 gave a Gram-variable result (total 328).

The ability to utilise glucose and the manner of utilisation allows further separation into three major subgroups: fermentative; oxidative, and those which showed no colour change in either tube which may be either non-utilisers or alkali producers.

A further important taxonomic sub-division is denoted by the reaction with oxidase reagent. Table 2.2 gives the summary of isolates which were Gram-negative, fermentative and oxidase positive (74 out of a total of 396 Gram-negtive isolates). Of these isolates 69 were Gram-negative bacilli. Table 2.3 gives the summary of isolates which were Gram-negative, fermentative and oxidase negative (34 from 396 Gram-negative isolates). Of the remaining isolates 86 were Gram-negative and oxidative in the Hugh and Leifsons medium, of these 59 were also oxidase positive (Table 2.4) and 27 were Gram-negative, oxidative and oxidase negative (Table 2.5).

Table 2.6 shows the frequency of identifications at each temperature and also between preflush and postflush samples. Commercial biochemical identification strips were used for 261 of the isolates, 143 were inoculated into BBL Crystal E/NF strips and 119 into API 20NE strips.

Two strains were identified by the API 20NE strips as belonging to recognised enteropathogenic species. These were strain 27 *Shigella* sp. isolated from a water sample after disinfecting the tap and strain 371 *V. parahaemolyticus* isolated from a pre-flush sample. Strain 27 gave no positive responses in the virulence tests (see table 2.7). When an attempt was made to repeat the identification and confirm it by further tests it was discovered that the stored cultures of this strain had died. Strain 371 was haemolytic to horse, human and sheep erythrocytes.

The most frequent tentative identification was *Listonella damsela* which accounted for 46 of the isolates. Of these 26 were from single occupancy sites, 18 of which were from preflush samples (9 incubated at 30 °C and 9 at 37 °C) and 8 from post flush samples (2 incubated at 30 °C and 6 at 37 °C). From multiple occupancy sites 20 tentative *L. damsela* were isolated, 11 of these were from preflush samples, 9 of which had been incubated at 30°C, of the remaining 9 isolates from postflush samples, 8 had been incubated at 30 °C.

The results from the O/F tests show that 35 of these isolates were oxidative in Hugh and Leifson's medium and 11 showed no change. Of the 46 isolates, all were acid and gas negative in glucose peptone water and 30 were oxidase positive. All the *L. damsela* were tentatively identified using the API 20NE test strips. For this species, there were 24 different profiles. The most frequent profile (14 from 46) was 4100004. The second most frequent tentative identifications (8) fell within the group termed Miscellaneous Gram Negative Bacilli (MGM-ve) [Appendix 3] and *Weeksella virosa* (8).

Six of the 29 oxidative, oxidase negative cultures incubated at 37 °C were also inoculated into API test strips and two of these also gave *Listonella damsela* as a tentative identification.

Table 2.1. Summary of preflush and postflush isolates from single and multiple occupancy sites.

Laboratory Site Code	occupancy	total no. isolates	pre-flush	postflush
B1	single	75	57	18
B2	multi	62	26	36
G1	single	90	60	30
G2	multi	91	61	30
M1	single	74	43	31
M2	multi	70	40	30
N1	single	59	29	30
N2	multi	36	24	12
P1	single	56	36	20
P2	multi	63	58	5
R1	single	65	60	35
R2	multi	24	14	10
S1	single	57	31	26
S2	multi	95	53	42
T1	single	63	18	45
T2	single	58	21	37
D1	single	78	45	33
D2	multi	10	9	1
		1127	686	441

9 laboratories each sampled two sites (1 and 2), 9 samples were from single dwelling sites (single) and seven from multiple occupancy sites e.g. student residences or nurses homes (multi).

Table 2.2 Gram negative, fermentative, oxidase positive isolates

SERIAL No	LAB	OCCUPANCY	°C	SHAPE	GAS	ACID	BBL CODE	IDENTIFICATION
6	T1	single	37	bacilli	-	+	2210100140	*Flavobacterium indologenes*
9	T1	single	37	bacilli	-	-	2000100004	*Weeksella virosa*
12	T1	single	37	bacilli	-	-	2111200000	unacceptable
18	T1	single	37	bacilli	+	+	2211120000	*Flavobacterium indologenes*
21	T1	single	37	bacilli	-	-	0000000000	no growth
31	T1	single	37	bacilli	-	-	2110100000	unacceptable
47	T2	single	37	bacilli	-	-	2233100000	*Pseudomonas stutzeri*
48	T2	single	37	bacilli	-	-	3233120000	unacceptable
49	T2	single	37	bacilli	-	-	3233120110	unacceptable
52	T2	single	37	bacilli	-	-	0000000000	no growth
59	T1	single	30	bacilli	+	+	0000000000	no growth
70	T2	single	30	bacilli	-	+	3331320240	unacceptable
76	T2	single	30	bacilli	+	+	0000000000	no growth
79	T2	single	30	bacilli	-	-	0000000000	no growth
95	T1	single	30	bacilli	+	+	3200100240	unacceptable
106	T2	single	30	bacilli	-	-	2310000000	*Pseudomonas vesicularis*
122	T2	single	30	bacilli	-	-	0000000000	no growth
124	P1	multi	30	bacilli	-	-	1020000000	Miscellaneous Gram negative bacilli
130	P1	multi	30	bacilli	-	-	0000000000	no growth
134	P1	multi	30	bacilli	-	-	0000000000	no growth
145	P1	multi	30	bacilli	-	-	1000000000	Miscellaneous Gram negative bacilli
151	P1	multi	30	bacilli	-	-	0000000000	no growth
163	P1	multi	30	bacilli	-	-	0110100004	unacceptable
165	P1	multi	30	bacilli	-	-	1010100004	Miscellaneous Gram-negative bacilli
167	P1	multi	30	bacilli	-	-	0000000000	no growth
182	P2	single	30	cocci	-	-	3321320122	*Flavobacterium meningosepticum*
189	P2	single	30	cocci	-	-	3211300320	unacceptable
199	P2	single	30	bacilli	+	-	3303310313	unacceptable
201	P2	single	30	bacilli	+	+	3331320320	unacceptable
203	P2	single	30	bacilli	+	+	3012010010	unacceptable
205	P2	single	30	bacilli	-	-	3212210130	unacceptable
206	P2	single	30	bacilli	-	-	0000000000	no growth
207	P2	single	30	cocci	+	+	3332021133	unacceptable
243	P2	single	37	bacilli	-	-	3212210330	unacceptable
521	N1	single	30	bacilli	-	-	0000000000	no growth
545	N1	single	30	bacilli	-	-	0000000000	no growth
305	N2	multi	30	bacilli	-	-	0101100011	Miscellaneous Gram-negative bacilli
321	R1	single	37	bacilli	-	-	2331300000	*Sphingobacterium multivorum*
323	R1	single	37	bacilli	-	-	3331300000	*Flavobacterium indologenes*
328	R1	single	37	bacilli	-	-	3331300000	*Flavobacterium indologenes*
554	R1	single	30	bacilli	-	-	3321120013	*Pseudomonas paucimobilis*
556	R1	single	30	bacilli	-	-	7310000000	unacceptable
557	R1	single	30	cocci	-	-	3302000000	*Pseudomonas vesicularis*
559	R1	single	30	cocci	-	-	3332022300	unacceptable

Table 2.2 (continued) Gram negative, fermentative, oxidase positive isolates

SERIAL No	LAB	OCCUPANCY	°C	SHAPE	GAS	ACID	BBL CODE	IDENTIFICATION
566	R1	single	30	bacilli	-	-	3221100000	unacceptable
790	B2	multi	37	bacilli	+	+		
794	B2	multi	37	bacilli	+	+	3131102200	unacceptable
802	B1	single	37	bacilli	+	+	0000000000	no growth
811	B1	single	37	bacilli	-	-	5601100024	unacceptable
830	B1	single	30	bacilli	+	+	4000140000	unacceptable
390	M1	single	30	bacilli	+	+		
405	M1	single	37	bacilli	-	-	0000000000	no growth
408	M1	single	37	bacilli	-	-		
419	M1	single	30	bacilli	-	-		
875	S2	multi	30	bacilli	+	+	0000000000	no growth
896	S2	multi	37	bacilli	+	+	0221124002	*Agrobacter tumifaciens*
926	S1	single	30	bacilli	-	-	0001320000	*Acinetobacter lwoffi*
933	S1	single	30	bacilli	-	-		
1056	G2	multi	37	bacilli	-	-	1003110010	*Pseudomonas flourescens*
1057	G2	multi	37	bacilli	-	-		
1058	G2	multi	37	bacilli	-	-	1203110010	*Pseudomonas stutzeri*
1064	G2	multi	37	bacilli	-	-	0000000000	no growth
1076	G2	multi	37	bacilli	-	-	1003000000	Miscellaneous Gram-negative bacilli
1077	G2	multi	37	bacilli	-	-	2323300000	*Sphingobacterium multivorum*
1078	G2	multi	37	bacilli	-	-	2323120202	*Sphingobacterium multivorum*
1080	G2	multi	37	bacilli	-	-	0000000000	no growth
1084	G2	multi	30	bacilli	-	-		
1156	G1	single	37	bacilli	-	-		
1167	G1	single	37	bacilli	-	-	0000000000	no growth
1186	G1	single	30	bacilli	-	-	2331100000	*Sphingobacterium multivorum*
585	D1	single	30	bacilli	-	-	0000000000	no growth
614	D1	single	37	bacilli	-	-	0000000000	no growth
624	D1	single	37	bacilli	-	-		
626	D1	single	37	bacilli	-	-	0000000000	no growth

52 isolates were from single occupancy sites of which 34 were from preflush samples and 18 from post flush samples (single). 22 isolates were from multiple occupancy sites (multi), of which 17 were from preflush samples and 5 from post flush samples.

Table 2.3 Gram negative, fermentative, oxidase negative isolates

SERIAL	LABCODE	OCCUPANCY	TEMP	SHAPE	GAS	ACID	BBL	IDENTIFICATION
3	T1	single	37	bacilli	-	-	2110211020	unacceptable
8	T1	single	37	bacilli	-	-	2110011000	unacceptable
10	T1	single	37	bacilli	-	+	2210320000	unacceptable
27	T1	single	37	bacilli	-	-	2010000000	*Shigella* sp.
29	T1	single	37	bacilli	+	+	2001304020	unacceptable
36	T1	single	37	bacilli	-	-	0000000000	no growth
42	T2	single	37	bacilli	+	+	0000000004	*Acinetobacter lwoffii*
43	T2	single	37	bacilli	-	-	3010000000	*Shigella* sp.
80	T2	single	30	bacilli	-	-	0000000000	no growth
125	P1	multi	30	bacilli	-	-	0010000000	*Acinetobacter lwoffii*
128	P1	multi	30	bacilli	-	-	1000000000	*Acinetobacter lwoffii* / *Shigella* sp.
136	P1	multi	30	bacilli	-	-	2210000000	unacceptable
146	P1	multi	37	bacilli	-	-	0000000000	no growth
158	P1	multi	30	bacilli	-	-	0001144004	unacceptable
190	P2	single	30	bacilli	-	-	3212010130	unacceptable
200	P2	single	30	bacilli	+	+	3201320320	unacceptable
526	N1	single	30	bacilli	-	-	1201100000	*Pseudomonas stutzeri / Acinetobacter*
304	N2	multi	30	cocci	-	-		
345	R1	single	37	bacilli	-	-	0011100000	*Acinetobacter lwoffii*
791	B2	multi	37	bacilli	+	+		
382	M1	single	30	bacilli	-	-		
384	M1	single	30	bacilli	+	+	0000000000	no growth
401	M1	single	30	bacilli	-	-	0000000000	no growth
404	M1	single	37	bacilli	+	+	0000000000	no growth
874	S2	multi	30	bacilli	+	+	0000000000	no growth
924	S1	single	30	bacilli	-	-	1301310010	*Xanthomonas maltophilia*
997	S1	single	37	bacilli	+	+	0000000000	no growth
1050	G2	multi	30	bacilli	-	-	0000000000	no growth
1059	G2	multi	37	bacilli	-	-	1003100000	Miscellaneous Gram-negative bacilli
1074	G2	multi	37	bacilli	-	-	3203110010	*Xanthomonas maltophilia*
644	D1	single	30	bacilli	+	+	0000000000	no growth
685	D1	single	30	bacilli	-	-	0000000000	no growth
731	D2	multi	37	bacilli	-	-	0000000000	no growth
755	D2	multi	30	bacilli	-	-		

21 isolates were from single occupancy sites of which 11 were from preflush samples. 13 isolates were from multiple occupancy sites of which 10 were from preflush samples.

Table 2.4 Gram negative, oxidative, oxidase positive isolates.

SERIAL	LABCODE	OCCUPANCY	FLUSH	TEMP	SHAPE	GAS	ACID	API	IDENTIFICATION
17	T1	single	postflush	37	bacilli	+	+	4141664	*Aeromonas*
113	T2	single	postflush	30	bacilli	+	+	0000000	no growth
126	P1	multi	preflush	30	bacilli	-	-	4100004	*Listonella damsela*
141	P1	multi	preflush	30	bacilli	-	-	4147004	*Aeromonas*
148	P1	multi	preflush	30	bacilli	-	-	4100004	*Listonella damsela*
149	P1	multi	preflush	30	bacilli	-	-	0000000	no growth
152	P1	multi	preflush	30	bacilli	-	-	0000000	no growth
193	P2	single	preflush	30	bacilli	-	-	4100004	*Listonella damsela*
221	P2	single	preflush	37	bacilli	-	-	1503056	*Pseudomonas putida*
245	N1	single	postflush	37	bacilli	-	-	1044444	*Pseudomonas stutzeri*
246	N1	single	postflush	37	bacilli	-	-	4150724	*Aeromonas*
247	N1	single	postflush	37	bacilli	-	-	4254360	*Vibrio metschnikovii* /
248	N1	single	postflush	37	bacilli	-	-	0100004	*Pasteurella* sp.
249	N1	single	postflush	37	cocci	-	-	4700204	*Listonella damsela*
250	N1	single	postflush	37	bacilli	-	-	4100004	*Listonella damsela*
252	N1	single	postflush	37	bacilli	-	-	0100004	*Pasteurella* sp.
254	N1	single	postflush	37	bacilli	-	-	4100004	*Listonella* sp
257	N1	single	postflush	37	bacilli	-	-	0110004	*Weeksella virosa*
261	N1	single	postflush	37	bacilli	-	-	4100004	*Listonella damsela*
264	N1	single	postflush	37	bacilli	-	-	4400004	*Aeromonas*
266	N1	single	postflush	37	bacilli	-	-	4000004	*Moraxella* sp.
267	N1	single	postflush	37	bacilli	-	-	0100004	*Pasteurella* sp.
268	N1	single	postflush	37	bacilli	-	-	4300004	*Listonella damsela*
520	N1	single	preflush	30	bacilli	-	-	4110004	unacceptable
530	N1	single	preflush	30	bacilli	-	-	4110004	unacceptable
532	N1	single	preflush	30	bacilli	-	-	0010004	*Weeksella virosa*
533	N1	single	preflush	30	bacilli	-	-	0110004	*Weeksella virosa*
536	N1	single	preflush	30	bacilli	-	-	4210004	*Weeksella zoohelicum*
541	N1	single	preflush	30	bacilli	-	-	4310004	*Listonella damsela*
291	N2	multi	preflush	30	bacilli	-	-	4010004	*Weeksella zoohelicum*
292	N2	multi	preflush	30	bacilli	-	-	4110004	*Weeksella virosa*
317	R1	single	preflush	37	bacilli	-	-	5057344	*Vibrio*
318	R1	single	preflush	37	bacilli	-	-	1247744	*Ochrobacter anthropi*
319	R1	single	preflush	37	bacilli	-	-	1647765	*Agrobacterium*
341	R1	single	preflush	37	bacilli	-	-	4100044	*Listonella damsela*
342	R1	single	preflush	37	bacilli	-	-	0300000	*Listonella damsela*
343	R1	single	preflush	37	bacilli	-	-	5147446	*Pseudomonas putida*
344	R1	single	preflush	37	bacilli	-	-	6210004	*Flavobacterium*
577	R1	single	preflush	30	bacilli	-	-	6340204	*Listonella damsela*
371	R2	multi	preflush	30	bacilli	-	-	4300000	*Listonella damsela*
373	R2	multi	preflush	37	bacilli	-	-	0743004	*Flavobacterium*
785	B1	single	preflush	30	bacilli	-	-	4240244	*Listonella damsela*
795	B2	multi	preflush	37	bacilli	-	-	4500004	*Listonella damsela*
812	B1	single	preflush	37	bacilli	-	-	5100004	*Listonella damsela*
813	B1	single	preflush	37	bacilli	-	-	4640044	*Listonella damsela*
814	B1	single	preflush	37	bacilli	-	-	4440244	*Pseudomonas*
815	B1	single	preflush	37	bacilli	-	-	4100044	*Listonella damsela*
817	B1	single	preflush	37	bacilli	-	-	4100044	*Listonella damsela*
819	B1	single	preflush	37	bacilli	-	-	4563204	*Sphingobacterium*
826	B1	single	preflush	37	bacilli	-	-	4100004	*Listonella damsela*
873	S2	multi	preflush	30	bacilli	-	-	5577741	*Neisseria cinerea*
934	S1	single	preflush	30	bacilli	-	-	1000004	*Moraxella* sp.
995	S1	single	postflush	37	bacilli	-	-	0000000	no growth
1068	G2	multi	preflush	37	bacilli	-	-	1047750	*Ochrobacter anthropi*
1069	G2	multi	preflush	37	bacilli	-	-	1247754	*Ochrobacter anthropi*
1130	G1	single	postflush	30	bacilli	-	-	0411001	*Flavobacterium*
1160	G1	single	preflush	37	bacilli	-	-		
1164	G1	single	preflush	37	bacilli	-	-		
583	D1	single	preflush	30	bacilli	-	-		
586	D1	single	preflush	30	bacilli	-	-	0000000	no growth
610	D1	single	preflush	37	bacilli	-	-	0000000	no growth

46 isolates were from single occupancy sites of which 29 were from preflush samples and 17 from post flush samples (single). 13 isolates were from multiple occupancy sites (multi), all of which were isolated from preflush samples

Table 2.5 Gram negative, oxidative, oxidase negative isolates.

SERIAL	LABCODE	OCCUPANCY	FLUSH	TEMP	SHAPE	GAS	ACID	API	IDENTIFICATION
115	T2	single	postflush	30	bacilli	+	+	0000000	no growth
118	T2	single	postflush	30	bacilli	-	-	0000000	no growth
123	P1	multi	preflush	30	bacilli	-	-	4100000	*Listonella damsela*
129	P1	multi	preflush	30	bacilli	-	-	4100000	*Listonella damsela*
218	P2	single	preflush	37	bacilli	-	-	5400050	*Shewanella putrefaciens*
238	P2	single	preflush	37	bacilli	-	-	5100000	*Listonella damsela*
251	N1	single	postflush	37	bacilli	-	-	4100000	*Listonella damsela*
256	N1	single	postflush	37	bacilli	-	-	0410000	unacceptable
269	N1	single	postflush	37	bacilli	-	-	0210000	*Weeksella zoohelicum*
542	N1	single	preflush	30	bacilli	-	-	4300000	*Listonella damsela*
289	N2	multi	preflush	30	bacilli	-	-	4310000	*Listonella damsela*
308	N2	multi	preflush	30	bacilli	-	-	4310000	*Listonella damsela*
312	N2	multi	preflush	30	bacilli	-	-	0110000	*Weeksella virosa*
313	N2	multi	preflush	30	bacilli	-	-	4300000	*Listonella damsela*
338	R1	single	preflush	37	bacilli	-	-	0000000	no growth
1204	B2	multi	postflush	30	bacilli	-	-	4100000	*Listonella damsela*
1205	B2	multi	postflush	30	bacilli	-	-	0100000	*Listonella damsela*
1221	B2	multi	postflush	30	bacilli	-	-	4563204	*Sphingobacterium multivorum*
1222	B2	multi	postflush	30	bacilli	-	-	4350204	*Listonella damsela*
1224	B2	multi	postflush	30	bacilli	-	-	4541260	*Chryseomonas luteola*
1225	B2	multi	postflush	30	bacilli	-	-	4100004	*Listonella damsela*
1230	B2	multi	postflush	30	bacilli	-	-	4100004	*Listonella damsela*
923	S1	single	preflush	30	bacilli	+	+	0477741	*Neisseria cinerea*
1133	G1	single	postflush	30	bacilli	-	-	0010000	*Weeksella virosa*
1176	G1	single	preflush	30	bacilli	-	-	0450240	*Pseudomonas vesicularis*
581	D1	single	preflush	30	bacilli	-	-	0000000	no growth
584	D1	single	preflush	30	bacilli	-	-		

14 isolates were from single occupancy sites of which 8 were from preflush samples and 6 from post flush samples (single). 13 isolates were from multiple occupancy sites (multi), 6 were from preflush samples and 7 from postflush samples.

Table 2.6. Frequency of tentatively identified bacterial isolates

IDENTIFICATION	FREQUENCY				
	total	Pre	Post	30 °C	37 °C
Acinetobacter lwoffii	6	5	1	4	2
Acinetobacter lwoffii / Shigella sp.	2	2	0	2	0
Aeromonas salmonicida	7	3	4	3	4
Agrobacter tumefaciens	1	1	0	0	1
Agrobacterium radiobacter	1	1	0	0	1
Chromobacterium violaceum	1	0	1	0	1
Chryseomonas luteola	1	0	1	1	0
Flavobacterium breve	2	3	0	3	0
Flavobacterium gleum	1	1	0	1	0
Flavobacterium indologenes	7	4	3	2	5
Flavobacterium meningosepticum	2	2	0	2	0
Flavobacterium odoratum	1	1	0	0	1
Listonella damsela[1]	46	29	17	28	18
Listonella sp.	1	0	1	0	1
Miscellaneous Gram-negative bacilli	8	7	1	6	2
Moraxella phenylpyruvica	1	1	0	1	0
Moraxella sp.	3	1	2	1	2
Neisseria cinerea	2	2	0	2	0
Ochrobacter anthropi	3	3	0	0	3
Pasteurella sp.	4	1	3	1	3
Pseudomonas fluorescens	1	1	0	0	1
Pseudomonas paucimobolis	1	1	0	1	0
Pseudomonas putida	2	2	0	0	2
Pseudomonas stutzeri	4	2	2	1	3
Pseudomonas stutzeri / Acinetobacter	1	1	0	1	0
Pseudomonas vesicularis	7	6	1	3	4
Shewanella putrefaciens	1	1	0	0	1
Shigella sp	2	0	2	0	2
Sphingobacterium multivorum	7	6	1	3	4
Sphingomonas paucimobilis	1	0	1	1	0
Vibrio metschnikovii / parahaemolyticus	1	0	1	0	1
Vibrio parahaemolyticus	1	1	0	0	1
Weeksella virosa	8	4	4	6	2
Weeksella zoohelicum	5	3	2	3	2
Xanthomonas maltophilia	3	3	0	2	1
no growth	75	30	15	26	19
unacceptable	40	23	16	21	18

262 of bacterial isolates were identified with commercial identification kits, of these 168 isolates were from preflush samples, 92 were from postflush samples, 142 were from samples grown at 30°C and 118 from samples incubated at 37°C.

[1] in view of the natural ecological niche of *Listonella damsella* these identifications are unlikely to be correct (see discussion in section 4.1).

2.2.2 Haemolysin, Cytotoxin and Virulence marker assays

The assays for haemolysis, verotoxins, adhesiveness and invasiveness all proved relatively easy to set up and perform. In contrast there were considerable problems initially in setting up the Y1 cell assay. Initial cells purchased from two sources failed to grow adequately because they were contaminated with mycoplasma. Eventually a mycoplasma free culture was obtained but these cells still proved difficult to maintain in the medium specified in the original method. After changing the medium to MEM there were no further problems and the Y1 cells grew well.

The results for those strains that displayed any activity in the tests for haemolysins, cytotoxins and virulence markers are summarised in table 2.7. The full set of results are included in Appendix 4. A total of 469 isolates were tested for cytotoxic activity in Vero cells and for invasiveness and adhesiveness in HEp-2 cells. A total of 151 were tested in the Y1 cell assay and 585 isolates were tested for haemolysin activity. Of these isolates, 127 showed some toxin or haemolysin activity (Table 2.7).

Of the isolates tested for haemolysin activity 95/585 strains displayed some haemolytic activity and 43 were haemolytic against all three cell types. The majority (25/36) of the β-haemolytic isolates were isolated from taps that had not been disinfected (preflush samples).

A total of 21 out of 469 strains displayed verotoxic activity. Interestingly only 3 of 19 verotoxic strains examined displayed any haemolytic activity. One with human erythrocytes only and 2 in all three cell types. The majority (15/21) of the verotoxic isolates were isolated from taps that had not been disinfected.

Two of the 469 isolates tested showed adhesion to HEp-2 cells, this strain showed no haemolysin activity.

Twelve isolates were invasive into Hep-2 cells, none of these showed any haemolytic activity, although two of these were also VT positive. The majority (8/12) of these invasive strains were isolated from taps that had not been disinfected.

A total of 151 strains were examined for LT type activity in Y1 cells and the test was repeated on 81 of these to check the repeatability of the results. Of the 81 strains tested on two occasions 76 were unreactive on both occasions. Two strains gave a positive reaction on one occasion only and 3 were positive on both occasions. Thus 79 out of 81 reactions were the same on both occasions. Of those not examined in duplicate 2 were positive as heated and unheated preparations of culture supernatant but not as whole cells. Only four strains, numbers 276, 790, 786, and 797 gave convincing positives. Strain 276 only showed activity as whole cells but the other strains gave a

reaction as culture supernatant or whole cells which was eliminated by heating indicating that they had a heat labile toxin. In contrast to the strains showing activity on Vero or Hep2 cells, the majority (4/5) of strains that displayed any LT type activity also displayed some haemolytic activity.

Excluding the haemolysin activities 38 isolates displayed biological activity in one or more tests. Of these 60% (23/38) were isolated from taps before disinfection. Most (28/38) of these isolates were gram -negative but 3 of the 6 strains showing LT type activity were gram positive. Only two strains, 364 and 630, displayed activity in two or more tests. Strain 364 was both Verotoxic and invasive in Hep 2 cells and strain 630 was Verotoxic and both adhesive and invasive to Hep2 cells. Both 364 and 630 were gram-negative, oxidase-positive and unreactive in the Hugh and Leifsons O/F test. These reactions place them in the genera *Moraxella, Alkaligenes* or *Pseudomonas* none of which include any recognised enteropathogens. Members of these genera are common environmental organisms. Both 364 and 630 were isolated from taps that had not been disinfected.

Strains from 14 of the 18 outlets (78%) examined were active in at least one of the tissue culture assays. Of these all 14 had strains with activity in the sample taken before disinfecting the tap and only 7 after disinfection of the tap.

Table 2.7. Haemolysin and cytotoxin positive isolates

SERIAL	LAB	OCC	FLUSH	°C	GRAM	OXID	HS	HH	HM	VT	LT	ADH	INV	API	BBL	IDENTIF
12	T1	s	post	37	–	+	–	–	–	–	—	–	+		2111200000	unacceptable
18	T1	s	post	37	–	+	–	–	–	+	—	–	–		2211120000	*Flavobacterium indologenes*
24	T1	s	post	37	–	+	–	–	–	–	—	–	+			
28	T1	s	post	37	+	+	–	–	–	+	—	–	–	0		no growth
39	T1	s	pre	37	–	+	–	α	–	–	—	–	–			
41	T2	s	post	37	+	+	–	α	–	–		–	–		0	no growth
47	T2	s	post	37	–	+	–	–	α	–	–	–	–		2233100000	*Pseudomonas stutzeri*
48	T2	s	post	37	–	+	–	–	α	+	—	–	–		3233120000	unacceptable
49	T2	s	post	37	–	+	–	–	α	–		–	–		3233120110	unacceptable
76	T2	s	post	30	–	+	–	–	–	+		–	–		0	no growth
86	T2	s	pre	30	–	–	–	–	–	–	+–	–	–			
93	T1	s	pre	30	–	+	–	–	–	+	—	–	–			
129	P1	m	pre	30	–	–	–	–	–	+		–	–	4100000		*Listonella damsella*
130	P1	m	pre	30	–	+	–	–	–	+		–	–		0	no growth
137	P1	m	pre	30	v	–	α	α	α	–		–	–		10200000	*A. lwoffii/Shigella sp.*
141	P1	m	pre	30	–	+	–	–	–	+	—	–	–	4147004		*Aeromonas salmonicida*
167	P1	m	post	30	–	+	α	α	α	–		–	–		0	no growth
189	P2	s	pre	30	–	+	–	–	–	+		–	–		3211300320	unacceptable
215	P2	s	pre	37	v	+	–	–	–	+		–	–			
228	P2	s	pre	37	+	+	α	α	α							
244	N1	s	post	37	+	–	–	–	α	–	–	–	–	4144440		*Chromobacterium violaceum*
246	N1	s	post	37	–	+	–	–	α	–	–	–	–	4150724		*Aeromonas salmonicida*
250	N1	s	post	37	–	+	α	α	α	–		–	–	4100004		*Listonella damsella*
260	N1	s	post	37	+	+	β	α	β	–	–	–	–	4210004		*Weeksella zoohelicum*
261	N1	s	post	37	–	+	α	α	α	–	—	–	–	4100004		*Listonella damsella*
262	N1	s	post	37	–	+	α	α	α	–	—	–	–	4100004		*Listonella damsella*
263	N1	s	post	37	–	+	α	α	α	–		–	–			
265	N1	s	post	37	–	+	β	–	–	–		–	–	4		*Moraxella* sp.
268	N1	s	post	37	–	+	β	β	β	–		–	–	4300004		*Listonella damsella*
269	N1	s	post	37	–	–	β	β	β	–	—	–	–	210000		*Weeksella zoohelicum*?
270	N1	s	post	37	v	–	β	β	β	–	+–	–	–	400000		*Pseudomonas vesicularis*
271	N1	s	post	37	–	+	β	β	β	–	–	–	–			
276	N2	m	post	37	+	+					++					
302	N2	m	pre	30	+	–	α	α	α							
303	N2	m	pre	30	+	–	α	α	α							
304	N2	m	pre	30	–	–	β	β	β							
305	N2	m	pre	30	–	+	α	α	α	+	–	–	–		101100011	Misc Gram neg bacillus
310	N2	m	pre	30	+	–	β	β	β							
311	N2	m	pre	30	+	–	β	β	β							
314	N2	m	pre	30	+	+	–	–	–	+		–	–			
316	R1	s	pre	37	+	+	α	α	α	–		–	–			
317	R1	s	pre	37	–	+	α	α	α	–		–	–	5057344		*Vibrio parahaemolyticus*
318	R1	s	pre	37	–	+	–	–	–	–		–	+	1247744		*Ochrobacter anthropi*
330	R1	s	pre	37	+	–	α	α	α							

Table 2.7. (continued) Haemolysin and cytotoxin positive isolates

SERIAL	LAB	OCC	FLUSH	°C	GRAM	OXID	HS	HH	HM	VT	LT	ADH	INV	API	BBL	IDENTIFICATION
345	R1	s	pre	37	–	–	–	–	–	–		–	+		11100000	*Acinetobacter lwoffii*
346	R1	s	post	30	+	–	α	–	α							
347	R1	s	post	30	+	–	α	–	α							
363	R2	m	pre	30	+	–	α	–	–							
364	R2	m	pre	30	–	+	–	–	–	+		–	+			
371	R2	m	pre	30	–	+	–	–	–	–		–	+	4300000		*Listonella damsella*
373	R2	m	pre	37	–	+	–	–	–	–		–	+	743004		*Flavobacterium indologenes*
375	M1	s	pre	30	+	–	β	–	–							
380	M1	s	pre	30	+	–	β	–	–							
381	M1	s	pre	30	+	–	β	–	–							
382	M1	s	pre	30	–	–	α	–	–	–		–	–			
383	M1	s	pre	30	–	–	β	–	–	–		–	–			
384	M1	s	pre	30	–	–	–	–	–	+		–	–		0	no growth
388	M1	s	pre	30	+	–	β	–	–							
394	M1	s	pre	30	–	–	β	–	–	–		–	–			
400	M1	s	pre	30	+	–	β	–	–							
401	M1	s	pre	30	–	–	β	–	–						0	no growth
402	M1	s	pre	30	+	–	α	–	–	–	–	–	–			
405	M1	s	post	37	–	+	–	–	–	–		–	+		0	no growth
410	M1	s	post	30	–	+	–	–	–	–		+	–			
421	M1	s	post	30	v	–	–	–	–	+		–	–			
526	N1	s	pre	30	–	–	α	α	α	–	–	–	–		1201100000	*Ps. stutzeri / A.lwoffii*
527	N1	s	pre	30	+	+	α	α	α			–	–	0		no growth
528	N1	s	pre	30	+	+	α	α	α							
529	N1	s	pre	30	+	+	α	α	α						0	no growth
533	N1	s	pre	30	–	+	α	–	–	–	–	–	–	110004		*Weeksella virosa*
534	N1	s	pre	30	v	+	β	–	–	–	—	–	–		2301100000	unacceptable
535	N1	s	pre	30	+	+	β	–	–		–					
536	N1	s	pre	30	–	+	β	–	–	–		–	–	4210004		*Weeksella zoohelicum*
537	N1	s	pre	30	v	+	β	–	–	–		–	–	4320004		*Listonella damsella*
538	N1	s	pre	30	–	+	α	–	–	–		–	–	4100004		*Listonella damsella*
539	N1	s	pre	30	+	+	–	–	–	–		–	+			
547	N1	s	pre	30	+	–	α	–	–	–		–	–			
557	R1	s	pre	30	–	+	–	–	–	–		–	+		3302000000	*Pseudomonas vesicularis*
560	R1	s	pre	30	v	+	–	–	–	+		–	–		2321100000	*Sphingobacterium multivorum*
580	R1	s	pre	30	+	+	α	–	α							
630	D1	s	pre	37	–	+				+		+	+			
772	B1	s	pre	30	+	+	α	–	–							
773	B1	s	pre	30	+	+	α	–	–							
786	B2	m	post	37	+	+	α	α	–	–	++	–	–		6763306211	unacceptable
787	B2	m	post	37	+	–	α	–	–							
788	B2	m	post	37	+	–	α	–	α		–					

Table 2.7. (continued) Haemolysin and cytotoxin positive isolates

SERIAL	LAB	OCC	FLUSH	°C	GRAM	OXID	HS	HH	HM	VT	LT	ADH	INV	API	BBL	IDENTIFICATION
789	B2	m	post	37	v	+	–	α	α	–	–	–	–		771106440	unacceptable
790	B2	m	post	37	–	+	α	α	–	–	++	–	–			
791	B2	m	post	37	–	–	α	α	–	–	—	–	–			
794	B2	m	pre	37	–	+	α	α	α	–		–	–		3131102200	unacceptable
797	B2	m	pre	37	+	–	α	α	α		+					
801	B1	s	pre	37	+	+	α	α	α		–					
803	B1	s	pre	37	+	+	α	α	α							
804	B1	s	pre	37	+	–	α	α	α							
805	B1	s	pre	37	+	+	–	–	α							
806	B1	s	pre	37	+	+	–	–	α							
807	B1	s	pre	37	+	+	–	α	–							
814	B1	s	pre	37	–	+	α	α	α	+	–	–	–	4440244		*Pseudomonas vesicularis*
819	B1	s	pre	37	–	+	α	–	–	–		–	–	4563204		*Sphingobacterium multivorum*
820	B1	s	pre	37	–	+	α	–	–	–		–	–	4350204		*Listonella damsella*
822	B1	s	pre	37	+	+	β	β	β		–					
823	B1	s	pre	37	+	+	β	β	β							
825	B1	s	pre	37	+	+	–	β	β							
836	B1	s	post	30	–	+	–	–	–	+						
846	B1	s	post	30	+	–	β	β	β							
848	B2	m	pre	30	+	–	β	β	β							
849	B2	m	pre	30	+	+	β	β	β							
853	B2	m	pre	30	+	–	–	β	–							
854	B2	m	pre	30	+	–	–	β	–							
857	B2	m	pre	30	v	–	–	β	–							
861	B2	m	pre	30	v	–	β	β	β	–	–	–	–		3363300100	*Flavobacterium meningosepticum*
862	B2	m	pre	30	+	–	–	β	–	–		–	–		0	no growth
1024	G2	m	pre	30	–	+	–	–	–	+	–	–	–		320311010	*Pseudomonas stutzeri*
1178	G1	s	pre	30	–	–				+		–	–			
1203	B2	m	post	30	+	+	–	β	–							
1206	B2	m	post	30	+	–	–	α	–							
1207	B2	m	post	30	+	–	–	α	–							
1208	B2	m	post	30	+	–	–	β	–							
1209	B2	m	post	30	v	–	α	α	α	–		–	–	4100000		*Listonella damsella*
1212	B2	m	post	30	v	–	–	β	–	–		–	–			
1216	B2	m	post	30	+	–	α	α	α							
1217	B2	m	post	30	+	–	α	α	α							
1218	B2	m	post	30	+	–	α	α	α							
1219	B2	m	post	30	+	–	α	α	α							
1224	B2	m	post	30	–	–	–	–	–	–		–	+	4541260		*Chryseomonas luteola*
1228	B2	m	post	30	+	–	β	β	β							

43 isolates were positive for haemolysin activity in all three cell types. 20 isolates were VT positive, 2 showed adhesion with Hep-2 cells and 12 were invasive in Hep-2 cells. (s= single occupancy site, m, multioccupancy site, pre = preflush, post = postflush, α = alpha haemolysis, β = beta-haemolysis).

3. Detection of *Campylobacter* species in potable water samples by PCR

3.1 METHODS

Full details of the methods are given in Appendix 3.

3.1.1 Evaluation of suitability of filter material for use with the PCR assay

Three filter materials were investigated for compatibility with the Campylobacter PCR assay. A suspension of 10^6 cfu/100ml of *Campylobacter jejuni* NCTC 11168 was prepared in sterile distilled water and filtered under vacuum through each of the following filter matrices; Gelman Supor 200 (Gelman 4702), Whatman Nylon (Whatman 1920-7009) and Sartorius cellulose nitrate (Sartorius 11307-47N) membranes. All filters were 47mm diameter and 0.2µm pore size. Filters were processed as described in Appendix 3, with the exception that two small segments from the centre of the filter were taken to perform direct PCR from the filter.

3.1.2 Examination of potable water samples with the PCR assay

Three sets of Pre-flush and Post-flush filter samples were submitted to Preston Public Health Laboratory for testing with the Campylobacter PCR assay.

3.2 RESULTS AND DISCUSSION

3.2.1 Limitations of the assay.

The Campylobacter PCR assay is a novel test for which we have only limited experience with naturally contaminated samples. A number of variables that may affect the sensitivity of the test have not yet been quantified.

The chemical nature of the filter matrix used for concentrating organisms from large volumes of water has been shown to affect the PCR reactions and in some cases will totally inhibit the reaction when the filter matrix is incorporated as part of the sample in the reaction tube. The results with different filters are presented in Table 3.1. These findings confirm that there was no amplification of the seeded water sample or the internal control with either any of the three filter types, when the filter comprised part of the sample inoculum added to the reaction mixture.

The extent to which chemical inhibitors such as humic acids, present in waters, will bind to or be concentrated by the filtration process, and the levels in filter washings has not been defined.

The extent to which organisms collected on filter matrices can be successfully recovered from the filter has not been quantified and this may represent a significant factor in the sensitivity of the assay. Our experience using this technique suggests that the sensitivity of this assay is significantly reduced by the ability to recover the organisms from the filter matrices. Using the present methodology greater than 10^3 cfu's per sample would be required to produce a positive signal under optimum conditions.

3.2.2 Examination of potable water samples with the PCR assay.

These results are summarised in Table 3.2.

Samples received from Bristol and Preston PHL contained inhibitory substances that were not inactivated or removed by the sample preparation procedure, therefore this assay was not a valid test for the presence or absence of Campylobacter cells in these samples.

The sample received from Nottingham PHL was negative and did not indicate the presence of Campylobacter cells.

Table 3.1. Evaluation of the suitability of filter material for use with the PCR assay.

Sample	Result
Gelman Filter Direct	Negative
Filter Direct + internal control	Negative
Prep A	POSITIVE
Prep A + internal control	POSITIVE
Prep B	POSITIVE
Prep B + internal control	POSITIVE
Whatman Filter Direct	Negative
Filter Direct + internal control	Negative
Prep A	Negative
Prep A + internal control	POSITIVE
Prep B	Negative
Prep B + internal control	POSITIVE
Sartorius Filter Direct	Negative
Filter Direct + internal control	Negative
Prep A	Negative
Prep A + internal control	POSITIVE
Prep B	Negative
Prep B + internal control	POSITIVE
Positive control	POSITIVE
Negative control	Negative
Reagent control	Negative

Filter direct - A portion of filter added to the reaction tube.

Prep A - Centrifuged deposit of filter washings.

Prep B - Boiled preparation of PrepA.

Table 3.2. Examination of potable water samples with the PCR assay.

Sample	Result
Bristol Pre-flush PrepA	negative
Pre-flush PrepA + internal control	negative
Pre-flush PrepB	negative
Pre-flush PrepB + internal control	negative
Post-flush PrepA	negative
Post-flush PrepA + internal control	negative
Post-flush PrepB	negative
Post-flush PrepB + internal control	negative
Positive control	POSITIVE
Negative control	negative
Nottingham Pre-flush PrepA	negative
Pre-flush PrepA + internal control	negative
Pre-flush PrepB	negative
Pre-flush PrepB + internal control	POSITIVE
Post-flush PrepA	negative
Post-flush PrepA + internal control	POSITIVE
Post-flush PrepB	negative
Post-flush PrepB + internal control	POSITIVE
Positive control	POSITIVE
Negative control	negative
Preston Pre-flush PrepA	negative
Pre-flush PrepA + internal control	negative
Pre-flush PrepB	negative
Pre-flush PrepB + internal control	negative
Post-flush PrepA	negative
Post-flush PrepA + internal control	negative
Post-flush PrepB	negative
Post-flush PrepB + internal control	negative
Positive control	POSITIVE
Negative control	negative

General Discussion

The work of Payment *et al*. 1991a & b suggested that there was a correlation between high or raised counts of HPC bacteria in drinking water and the incidence of gastrointestinal disease. Several possible explanations were discussed in section 1.1 but the practical work described in this report has concentrated on determining whether the population of HPC bacteria detectable by routine procedures contain previously unrecognised components that are potentially pathogenic.

4.1 IDENTIFICATION OF THE ISOLATES

The databases of both traditional identification keys and commercial identification systems are largely based on taxonomic studies of isolates of clinical origin. As a consequence their application to the identification of environmental isolates often produces unsatisfactory or improbable results. This study was no exception and many of the isolates failed to be identified. However, if any of the strains studied had belonged to recognised gram negative pathogenic species these should have been identified adequately by the systems used since, *a priori,* they would have been included in the data bases.

Two strains were identified as belonging to enteropathogenic genera / species , strain 27 as *Shigella* sp. and strain 317 as *Vibrio parahaemolyticus*.

It was not possible to confirm the identification of strain 27 as it had died. It was not active in any of the virulence tests. This strain was isolated from a water sample collected after disinfection of the tap which would suggest it was more likely to have been derived from the main supply rather than external contamination of the tap. However if the identification is correct this is unlikely as *Shigella* species can only grow in the intestines of man and apes and thus its presence would be associated with faecal contamination of the water. The outlet from which the water sample was collected was supplied with mains water that complied with all the usual regulatory criteria. Thus , if the identification of this strain was correct' it was more likely to have been derived from local contamination of the tap but the most likely explanation is that this was an incorrectly identified strain. The characteristics recorded would probably still place it in the Enterobacteriaceae.

The strain identified as *V. parahaemolyticus* was isolated from a sample collected without disinfecting the tap. *Vibrio parahaemolyticus* is a very common marine bacterium that is found in seawater and seafoods. Infection by it results from the ingestion of inadequately cooked or raw seafoods. It requires sodium chloride for growth, is incapable of growth or survival in clean fresh water and is very sensitive to chlorine. As would be expected for pathogenic strains of this species it was haemolytic to human blood. If the identification is correct the most likely explanation for its presence is the external contamination of the tap while preparing seafoods.

One other strain was identified as a possible strain of *V. parahaemolyticus* or *V. metschnikovii.* The latter identification is unlikely to be correct as strains of this species are oxidase-negative and this strain was oxidase positive. None the less *V. metschnikovii* is one of the few *Vibrio* species that can occur naturally in fresh water environments but it is not a recognised enteropathogen. It was non-haemolytic and unreactive in all the other virulence tests. If this strain was a true *Vibrio* species it is most likely to be a non-pathogenic environmental strain.

Listonella damsella (formerly *V. damsella*) was the most common identification recorded. However this is also a naturally occurring marine bacterium that can cause wound infections in man and aquatic animals. It is not a recognised enteropathogen. It is not a species that one would expect to find commonly in tap water although occasional contamination of taps during the washing of seafood is conceivable. *Listonella damsella, V. metschnikovii* and *V. parahaemolyticus* are all members of the Vibrionaceae. Members of this family are all natural inhabitants of marine environments and require higher levels of salts, in particular sodium chloride, than terrestrial bacteria. To obtain the correct reactions in normal biochemical tests such as the Hugh and Leifson test, the media require supplementing with additional sodium chloride. In the presence of sufficient salt they are all fermentative in the Hugh and Leifson O/F test. If there is too little sodium chloride in this test they will grow poorly and appear to give no reaction in this test. As a consequence it is quite common for them to be incorrectly identified. The API data base has been developed for isolates from clinical specimens and consequently assumes that the O/F test, as normally performed (no added salt), will frequently give an incorrect reaction for the Vibrionaceae. Most of the strains identified as *L. damsella* were oxidative. This fact combined with the non-marine origin of these strains means that these strains are probably incorrectly identified. They certainly represent one or more taxonomic groups of heterotrophs that commonly occur in our drinking water and attempts to identify them further would certainly be warranted. However this would require a formal taxonomic study which was beyond the scope of this project.

The API20NE system provided a name for 102 out of the 119 strains examined by this kit.. However 46 of these were identified as *L. damsella* and, as discussed above, this is likely to be an incorrect identification. Most of the remaining identifications by this kit were probably satisfactory. Thus this API20NE failed to identify satisfactorily 63/119 (53%) of the strains examined. Using the BBL Crystal system 98/143 (69%) of the strain failed to identify. In the absence of any systems designed for identifying environmental isolates, these two systems were chosen because trials had shown them to provide good identification of clinical non-fermenters and fermenters respectively. Unfortunately it is clear that they are completely incapable of providing identification to genus or species level for the majority of environmental isolates of gram-negative bacteria. Clearly much further taxonomic work is required before adequate identification systems can be developed for such isolates. The strains collected during this study would provide a good collection of strains for such studies.

Participating laboratories were asked to select colonies with different morphology irrespective of the relative numbers of each type on the culture plate. Consequently the relative numbers of Gram-positive to Gram-negative isolates does not accurately reflect the relative proportions in the total populations. The Gram-positive rod shaped bacteria represent species of the genus *Bacillus* which is also extremely difficult to identify accurately to species level. Similarly the Gram-positive cocci represent members of the Micrococcaceae which are also difficult to identify accurately to the species level. The organisms in these groups are common in the environment and are not recognised to cause enteric infections. However one or two species, notably *Bacillus cereus* and *Staphylococcus aureus,* can cause food poisoning. In this case the organisms proliferate to high numbers in contaminated foods and food poisoning results from preformed toxins in the food rather than proliferation in the intestine and vomiting is the common result.

4.2 VIRULENCE DETERMINANTS OF ISOLATES

Fourteen of the eighteen water samples collected contained bacteria that possessed activities towards the tissue cultures similar to those activities that are associated with pathogenicity in recognised enteropathogens. It must be emphasised that this does not necessarily mean that these strains were actually capable of expressing these characteristics and causing disease within the human intestine. Of these all 14 had strains with activity present before the tap was disinfected and only 7 after disinfection. This would suggest that a significant proportion of these isolates had been derived from colonisation within the tap itself. The source of this colonisation is equally likely, if not more likely, to have been derived from external contamination from within the household rather than the incoming mains water. The incidence of biologically active strains in our study is comparable to that found by Payment *et al.* 1994.

Whereas the ability to haemolyse erythrocytes often correlates with other types of pathogenicity, there is generally little or no correlation between haemolytic activity and enteropathogenicity. The clear exception is *Vibrio parahaemolyticus* in which the diarrhoeagenic ability of strains is clearly correlated with the possession of a thermostable haemolysin which is frankly (β) haemolytic for human erythrocytes. It is possible that similar haemolysins also play a role in the enteropathogenicity of some other *Vibrio* species and it has also been suggested that haemolysins could be an enteropathogenic mechanism in *Aeromonas* species. Where haemolysis has been shown or suggested to be correlated with enteropathogenicity it has always been β-haemolysis. Thus, although we recorded α haemolysis we do not believe it is likely to correlate with enterotoxigenicity. By analogy with *V parahaemolyticus* the β-haemolysis might have some significance for some of the Gram-negative isolates but the balance of probability is that these haemolysins are not enterotoxins. The majority of the β-haemolytic strains were isolated from pre-flush samples suggesting that they could have originated from external contamination of the taps

rather from the incoming water.

None of the isolates with activity against tissue cultures could be assigned to a recognised species of enteropathogenic bacteria and most of them could not be clearly identified. Although it was not possible to identify most of the active strains the range of phenotypic profiles obtained for them suggests that they belong to a range of taxa and no single phenotype dominated.

In this study we were not able to test for heat stable toxins similar to the ST toxin of *E. coli* because the only assays available for biological activity utilise animals and therefore were not suitable for screening large numbers of isolates. If we had been able to detect ST-type activity we may have found more biologically active strains but it is unlikely to have altered the overall conclusions of the study.

Nichols *et al.* 1995a reviewed the health significance of heterotrophic bacteria growing in water distribution systems. In relation to the potential enteropathogenicity of HPC bacteria that may be found in water they concluded that *Aeromonas* species and *Yersinia enterocolitica* may pose a risk to human health but are not major causes of disease in the United Kingdom. In a trial of presence /absence (PA) methods of determining coliforms and *E* coli in drinking water, Lightfoot *et al.* 1995 found that *Yersinia* species can occur in private water supplies in the UK and would be missed by the conventional membrane filtration methods although detected by methods based on the detection of β-galactosidase. However the incidence of infections with *Yersinia enterocolitica* in the UK is low (Nichols *et al.* 1995a) and not all strains can cause disease. The increasing application of methods based on β–galactosidase production will provide improved detection of *Yersinia* species in drinking water and the development of further techniques or standards for this species are not justified.

Nichols *et al.* 1995b reviewed the risk to health from *Aeromonas* in drinking water distribution systems. They concluded that: *Aeromonas* species can cause infection in humans including diarrhoea; water utilities cannot be confident that people are not being infected with *Aeromonas* species from the water distribution system and further work is required to demonstrate whether human infections with aeromonads are not being derived from organisms present within the distribution system. A few of the strains isolated in the current study were identified as *Aeromonas salmonicida.* This species is a fish pathogen and it is possible that the identification is incorrect as the taxonomy of the aeromonads is far from clear and accurate speciation is difficult without the use of molecular biological methods. It is probable that these strains were genuine *Aeromonas* but not *A salmonicida. Aeromonas* species certainly occur in water in the UK and some strains of *Aeromonas* have previously been shown to possess the markers of virulence that could be associated with enteropathogenicity. However they are not fastidious organisms and can readily be grown on simple media such as those commonly used to detect HPC. If these organisms are genuinely enteropathogenic the infectious dose is almost certainly large and would be associated

with a high heterotrophic count.

Versteeg *et al.* 1989 found that the addition of EDTA to water samples to chelate copper ions could improve the recovery of aeromonads from tap water. Studies at the PHLS WEMRU (Lee *et al.* unpublished) confirmed these conclusions using water artificially contaminated with laboratory grown and natural river derived aeromonads. Huys *et al.* 1995 observed good correlation between the counts of HPC and numbers of *Aeromonas* species present in raw and treated waters from five drinking water production plants in Belgium. Selective methods have been developed for the isolation of *Aeromonas* species from water by membrane filtration and one is included in Report 71 1994. The exact role of aeromonads in diarrhoea is still not entirely clear and these organisms are certainly to be found in water in distribution in the UK. Even if they were proven to be a significant risk it is certain that there would have to be relatively high numbers present to produce infection. The current methods used to determine the HPC count are capable of detecting aeromonads and relatively minor modifications to them may increase the recovery. There is no scientific evidence on which to base a numerical standard for *Aeromonas* at present. However, since the infectious dose is likely to be high and aeromonads are sensitive to chlorine, water meeting the current EC guidelines for HPC of less than 10 cfu/ml after 48 hours incubation at 37°C and containing some residual chlorine should give adequate protection against tap water being a source of infection.

The common practice within the UK is to measure the HPC count at 37°C after only 24 hours incubation. The results presented here show that this practice produces a considerable underestimate of the numbers of HPC present. The difference between 24 hours and 48 hours was far greater than the increase in count obtained by increasing the incubation time further. The use of R2A inoculated by the spread plate technique also gave higher counts than the standard pour plate procedure using YEA. A comparison of spread plates using YEA and R2A at 37°C was not performed and would be needed to confirm the effect of R2A although other workers have consistently reported R2A to give higher counts. Our results suggest using spread plates and incubating the plates for 48 rather than 24 hour would increase the numbers of HPC detected but whether this would be significant in relation to improving the assessment of risk is uncertain and would require further studies.

Although a number of strains were detected that possessed virulence markers associated with known pathogens it is unknown if these activities are due to toxins related to those already recognised or are due to other factors. Molecular biological techniques could be used on these isolates to determine if any of them possess gene sequences allied to those corresponding to the recognised toxins. If this proved to be the case it would still only be circumstantial evidence that these organisms could be potential pathogens. Much further work would be required to confirm their enteropathogenicity. Before this could take place it would be essential to have fully characterised the strains. At this stage they cannot even be adequately identified because of the inadequate state of the taxonomy of environmental heterotrophs. It is essential that extensive

taxonomic studies are performed on a wide range of isolates of environmental origin in order to generate a more extensive data base and improved identification schemes for this increasingly important group of organisms.

There are some known pathogens that cannot be readily detected in water by cultural methods either because they are too fastidious or are overgrown by other heterotrophs. This group of organisms includes *Campylobacter* species. Potentially, such organisms can be detected by direct analysis of concentrated water samples using PCR technology. The initial study by the Preston laboratory shows that detection of *Campylobacter* species, for example, by PCR is feasible. However further study is necessary to determine the best method to reduce the level of inhibitors present in direct water samples which, for example, may be due to humic and fulvic acids, metal ions, and enzymatic activity of viable micro-organisms.. When the problems of inhibition can be overcome, the potential for PCR based techniques is considerable for they could not only be used to detect specific pathogenic bacteria but they could also be used to look for gene sequences related to recognised toxin genes in filtrates from samples of water.

Outbreaks of enteric disease associated with drinking water are relatively rare in the UK. When such outbreaks have been reported a known pathogen has been incriminated or there has been evidence of sewage or other relevant contamination which could have introduced a recognised pathogen or pathogens. In this study, therefore, we have only been able to examine strains isolated from water in the absence of known illness associated with drinking that water. In the future, if an unexplained outbreak occurs it would be worthwhile collecting samples and testing them with methods used in this study and any appropriate molecular biological techniques that may be available. To design a study to isolate HPC organisms in the presence of an excess of illness associated with drinking the water would require setting up an epidemiological study comparable to that of Payment *et al.* 1981a & b. This would have to be a very large study to have even a small chance of success and on the evidence available would be hard to justify.

5. Conclusions and recommendations for future work

1. The population of HPC organisms in normal tap water includes small numbers of organisms that possess activities on tissue cultures (markers) similar to those associated with the virulence markers of some recognised enteropathogenic species of bacteria.

2. These activities are not associated with any particular species or phenotypic group of bacteria or recognised enteropathogenic species.

3. If these organisms can produce disease they would need to be present in water in high numbers in excess of the guideline values for the HPC at 37°C after 48 hours incubation.

4. More extensive trials should be undertaken to compare the use of R2A medium and YEA and spread versus pour plates for the determination of the HPC count at 37°C and the effect of using chelating agents to improve recovery of HPC bacteria.

5. There is insufficient evidence at present to indicate a need for the development of standards for individual species but this should be continually reviewed in the light of new epidemiological information.

6. There is an urgent need for taxonomic studies on HPC bacteria in order to improve the ability to identify them and further our understanding of any role, if any, they may have in the epidemiology of gastrointestinal disease. The strains collected in this study could form the basis of such studies.

7. Although appropriate for research projects the tissue culture based assays are too laborious for the application as routine test methods in water laboratories

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APPENDIX 1

7. PILOT STUDY: PRACTICAL COMPARISON OF METHODS FOR DETERMINING THE HETEROTROPHIC COLONY COUNT

7.1 MATERIALS

7.1.1 Sampling equipment

1 litre sterile sample bottles (borosilicate glass Duran or polyethylene) containing 1 ml of sterile 1.8% sodium thiosulphate solution.

500ml sterile bottles (Glass Duran or polyethylene).

500ml sterile bottles containing 1 ml of 2.5% Na_2EDTA solution

Thermometer (0-60°C)

Disposable rubber / plastic gloves

Protective goggles

Insulated container (coolbox) with refrigerant block or ice packs.

Sodium hypochlorite solution (10%) in a wash-bottle.

Cotton wool or equivalent for cleaning taps

7.1.2 Bacterial culture

Sterile plastic petri dishes

Membranes cellulose ester 47 mm diameter 0.45µm pore size

Yeast extract agar. Yeast extract 3g; peptone 5g; agar 12 g; distilled water 1 litre pH to 7.3. (Unipath, Oxoid CM19. Prepared media was stored at 2 - 8^{o}C and used within 4 weeks.

For each water sample the following were used: 9 pre-poured plates containing about 20ml of YEA and 3 tubes/bottles containing 15ml of melted YEA.

R2A Agar (R_2A, Difco 1826-17-1). Bacto Yeast extract 0.50g, proteose peptone #3,Difco 0.50g; Bacto casamino acids 0.50g; Bacto dextrose 0.50g; soluble starch 0.50g; sodium pyruvate 0.30g; potassium phosphate,dibasic 0.30g; magnesium sulphate 0.05g; Bacto agar 15.00g; distilled water 1 litre; pH 7.2±0.2. The medium was prepared according to the manufacturers instructions and plates poured containing about 20ml of medium. Poured plates were stored at 2 - 8^{o}C and used within 4 weeks.

7.2 METHOD

In order to obtain samples from a range of types of water and different geographical regions samples were collected and processed by Public Health Laboratories in Bristol, Guildford, Manchester, Reading, Newcastle, Nottingham, Rhyl, Swansea and Preston. Following the methods described below, samples were collected from two households preferably served by different water sources but at least one mile apart. For each household there were four samples: one pre-flush

sample containing thiosulphate and EDTA; one pre-flush sample with only thiosulphate; one post-flush sample containing thiosulphate and EDTA; and one post-flush sample with only thiosulphate.

7.2.1 Choice of tap

A single tap was selected that was known to be connected to the cold rising main supply and not fed via a break tank. Laboratories were instructed not to use mixer taps, dripping taps or taps with leaking glands and no fittings attached to it such as anti-splash hoses etc. The material of construction of the pipe feeding the tap was recorded.

7.2.2 Collection of pre-flush sample

First thing in the morning, before the tap had been used, a sample of water was collected, without disinfection or preflushing into a one litre container containing sodium thiosulphate. The container was shaken well and then 500ml was poured into a bottle containing EDTA and the remaining 500ml was poured into a bottle containing no EDTA.

7.2.3 Collection of post-flush sample

After taking the pre-flush sample the tap was left running for two to three minutes. The outside of the tap and nozzle were cleaned with a cotton wool swab soaked in a 1% solution of sodium hypochlorite. and the outside of the tap and inside the nozzle were sprayed with the 1% hypochlorite solution. After two to three minutes the tap was opened and water run to waste for two to three minutes to remove all disinfectant. A one litre sample was then collected and divided into 500 ml portions with and without EDTA as described above. The samples were transported to the laboratories in an insulated cooled container and processed within six hours of collection.

7.2.4 Sample processing

For each 500ml of water the following were inoculated:

7.2.4.1 Spread Plates - R2A

The sample was mixed well and 0.1ml amounts of water were spread onto three plates of R2A previously dried just sufficiently to remove all surface moisture. One plate was incubated at 37^{o}C, the second at 30^{o}C and the remaining one at 22^{o}C. The procedure was repeated using 0.5ml volumes.

7.2.4.2 Pour Plates - Yeast Extract Agar

1ml volumes of water were dispensed into each of three petri dishes and about 15ml of molten YEA, previously melted and cooled to 45 ± 1°C, was poured into each petri dish. The samples and agar were immediately mixed and allowed to cool and set on a level bench. The time from when the water was pipetted into the petri dish and the moment when the molten medium was added did not exceed 15 minutes. After setting, for each subsample, one plate was incubated at 37^{o}C, one at 30^{o}C and one at 22^{o}C.

7.2.4.3 Membrane Filtration

Using standard membrane filtration techniques three 1ml, three 10ml and three 100ml volumes were filtered through 47mm diameter 0.45ml pore-size cellulose ester membranes. Each membrane was placed on a plate of yeast extract agar being careful to ensure there were no bubbles underneath the membrane. For each subsample, one set of membranes was incubated at 22^{o}C, one set at 30^{o}C and one set at 37^{o}C.

7.2.5 Incubation

All plates were incubated in sealed plastic bags to prevent the drying out of the agar during the two week incubation period.

7.2.6 Counting Colonies

Plates were examined with a hand lens or low power binocular microscope and the numbers of colonies were recorded for each plate and membrane after 1, 2, 3, 5, 7, 10 and 14 days. The results were recorded on a standard form and returned to the PHLS WEMRU in Nottingham for the analysis of the results.

7.2.7 Analysis

A data base was constructed and the data entered using Epi Info version 6 and analysis performed using Excel version 5, and Minitab for Windows version 10 .2 .

7.3 RESULTS

7.3.1 The effect of incubation time

The effect of the length of incubation is summarised in tables 7.1 and .2 and figure 7.1 below. It can be seen that the count continues to increase throughout the incubation period at all temperatures but that this effect is most marked at 20°C. At 30°C and 37°C the largest change in count occurred between the 24 and 48 hour readings. By 7 days the mean count at 30°C was 87% of the final mean count and at 37°C it was 94% of the final mean count. In contrast, the mean count at 20°C had only reached 62% of the final mean count by 7 days.

In the UK the standard method is to use pour plates with YEA and incubate the plates for only 24 hours rather than the 48 which is specified in the EC Drinking Water Directive. The counts using the pour plate method at 37°C after 24 and 48 hours incubation were compared using a simple two factor analysis of variance. The counts after 48 hours were significantly higher than the counts after 24 hours (p=0.02)

Table 7.1 Overall comparison of counts after different periods of incubation combining data for all methods**.**

	Count (cfu/ml)						
	1 day	2 day	3 day	5 day	7 day	10 day	14 day
Incubated at 20°C							
Mean	6	76	227	444	906	1264	1464
Median	0	1	30	127	162	200	270
Minimum	0	0	0	0	0	1	1
Maximum	170	1160	8900	10000	40000	51000	53000
No. of readings	209	209	210	208	201	189	187
Incubated at 30°C							
Mean	17	373	411	688	995	1073	1146
Median	0	13.5	58.5	90	120	120	150
Minimum	0	0	0	0	0	0	0
Maximum	612	44040	12000	43000	70000	78000	80000
No. of readings	207	208	204	197	191	183	180
Incubated at 37°C							
Mean	18	111	133	171	211	222	224
Median	0	4	8	10	18	22.5	21
Minimum	0	0	0	0	0	0	0
Maximum	400	3730	4320	3680	4400	4780	3520
No. of readings	211	209	203	197	197	192	191

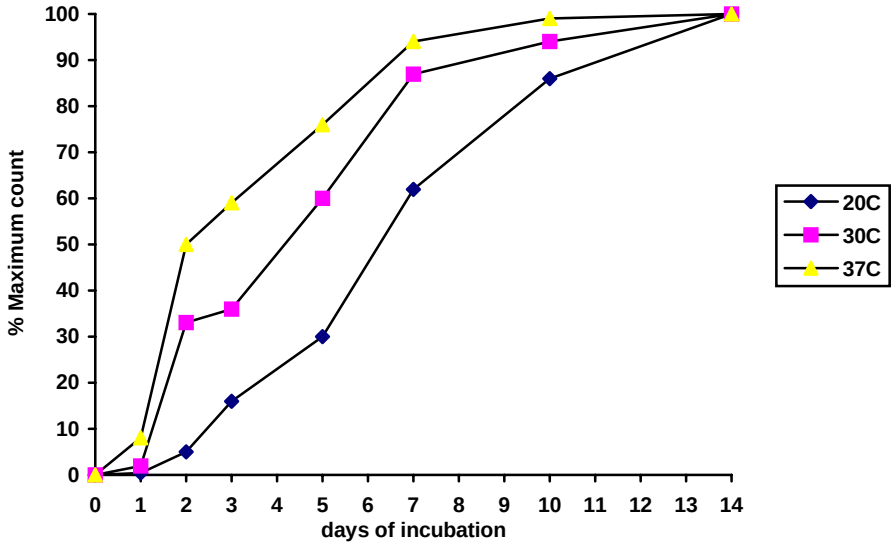

Figure 7.1 Mean count expressed as a percentage of the maximum count achieved for each temperature of incubation and combining data for each method.

Table 7.2 Relationship between median colony count and method

Incubation temperature	Inoculation Method	Median count (cfu/ml)						
		day 1	day 2	day 3	day 5	day 7	day 10	day 14
No EDTA added to sample								
20C	Spread	0	10	70	380	630	1890	2280
	Pour	0	2	26	140	181	188	200
	Filter	0	0	11	19	54	65	65
30C	Spread	0	45	175	380	455	545	670
	Pour	0	11	75	120	136	120	148
	Filter	0	7	14	10	19	19	25
37C	Spread	0	10	40	60	80	90	100
	Pour	1	5	9	9	16	16	16
	Filter	1	3	6	5	5	6	5
EDTA added to sample								
20C	Spread	0	20	150	410	740	1800	2960
	Pour	0	3	27	151	139	143	270
	Filter	0	0	8	24	46	57	58
30C	Spread	0	20	200	340	450	500	560
	Pour	1	13	91	110	145	163	171
	Filter	0	6	12	16	28	34	20
37C	Spread	10	20	30	50	60	130	130
	Pour	1	2	10	14	17	17	17
	Filter	1	2	4	4	4	3	3

7.3.2 Comparison of counts by spread plates (R2A), pour plates (YEA) and filtration (YEA)

The spread plate technique using R2A medium consistently gave the highest counts at all temperatures in the presence or absence of EDTA. The pour plate method also gave higher counts than the filtration method. The spread plate method on R2A often gave counts that were one or even two orders of magnitude higher than the counts obtained by the filtration method. The counts on R2A by the spread plate method were generally higher than on YEA by the standard pour plate method. A comparison was made of the counts determined by the two methods on the same samples after all missing data had been excluded. The results for 30°C and 37°C are summarised in table 7.3. It can be seen that the count determined by the spread plate method was higher for 44/51 paired results at 30°C and for. 37/52 paired results at 37°C.

Several laboratories noted difficulties in counting colonies on the membranes because they were difficult to see and often became overgrown. In addition the number of colonies recorded on membranes with discrete colonies and not suffering from overgrowth sometimes declined after seven days incubation. This appeared to be caused by colony lysis

Table 7.3 Comparison of counts by the spread and pour plate methods for pairs of results for the same samples after 7 days incubation at 30 and 37°C.

Result of comparison	30°C	37°C
Spread > pour	44	37
Spread = pour =>0	1	1
Spread = pour = 0	0	2
Pour > spread count	5	12
Total comparisons	51	52

7.3.3 The effect of incubation temperature

As might have been expected, there was a considerable difference between the counts recorded at the different temperatures and this was significantly different. The counts at 20°C exceeded those at 30°C and in turn these were greater than the counts at 37°C

7.3.4 The effect of EDTA

After eliminating all missing data the results were compared for pairs of subsamples that were from the same source and otherwise treated exactly the same way except one had been supplemented with EDTA. The results are summarised in figure 7.1 and table 7.4 where it can clearly be seen that the number of occasions when the count in the presence of EDTA exceeded that in its absence was actually less than the number of times that the count in the absence of EDTA was greatest. A comparison of the two sets of counts by a two factor analysis of variance confirmed that there was no significant difference in the means of the two sets of results and that this was true for both immediate and post-flush samples

Figure 7.2 Bar and whisker plot of the results for samples with and without EDTA. The box shows the range in which 95% of results fall, the horizontal line in each box indicates the median and the vertical line indicates the range of results

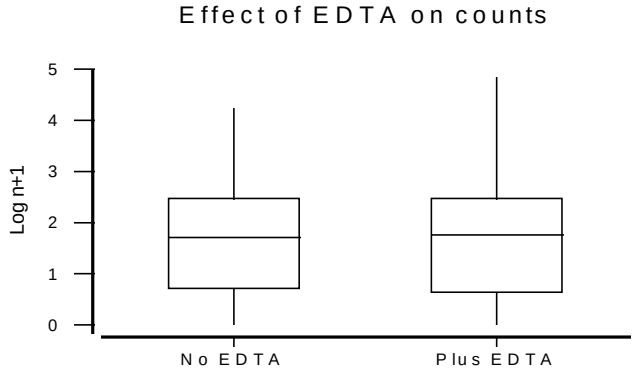

Table 7.4 Comparison of counts of pairs of subsamples treated with or without EDTA for all combinations of methods and incubation conditions

Result of comparison	Number of comparisons with specified result
Count higher with EDTA	258
Results the same and >0	22
Results the same and = 0	4
Count higher without EDTA	270
Total comparisons	554

7.3.5 Comparison of immediate and post flushed samples

The counts of the pre flushed samples, that is the samples of water withdrawn from the taps without previously disinfecting or running them, were consistently higher than the counts on samples collected after the taps had been disinfected and flushed.

APPENDIX 2

8. ISOLATION OF STRAINS AND VIRULENCE TESTING

8.1 MATERIALS AND METHODS

8.1.1 Sample collection

Participating laboratories were asked to collect samples from two sites preferably served by different water sources. One sample was taken from a single dwelling e.g. a family house. Where possible the second sample was taken from a site located in a multiple occupancy building e.g. a nurse or student residence.

At each site two samples, one preflush and one postflush, were collected from a single tap known to be connected to the cold rising main supply and not fed via a break tank. Mixer taps, dripping taps or taps with leaking glands were not used. preflush samples are samples of water collected first thing in the morning before the tap has been used and without prior disinfection and are an indication of tap contamination which could affect the water quality. Postflush samples are taken following tap disinfection and flushing with water, these samples therefore, give a better indication of water quality.

8.1.2 Preflush samples

The temperature of the water at the time of collection was noted. A sample of water was collected directly into a 500 ml container (Glass Duran or polyethylene) containing 0.5 ml sterile 1.8 % sodium thiosulphate solution to neutralise residual chlorine. Site details and the time of collection were recorded.

8.1.3 Postflush samples

After collection of the preflush sample, the tap was left running for 2-3 minutes and the temperature of the water was recorded. Prior to the collection of the postflush sample the ouside of the tap and the nozzle were disinfected with a 1 % solution of sodium hypochlorite. The tap was allowed to run for a further 2-3 minutes to remove all traces of the disinfectant. The postflush sample was collected as described above and the temperature of the water was noted.

The samples were then transported to the appropriate laboratory in an insulated cooled container and processed within 6 h of collection.

8.1.4 Sample processing

The samples were mixed thoroughly before 1:10 and 1:100 dilutions were made using sterile quarter strength Ringers. Two plates of R2A (Reasoner and Geldreich, 1985) for each dilution were then inoculated with 0.1 ml of neat, 1:10 and 1:100 dilutions and spread with a sterile spreader. One plate of each dilution was incubated at 30 °C and the other at 37 °C.

The plates were incubated in sealed plastic bags or containers to prevent the drying out of the agar during the seven day incubation period.

8.1.5 Counting colonies

A plate from each temperature was selected which gave the highest number of discreet colonies (>200 colonies) and which also included at least one representative of each colony type. Thirty colonies were selected from each of these plates or all of the colonies isolated if this was less than

30 *eg.* for plates containing >30 colonies; if there were 15 different colony types two were subcultured from each, if there were 5 colony types six were subcultured.

Each colony selected was inoculated onto a fresh R2A slope, the temperature of incubation and description of the colony appearance recorded. The isolates were then sent to the PHLS Water and Environmental Microbiology Research Unit (WEMRU) for identification and toxin studies.

On receipt each slope was subcultured onto R2A agar and incubated at the temperature corresponding to that of the original isolation. To maintain a stock culture and in order to avoid the loss of any virulence / toxin activity which may result from repeated subculture of strains, each isolate was then inoculated onto two fresh R2A slopes and also into "Microbank" cryovials (Pro-Lab Diagnostics (UK) Ltd.). One of the slopes was stored at room temperature and the other at 4-8 °C. the cryovials were stored at -40 °C.

8.2 PRESUMPTIVE IDENTIFICATION OF ISOLATES

The Gram-staining reaction is the first step in the preliminary identification of strains, the method used was as described by Collins *et al.,* 1995. The Oxidation -Fermentation (O / F) is the next most important step in further identification, it allows the differentiation of bacteria which are able to utilise glucose in the test of Hugh and Leifson 1953 into three groups; glucose oxidisers or glucose fermenters and non-utilisers / alkali producing strains. Further identification steps were then based on the first stage diagnostic tables as described in Cowan and Steel's Manual for the Identification of Medical Bacteria (Barrow and Feltham, Eds, 1993) i.e. oxidase and glucose (acid / gas). In the absence of suitable kits for environmental strains, two commercial kits for medical isolates, BBL Crystal, (Becton Dickinson Diagnostic Systems UK Ltd.) for identifying enteric and nonfermenting medical bacteria and API 20 NE for non-enteric Gram-negative rods (API-Biomerieux Basingstoke) were used to try and further identify the isolates.

8.3 TOXIN ASSAYS

8.3.1 Haemolysin test

Evidence of haemolysin activity is detected by the complete clearing of the medium surrounding the colony due to erythrocyte lysis (beta-haemolysis) or as a green colouration in the agar which is due to incomplete red cell lysis and which is termed alpha-haemolysis. The latter form of haemolysis may not extend beyond the colony boundaries, it is therefore necessary to remove the colony from the agar surface to allow accurate detection.

Sterile defibrinated horse, human, or sheep blood (Bradsure Biologicals) was washed in sterile peptone buffered saline (PBS) by centrifugation at 3000g for 15 min followed by two 10 min spins at 3000 g. The appropriate amount of red blood cells were added aseptically to molten sterile blood base agar to give a final concentration of 6%. Samples were prepared by inoculating 1 ml of sterile (PBS) with a single colony of a day old culture. The plates were inoculated with a 21-point multi-inoculator and incubated at the appropriate temperature for 24 h. The production of alpha or beta-haemolysin was recorded.

8.3.2 Test for vero cytotoxin

The method is based on those of Konowalchuk *et al.* 1977 and Scotland *et al*. 1980. Vero cells (African green monkey kidney) were grown and maintained as described by Scotland *et al.,* 1985. Vero cells which had been grown in Minimum Essential Medium Eagles (MEM) and incubated at 37 °C for 7 days in a CO_2 incubater were resuspended in MEM after trypsinisation to give a final concentration of approximately 5 x 104 cells / ml. A 0.2 ml portion of the diluted sample was distributed in each well of a 96-well tissue culture plate (Falcon Micro Test II). The plate was then sealed with pressure sensitive film and incubated at 37 °C in CO_2 for approximately 3 days until the cell monolayer was confluent.

The strains to be tested were grown in trypticase soy broth, 10 ml, for 18-24 h at 37 °C whilst shaking (Scotland *et al.,* 1985). The cultures were centrifuged for 15 min at 3000 g and the supernatant sterilised by filtration through a Millipore filter (0.45 um). A second aliquot of the sample of the supernatant was boiled for 15 min. A portion 0.02 ml of the filtered supernatant and the heat treated samples were each added in duplicate to wells without changing the media. The plates were resealed and incubated at 37 °C for 4 days. The media was removed and the cells fixed with methanol for 5 min and then stained with Giemsa stain (5%) for 45 min. The monolayers were then washed, the plates dried and the cells were examined microscopically. In the presence of the cytotoxin the monolayer became detatched and very few cells were visible.

A positive control of a known verocytotoxin producer which was obtained from the PHLS Laboratory of Enteric Pathogens LEP E3787 *E. coli* 026:H11 and a negative control were inoculated with each batch of bacteria tested.

8.3.3 Test for adhesion

This assay was based on that of Cravioto *et al.,* 1979. HEp-2 cells which were grown and maintained as described by Scotland *et al.,* 1985, were resupended in complete growth media (MEM) without any antibiotics to a final concentration of 106 cells / ml. Tissue culture trays with 4 cm wells containing a sterile coverslip were inoculated with 2 ml of the suspension. The plates were incubated at 37 °C for 48 h until the monolayer was confluent.

The bacterial strain to be tested was grown overnight without shaking at 37 °C in peptone water with added D-mannose (1%) . Immediately prior to the test the HEp-2 monolayer was washed twice with Earle's balanced salt solution (EBSS). Each culture to be tested was diluted 1:50 in MEM medium containing mannose (1%) to give a final concentration of approximately. 107-108 bacteria / ml. To each well 1 ml of the bacterial suspension was added and the plate was resealed and incubated at 37 °C for 3 h.

After 3 h the media was removed and the monolayer was washed three times with EBSS, 2 ml of MEM containing mannose (1%) was then added to each well. After a further 3 h incubation period the media was removed and the monolayer was washed three times with EBSS. The cells were then fixed with methanol for 5 min, stained with Giemsa stain (10%) for 45 min and de-stained as described by Scotland *et al.,* 1985. The coverslips were then mounted onto microscope slides and viewed under oil immersion. Positive results for attatchment were described as; the attachment of at least 10 cells to 40% of the HEp-2 cells. A negative result was denoted by no evidence of attatchment of 1-5 bacteria to less than 5% of the cells.

A positive control strain for localised adhesion on Hep-2 cells; (LEP E2347) E.coli 0127:H6 and a negative control strain were inoculated with batch of isolates.

8.3.4 Invasive assay

The method is based on those of Mehlman *et al* ., 1977 and Day *et al.,* 1981. Sterile coverslip were placed in wells in a tissue culture plate and then inoculated and incubated as described above. The bacterial strains to be tested were grown overnight in nutrient broth without shaking. The bacterial cells were diluted (1:20) in an infection medium (70 ml EBSS, 10 ml brain heart infusion and 20 ml heat-inactivated foetal calf serum). The monolayer was washed once with EBSS and each well was then inoculated with the bacterial suspension. The plate was resealed and incubated for 2 h at 37°C.

The monolayer was then washed twice in EBSS and 2 ml of an intracelluar media (45 ml complete growth media without antibiotics, 0.5 ml gentamicin, 2mg/ml, and 5ml lysozyme, 3mg/ml) was added to each well. The plate was resealed and incubated at 37 °C for 3 h. The cells were fixed, stained and destained as described in the assay for bacterial adhesion. At least 300 healthy cells were examined and each cell containing more than one bacterium was counted as a positive.

Positive control strains for invasion; E. coli , LEP 66438 DA and a negative control strain were inoculated with each batch of isolates tested.

8.3.5 LT Assay

LT type toxic activity in whole cells and culture supernatants was detected by the Y1 mouse adrenal cell test as described by Scotland *et al.* (1980) except that it was found that the the growth medium recommended, Ham's F10, did not suppport more than one or two passages. It was thought that this was probably caused by some component in the horse serum and consequently the growth medium was changed to MEM which proved to give good growth of the Y1 cells and was therefore used thereafter. *Escherichia coli* O7 H18 strain E 5798 was included as a positive control in each batch of strains tested.

APPENDIX 3

9. TRIAL OF THE DETECTION OF CAMPYLOBACTER SPECIES IN POTABLE WATER SAMPLES BY PCR

9.1 BACKGROUND

The oligonucleotide primers for the Campylobacter PCR assay were designed to amplify a 256 base pair product from an open reading frame adjacent to and downstream of a *Campylobacter jejuni* two component regulator gene. The assay was developed at the Manchester Public Health Laboratory and the primer sequences are subject to an international patent application, No. PCT/GB94/01967.

The assay is specific for the thermophilic enteropathogenic species, *Campylobacter jejuni*, *Campylobacter coli* and *Campylobacter upsaliensis*. Differentiation between these species is achieved by restriction fragment length polymorphisms of PCR products with a panel of restriction enzymes. The assay has a sensitivity of detection of 10-25 cfu's in water.

9.2 MATERIALS AND METHODS

9.2.1 Sample collection

Samples of potable water from multiple occupancy buildings were collected according to the protocol supplied given in Appendix 2. Pre-flush and Post-flush filters were sent to Preston Public Health Laboratory for examination using the Campylobacter PCR assay.

Samples were received from Bristol, Nottingham and Preston Public Health Laboratories.

9.2.2 Sample Preparation

Sample filters were placed in sterile plastic universal containers (conical bottom) and 5ml of sterile distilled water was added to cover the filter. The contents were mixed by vortex for 1 minute and incubated in a water bath at 65^0C for one hour, with intermittent shaking. The filter was removed and the organisms detached from the filter were recovered by centrifugation at 4000 rpm in an IEC 8R centrifuge. The pellet was resuspended in 500µl of sterile distilled water (PrepA). Two hundred and fifty microlitres of this sample was pipetted into a microcentrifuge tube and placed in a boiling water bath for 5 minutes. The sample was snap cooled on ice and centrifuged at 14000 rpm in an Eppendorf refrigerated microcentrifuge (PrepB).

9.2.3 PCR assay

The amplification assay was performed in a volume of 50µl containing the following: 1x PCR buffer (Perkin-Elmer), 1.5mM $MgCl_2$ (Perkin-Elmer), 200µM each dATP, dTTP, dCTP, dGTP (Pharmacia Biotech), 0.2µM each primer (Immunogen International), 2.5 units Amplitaq DNA polymerase (Perkin-Elmer). Five microlitres of each sample preparation was added to the above reagents for each test. A duplicate tube was prepared for each test to which 5µl of sample preparation and 5µl of a DNA sample prepared from a boiled suspension of 1 x 10^6 cfu of *Campylobacter coli* NCTC 11366. This spiked sample served to act as a control for the inhibition of the PCR reaction by substances present in the sample preparation. Each batch of tests include a positive and negative control.

Each tube was overlaid with 50µl of sterile liquid paraffin to act as a vapour barrier and amplification was carried out using an MJ research PTC150 thermal cycler. The amplification cycle comprised, initial denaturation at 95^CC four five minutes followed by 40 cycles of primer annealing at 55^CC for 1 minute, extension at 72^CC for 1 minute and denaturation at 95^0C for 1 minute. A terminal extension step at 72^0C for five minutes completed the reaction.

PCR products were visualised by gel electrophoresis on 2% NuSieve agarose (FMC Bioproducts) containing 0.5µg/ml ethidium bromide. Photographs were taken with a Polaroid CU-5 Land Camera fitted with a Wratten 3A filter and using Polaroid Type 667 black and white film.

9.3 EVALUATION OF SUITABILITY OF FILTER MATERIAL FOR USE WITH THE PCR ASSAY

Three filter materials were investigated for compatibility with the Campylobacter PCR assay.

A suspension of 10^6 cfu/100ml of *Campylobacter jejuni* NCTC 11168 was prepared in sterile distilled water and was filtered under vacuum through each of the following filter matrices; Gelman Supor 200 (Gelman 4702), Whatman Nylon (Whatman 1920-7009) and Sartorius cellulose nitrate (Sartorius 11307-47N) membranes. All filters were 47mm diameter and 0.2µm pore size. Filters were processed as described in 2.2, with the exception that two small segments from the centre of the filter were taken to perform direct PCR from the filter.

9.4 EXAMINATION OF POTABLE WATER SAMPLES WITH THE PCR ASSAY

A.Three sets of Pre-flush and Post-flush filter samples were submitted to Preston Public Health Laboratory for testing with the Campylobacter PCR assay.

APPENDIX 4

10 COMPLETE SET OF RESULTS FOR ALL STRAINS EXAMINED

KEY

Serial, strain serial number

Labcode
B1, Bristol address 1; B2, Bristol address 2; D1, Reading address 1; D2, Reading address 2;
G1, Guildford address 1; G2, Guildford address; M1, Manchester address 1; M2, Manchester address 2;
N1, Newcastle address 1; N2, Newcastle address 2; T1, Nottingham address 1; T2, Nottingham address 2;
P1, Preston address 1; P2, Preston address 2; R1, Rhyl address 1; R2, Rhyl address 2;
S1, Swansea address 1; S2, Swansea address 2;

Occ 0, single occupancy; 1, multiple occupancy:
Flush 0, preflush; 1, postflush:
ºC 0, 30°C, 1, 37°C:
Gram 0, Gram negative; 1, Gram positive; 2, Gram variable:
Rods 0, cocci; 1, bacilli:
O/F 0, no growth ; 1, fermentative; 2, oxidative:
Gas 0, no gas produced; 1, gas produced;
Acid 0, no acid produced; 1, acid produced;
Oxid 0, oxidase negative; 1, oxidase positive;
HS haemolytic acitivity on sheep blood - 0, none; 1, alpha-haemolytic; 2, beta-haemolytic:
HH haemolytic activity on horse blood - 0, none; 1, alpha-haemolytic; 2, beta-haemolytic;
HM haemolytic activity on human blood - 0, none; 1, alpha-haemolytic; 2, beta-haemolytic.
VT action on Vero cells 0, no cytotoxic activity; 1, vero cytoxic activity
LT heat labile toxin produced: OO, none on either occassion tested; 1O or O1, positive on the first or second time of testing; 11, positive on both ocassions
Adhes 0, no adhesive activity; 1, adhesive activity:
Inv 0, no invasive activity; 1 = invasive activity

Miscellaneous Gram-negative bacilli

Miscelllaneous Gram-negative bacteria refers to a group of oxidase positive species that are relatively indistinguishable from each other in the BBL Crystal Enteric/Nonfermenter System. They include:

Acaligenes faecalis
Alcaligenes piechaudii
*Alcaligenes xylosoxidan*s subsp. *denitrificans*
Alcaligenes xylosoxidans subsp. xylosoxidans
Bordetella bronchiseptica
Burkholderia (Pseudomonas) pickettii
CDC Group IV C-2
Comomonas acidovorans
Comamonas testosteroni
Eikenella corrodens
Methylobacterium species
Moraxella lacunata
Moraxella osloensis
Ochrobactrum anthropi
Oligella urethralis
Pseudomonas alcaligenes
Pseudomonas fluorescens
Pseudomonas mendocina
Pseudomonas- pseudoalcaligenes
Pseudomonas putida

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1	T1	0	1	1	1	0	1	1	1	1		0	0	0	0	00	0	0		0000000000	unacceptable
2	T1	0	1	1	1	0	0	0	0	0		0	0	0	0	00	0	0			
3	T1	0	1	1	0	1	1	0	0	0		0	0	0	0	00	0	0		2110211020	unacceptable
4	T1	0	1	1	1	0	1	0	0	1		0	0	0	0		0	0		2251100240	unacceptable
5	T1	0	1	1	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
6	T1	0	1	1	0	1	1	0	1	1		0	0	0	0	00	0	0		2210100140	*Flavobacterium indologenes*
7	T1	0	1	1	0	1	0	0	0	1		0	0	0	0		0	0			
8	T1	0	1	1	0	1	1	0	0	0		0	0	0	0	00	0	0		2110011000	unacceptable
9	T1	0	1	1	0	1	1	0	0	1		0	0	0	0	00	0	0		2000100004	*Weeksella virosa*
10	T1	0	1	1	0	1	1	0	1	0		0	0	0	0	00	0	0		2210320000	unacceptable
11	T1	0	1	1	0	1	0	0	0	1		0	0	0	0	00	0	0			
12	T1	0	1	1	0	1	1	0	0	1		0	0	0	0	00	0	1		2111200000	unacceptable
13	T1	0	1	1	2	0	0	0	0	1		0	0	0	0	00	0	0			
14	T1	0	1	1	0	1	0	0	0	1		0	0	0	0		0	0			
15	T1	0	1	1	2	0	0	0	0	1		0	0	0	0	00	0	0			
16	T1	0	1	1	2	0	0	0	0	1		0	0	0	0	00	0	0			
17	T1	0	1	1	0	1	2	1	1	1		0	0	0	0	00	0	0	4141664		*Aeromonas salmonicida*
18	T1	0	1	1	0	1	1	1	1	1		0	0	0	1	00	0	0		2211120000	*Flavobacterium indologenes*
19	T1	0	1	1	2	0	0	0	0	1		0	0	0	0	00	0	0			
20	T1	0	1	1	2	0	0	0	0	0		0	0	0	0	00	0	0			
21	T1	0	1	1	0	1	1	0	0	1		0	0	0	0	00	0	0		0000000000	no growth
22	T1	0	1	1	0	1	0	0	0	1		0	0	0	0	00	0	0			
23	T1	0	1	1	0	1	0	0	0	1		0	0	0	0	00	0	0			
24	T1	0	1	1	0	1	0	0	0	1		0	0	0	0	00	0	1			
25	T1	0	1	1	0	1	0	0	0	0		0	0	0	0	00	0	0			
27	T1	0	1	1	0	1	1	0	0	0		0	0	0	0	00	0	0		2010000000	*Shigella sp*
28	T1	0	1	1	1	0	2	0	0	1		0	0	0	1	00	0	0	0000000		no growth
29	T1	0	1	1	0	1	1	1	1	0		0	0	0	0	00	0	0		2001304020	unacceptable
30	T1	0	1	1	0	1	0	0	1	0		0	0	0	0	00	0	0			
31	T1	0	1	1	0	1	1	0	0	1		0	0	0	0	00	0	0		2110100000	unacceptable
32	T1	0	1	1	0	1	0	0	1	1		0	0	0	0		0	0			
33	T1	0	0	1	2	0	0	0	0	1		0	0	0	0		0	0			
34	T1	0	0	1	0	1	0	0	0	0		0	0	0	0		0	0			
35	T1	0	0	1																	
36	T1	0	0	1	0	1	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
37	T1	0	0	1	2	0	1	0	0	0		0	0	0	0		0	0		1100000040	unacceptable
38	T1	0	0	1												00					
39	T1	0	0	1	0	1	0	0	0	1		0	1	0	0	00	0	0			
40	T1	0	0	1	1	0	1	1	1	1		0	0	0	0	00	0	0		0000000000	no growth
41	T2	0	1	1	1	0	1	0	0	1		0	1	0	0		0	0		0000000000	no growth
42	T2	0	1	1	0	1	1	1	1	0		0	0	0	0		0	0		4	*Acinetobacter lwoffii*

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
43	T2	0	1	1	0	1	1	0	0	0		0	0	0	0		0	0		3010000000	Shigella sp
44	T2	0	1	1																	
45	T2	0	1	1												00					
46	T2	0	1	1												00					
47	T2	0	1	1	0	1	1	0	0	1		0	0	1	0	0	0	0		2233100000	*Pseudomonas stutzeri*
48	T2	0	1	1	0	1	1	0	0	1		0	0	1	1	00	0	0		3233120000	unacceptable
49	T2	0	1	1	0	1	1	0	0	1		0	0	1	0		0	0		3233120110	unacceptable
50	T2	0	1	1	1	0	1	0	0	1		0	0	0	0		0	0	0000000	0000000000	no growth
51	T2	0	1	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
52	T2	0	1	1	0	1	1	0	0	1		0	0	0	0	00	0	0		0000000000	no growth
53	T1	0	1	0	0	1	0	0	0	0		0	0	0	0	00	0	0			
54	T1	0	1	0	0	1	0	0	0	0		0	0	0	0	00	0	0			
55	T1	0	1	0	1	0	1	1	1	1		0	0	0	0	00	0	0		0000000000	no growth
56	T1	0	1	0	1	0	2	1	1	0		0	0	0	0	00	0	0	0000000		no growth
57	T1	0	1	0																	
58	T1	0	1	0	1	0	0	0	1	0		0	0	0	0		0	0			
59	T1	0	1	0	0	1	1	1	1	1		0	0	0	0	00	0	0		0000000000	no growth
60	T1	0	1	0	0	1	0	0	1	1		0	0	0	0	00	0	0			
61	T1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
62	T1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
63	T1	0	1	0	0	1	0	0	0	1		0	0	0	0	00	0	0			
64	T1	0	1	0																	
65	T1	0	1	0	1	0	0	0	0	1		0	0	0	0	00	0	0			
66	T2	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
67	T2	0	1	0												00					
68	T2	0	1	0	1	1	0	0	0	1		0	0	0	0		0	0			
69	T2	0	1	0	1	0	1	1	1	1		0	0	0	0	00	0	0		0000000000	no growth
70	T2	0	1	0	0	1	1	0	1	1		0	0	0	0	00	0	0		3331320240	unacceptable
71	T2	0	1	0	0	1	0	0	0	1	0	0	0	0	0	00	0	0			
72	T2	0	1	0	0	1	0	1	1	1		0	0	0	0		0	0			
73	T2	0	1	0	0	1	0	1	1	0		0	0	0	0		0	0			
74	T2	0	1	0	0	1	0	0	0	0		0	0	0	0		0	0			
75	T2	0	1	0	0	1	0	0	0	1		0	0	0	0	00	0	0			
76	T2	0	1	0	0	1	1	1	1	1		0	0	0	1		0	0		0000000000	no growth
77	T2	0	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
78	T2	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0			
79	T2	0	0	0	0	1	1	0	0	1		0	0	0	0	00	0	0		0000000000	no growth
80	T2	0	0	0	0	1	1	0	0	0		0	0	0	0	00	0	0		0000000000	no growth
81	T2	0	0	0																	
82	T2	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0			
83	T2	0	0	0	1	0	0	0	0	1		0	0	0	0		0	0			

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
84	T2	0	0	0	1	0	0	0	1	1		0	0	0	0		0	0			
85	T2	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
86	T2	0	0	0	0	1	0	0	0	0		0	0	0	0	10	0	0			
87	T2	0	0	0																	
88	T2	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
89	T2	0	0	0	1	0	1	1	1	1		0	0	0	0		0	0		0000000000	no growth
90	T2	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0			
91	T2	0	0	0																	
92	T1	0	0	0	0	1	0	0	0	0		0	0	0	0	00	0	0			
93	T1	0	0	0	0	1	0	0	0	1		0	0	0	1	00	0	0			
94	T1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
95	T1	0	0	0	0	1	1	1	1	1		0	0	0	0		0	0		3200100240	unacceptable
96	T1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
97	T1	0	0	0												00					
98	T1	0	1	0																	
99	T1	0	0	0																	
100	T1	0	0	0	0	1	0	0	0	1		0	0	0	0	00	0	0			
101	T1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
102	T1	0	0	0	0	1	0	0	0	0		0	0	0	0	00	0	0			
103	T2	0	0	0												00					
104	T2	0	0	0	0	1	0	0	0	0		0	0	0	0	00	0	0			
105	T2	0	0	0	0	1	0	0	0	1		0	0	0	0	00	0	0			
106	T2	0	0	0	0	1	1	0	0	1		0	0	0	0	00	0	0		2310000000	*Pseudomonas vesicularis*
107	T2	0	0	0	1	0	0	0	0	0		0	0	0	0	00	0	0			
108	T2	0	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
109	T2	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
110	T2	0	1	0	0	1	0	0	0	1		0	0	0	0	00	0	0			
111	T2	0	1	0	0	0	0	0	0	0		0	0	0	0	00	0	0			
112	T2	0	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
113	T2	0	1	0	0	1	2	1	1	1		0	0	0	0		0	0	0000000		no growth
114	T2	0	1	0																	
115	T2	0	1	0	0	1	2	1	1	0		0	0	0	0		0	0	0000000		no growth
116	T2	0	1	0																	
117	T2	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
118	T2	0	1	0	0	1	2	0	0	0		0	0	0	0		0	0	0000000		no growth
119	T2	0	1	0																	
120	T2	0	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
121	T2	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
122	T2	0	1	0	0	1	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
123	P1	1	0	0	0	1	2	0	0	0		0	0	0	0		0	0	4100000		*Listonella damsella*
124	P1	1	0	0	0	1	1	0	0	1		0	0	0	0		0	0		1020000000	Misc Gram neg bacillus

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
125	P1	1	0	0	0	1	1	0	0	0		0	0	0	0		0	0		1000000000	*Acinetobacter lwoffii*
126	P1	1	0	0	0	1	2	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
127	P1	1	0	0																	
128	P1	1	0	0	0	1	1	0	0	0		0	0	0	0		0	0		1000000000	*A. lwoffii/Shigella sp.*
129	P1	1	0	0	0	1	2	0	0	0		0	0	0	1		0	0	4100000		*Listonella damsella*
130	P1	1	0	0	0	1	1	0	0	1		0	0	0	1		0	0		0000000000	no growth
131	P1	1	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
132	P1	1	0	0	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
133	P1	1	0	0	1	0	0	0	0												
134	P1	1	0	0	0	1	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
135	P1	1	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
136	P1	1	0	0	0	1	1	0	0	0		0	0	0	0		0	0		2210000000	unacceptable
137	P1	1	0	0	2	0	1	0	0	0		1	1	1	0		0	0		10200000	*A. lwoffii/Shigella sp.*
138	P1	1	0	0	2	0	1	0	0	1		0	0	0	0		0	0		10000000	Misc Gram neg bacillus
139	P1	1	0	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
140	P1	1	0	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
141	P1	1	0	0	0	1	2	0	0	1		0	0	0	1	00	0	0	4147004		*Aeromonas salmonicida*
142	P1	1	0	0	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
143	P1	1	0	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
144	P1	1	0	0	1	0	1	0	0	1		0	0	0	0	0	0	0		0000000000	no growth
145	P1	1	0	0	0	1	1	0	0	1		0	0	0	0	0	0	0		1000000000	Misc Gram neg bacillus
146	P1	1	0	1	0	1	1	0	0	0		0	0	0	0		0	0			no growth
147	P1	1	0	0	2	0	1	0	0	0		0	0	0	0		0	0		10000000	*Acinetobacter lwoffii*
148	P1	1	0	0	0	1	2	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
149	P1	1	0	0	0	1	2	0	0	1		0	0	0	0		0	0	0000000		no growth
150	P1	1	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
151	P1	1	0	0	0	1	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
152	P1	1	0	0	0	1	2	0	0	1		0	0	0	0		0	0	0000000		no growth
153	P1	1	1	0	1	0	0	0	0	1		0	0	0	0		0	0	4000004		
154	P1	1	1	0																	
155	P1	1	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
156	P1	1	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
157	P1	1	1	0	1	0	0	0	0	1		0	0	0	0		0	0			
158	P1	1	1	0	0	1	1	0	0	0		0	0	0	0		0	0		1144004	unacceptable
159	P1	1	1	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
160	P1	1	1	0																	
161	P1	1	1	0	1	0	1	0	0	0		0		0				0		0000000000	no growth
162	P1	1	1	0	2	0	2	0	0	0		0	0	0	0		0	0	700000		*Listonella damsella*
163	P1	1	1	0	0	1	1	0	0	1		0	0	0	0		0	0		110100004	unacceptable
164	P1	1	1	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
165	P1	1	1	0	0	1	1	0	0	1		0	0	0	0		0	0		1010100004	Misc Gram neg bacillus

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
166	P1	1	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
167	P1	1	1	0	0	1	1	0	0	1		1	1	1	0		0	0		0000000000	no growth
168	P1	1	1	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
169	P1	1	1	0																	
170	P1	1	1	0																	
171	P1	1	1	0																	
172	P1	1	1	0																	
173	P1	1	0	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
174	P1	1	0	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
175	P1	1	0	1	1	0	1	0	0	0		0	0	0	0	0	0	0		0000000000	no growth
176	P1	1	0	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
177	P1	1	0	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
178	P1	1	0	1	1	0	1	0	0	0		0	0	0	0		0	0		0000000000	no growth
179	P2	0	0	0	1	0	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
180	P2	0	0	0	2	0	1	0	0	1		0	0	0	0		0	0		3201300320	*Flavobacterium breve*
181	P2	0	0	0	1	0	0	0	0	0		0	0	0							
182	P2	0	0	0	0	0	1	0	0	1		0	0	0		0				3321320122	*Flavobacterium meningosepticum*
183	P2	0	0	0	1	0	1	0	0	1		0	0	0							
184	P2	0	0	0																	
185	P2	0	0	0																	
186	P2	0	0	0																	
187	P2	0	0	0	1	0	0	0	0	1		0	0	0							
188	P2	0	0	0												0					
189	P2	0	0	0	0	0	1	0	0	1		0	0	0	1		0	0		3211300320	unacceptable
190	P2	0	0	0	0	1	1	0	0	0		0	0	0	0		0	0		3212010130	unacceptable
191	P2	0	0	0																	
192	P2	0	0	0																	
193	P2	0	0	0	0	1	2	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
194	P2	0	0	0	1	1	0	0	0	0		0	0	0	0		0	0			
195	P2	0	0	0	1	0	1	0	0	1		0	0	0							
196	P2	0	0	0																	
197	P2	0	0	0	1	0	1	0	0	1		0	0	0	0		0	0			
198	P2	0	0	0	1	0	1	1	0	1		0	0	0							
199	P2	0	0	0	0	1	1	1	0	1		0	0	0	0	0	0	0		3303310313	unacceptable
200	P2	0	0	0	0	1	1	1	1	0		0	0	0	0	0	0	0		3201320320	unacceptable
201	P2	0	0	0	0	1	1	1	1	1		0	0	0	0	0	0	0		3331320320	unacceptable
202	P2	0	0	0	1	0	1	1	1	0		0	0	0		0					
203	P2	0	0	0	0	1	1	1	1	1		0	0	0	0		0	0		3012010010	unacceptable
205	P2	0	0	0	0	1	1	0	0	1		0	0	0	0	0	0	0		3212210130	unacceptable
206	P2	0	0	0	0	1	1	0	0	1		0	0	0	0	0	0	0			
207	P2	0	0	0	0	0	1	1	1	1		0	0	0	0	00	0	0		3332021133	unacceptable

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
208	P2	0	0	0	1	0	1	0	0	1						00					
209	P2	0	1	0	1	0	1	0	0	1											
210	P2	0	1	0	1	0	1	0	0	1											
211	P2	0	1	0	1	0	1	0	0	1		0	0	0	0		0	0			
212	P2	0	1	0	1	0	2	0	0	1		0	0	0	0	0	0	0	4100004		*Listonella damsella*
213	P2	0	1	0	1	0	1	0	0	1											
214	P2	0	0	1	1	0	0	0	0	0						0					
215	P2	0	0	1	2	0	1	0	0	1		0	0	0	1		0	0			
216	P2	0	0	1																	
217	P2	0	0	1	1	0	1	0	0	1											
218	P2	0	0	1	0	1	2	0	0	0		0	0	0	0	0	0	0	5400050		*Shewanella putrefaciens*
219	P2	0	0	1																	
221	P2	0	0	1	0	1	2	0	0	1		0	0	0	0	0	0	0	1503056		*Pseudomonas putida*
222	P2	0	0	1	1	0	0	0	0	0		0	0	0		0					
223	P2	0	0	1	1	0	1	0	0	1											
224	P2	0	0	1	1	0	1	0	0	1											
225	P2	0	0	1	1	1	0	0	0	0											
226	P2	0	0	1	1	0	1	0	0	1											
227	P2	0	0	1	1	0	1	0	0	1											
228	P2	0	0	1	1	0	1	0	0	1		1	1	1							
229	P2	0	0	1	2	0	1	0	0	1		0		0	0	00	0	0		3212010012	unacceptable
230	P2	0	0	1																	
231	P2	0	0	1												0					
232	P2	0	0	1	1	0	1	0	0	1		0	0	0	0		0	0			
233	P2	0	0	1	1	1	2	0	0	0		0	0	0							
234	P2	0	0	1	1	0	2	0	0	1											
235	P2	0	0	1	1	0	1	0	0	1						0					
236	P2	0	0	1	1	0	1	0	0	1						0					
237	P2	0	0	1	1	0	1	0	0	0						0					
238	P2	0	0	1	0	1	2	0	0	0		0	0	0	0	0	0	0	5100000		*Listonella damsella*
239	P2	0	0	1	2	0	1	0	0			0	0	0	0	0	0	0		3212210120	unacceptable
240	P2	0	0	1	1	0	1	0	0	1		0	0	0	0	00	0	0			
241	P2	0	0	1	1	0	1	0	0	1						0					
242	P2	0	0	1	1	0	1	0	0	1						0					
243	P2	0	0	1	0	1	1	0	0	1		0	0	0	0	00	0	0		3212210330	unacceptable
244	N1	0	1	1	1	0	2	0	0	0		0	0	1	0	0	0	0	4144440		*Chromobacterium violaceum*
245	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	1044444		*Pseudomonas stutzeri*
246	N1	0	1	1	0	1	2	0	0	1		0	0	1	0	0	0	0	4150724		*Aeromonas salmonicida*
247	N1	0	1	1	0	1	2	0	0	1		0	0	0	0	00	0	0	4254360		*Vibrio metschnikovii/parahaemolyticus*
248	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	100004		*Pasteurella sp.*

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
249	N1	0	1	1	1	0	2	0	0	1		0	0	0	0		0	0	4700204		*Listonella damsella*
250	N1	0	1	1	0	1	2	0	0	1		1	1	1	0		0	0	4100004		*Listonella damsella*
251	N1	0	1	1	0	1	2	0	0	0		0	0	0	0		0	0	4100000		*Listonella damsella*
252	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	100004		*Pasteurella sp.*
253	N1	0	1	1	1	0	0	0	0	1		0	0	0	0		0	0			
254	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	4100004		*Listonella sp*
255	N1	0	1	1	1	0	0	0	0	1		0	0	0	0		0	0			
256	N1	0	1	1	0	1	2	0	0	0		0	0	0	0		0	0	410000		unacceptable
257	N1	0	1	1	0	1	2	0	0	1		0	0	0	0	0	0	0	110004		*Weeksella virosa*
258	N1	0	1	1	2	0	0	0	0	0		0	0	0	0		0	0			
259	N1	0	1	1	1	0	0	0	0	1		0	0	0	0		0	0			
260	N1	0	1	1	1	0	2	0	0	1		2	1	2	0	0	0	0	4210004		*Weeksella zoohelicum*
261	N1	0	1	1	0	1	2	0	0	1		1	1	1	0	00	0	0	4100004		*Listonella damsella*
262	N1	0	1	1	0	1	0	0	0	1		1	1	1	0	00	0	0	4100004		*Listonella damsella*
263	N1	0	1	1	0	1	0	0	0	1		1	1	1	0		0	0			
264	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	4400004		*Aeromonas salmonicida*
265	N1	0	1	1	0	1	0	0	0	1		2	0	0	0		0	0	4		*Moraxella sp.*
266	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	4000004		*Moraxella sp.*
267	N1	0	1	1	0	1	2	0	0	1		0	0	0	0		0	0	100004		*Pasteurella sp.*
268	N1	0	1	1	0	1	2	0	0	1		2	2	2	0		0	0	4300004		*Listonella damsella*
269	N1	0	1	1	0	1	2	0	0	0		2	2	2	0	00	0	0	210000		*Weeksella zoohelicum?*
270	N1	0	1	1	2	0	2	0	0	0		2	2	2	0	10	0	0	400000		*Pseudomonas vesicularis*
271	N1	0	1	1	0	1	0	0	0	1		2	2	2	0	0	0	0			
272	N1	0	1	1	2	0	0	0		1		0	0	0	0	00	0	0	4043004		*Aeromonas salmonicida*
273	N1	0	1	1	0	1	0	0	0	0		0	0	0	0		0	0			
274	N2	1	1	1	0	1	0	0	1	0		0	0	0	0		0	0			
275	N2	1	1	1	0	0	0	0	0	0		0	0	0	0		0	0			
276	N2	1	1	1	1	0	0	0	0	1						11					
277	N2	1	1	1	1	0	0	0	0	0											
278	N2	1	1	1																	
279	N2	1	1	1																	
280	N2	1	1	1																	
281	N2	1	1	1	1	0	0	0	0	0											
282	N2	1	1	1	0	1	0	0	0	0		0	0	0	0		0	0			
283	N2	1	1	1	1	0	0	0	0	0		0	0	0	0		0	0			
284	N2	1	1	1	0	1	0	0	0	0		0	0	0	0		0	0			
285	N2	1	1	1	1	0	0	0	0	0											
286	N2	1	0	0	0	0	0	0	0	1		0	0	0	0	0	0	0	5010004		*Aeromonas salmonicida*
287	N2	1	0	0	0	0	0	0	0	1		0	0	0	0		0	0	4110004		*Aeromonas salmonicida*
288	N2	1	0	0	1	0	0	0	0	0	1	0	0	0							
289	N2	1	0	0	0	1	2	0	0	0		0	0	0	0	0	0	0	4310000		*Listonella damsella*

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
290	N2	1	0	0												00					
291	N2	1	0	0	0	1	2	0	0	1		0	0	0	0	00	0	0	4010004		*Weeksella zoohelicum*
292	N2	1	0	0	0	1	2	0	0	1		0	0	0	0	0	0	0	4110004		*Weeksella virosa*
293	N2	1	0	0	0	1	0	0	0	1		0	0	0	0	0	0	0	310004		*Weeksella zoohelicum*
294	N2	1	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
301	N2	1	0	0	1	0	0	0	0	1											
302	N2	1	0	0	1	0	0	0	0	0		1	1	1							
303	N2	1	0	0	1	0	0	0	0	0		1	1	1							
304	N2	1	0	0	0	0	1	0	0	0		2	2	2							
305	N2	1	0	0	0	1	1	0	0	1		1	1	1	1	0	0	0		101100011	Misc Gram neg bacillus
306	N2	1	0	0	1	0	1	0	0	1											
307	N2	1	0	0	1	0	0	0	0	0											
308	N2	1	0	0	0	1	2	0	0	0		0	0	0	0		0	0	4310000		*Listonella damsella*
309	N2	1	0	0	1	0	0	0	0	0											
310	N2	1	0	0	1	0	1	0	0	0		2	2	2							
311	N2	1	0	0	1	0	0	1	0	0		2	2	2							
312	N2	1	0	0	0	1	2	0	0	0		0	0	0	0		0	0	110000		*Weeksella virosa*
313	N2	1	0	0	0	1	2	0	0	0		0	0	0	0		0	0	4300000		*Listonella damsella*
314	N2	1	0	0	1	0	0	0	0	1		0	0	0	1		0	0			
315	N2	1	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
316	R1	0	0	1	1	0	0	0	0	1		1	1	1	0		0	0			
317	R1	0	0	1	0	1	2	0	0	1		1	1	1	0		0	0	5057344		*Vibrio parahaemolyticus*
318	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	1	1247744		*Ochrobacter anthropi*
319	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	1647765		*Agrobacterium radiobacter*
320	R1	0	0	1	1	0	1	0	0	1		0	0	0							
321	R1	0	0	1	0	1	1	0	0	1		0	0	0	0		0	0		2331300000	*Sphingobacterium multivorum*
322	R1	0	0	1	1	1	1	0	0	1		0	0	0							
323	R1	0	0	1	0	1	1	0	0	1		0	0	0	0		0	0		3331300000	*Flavobacterium indologenes*
324	R1	0	0	1	1	0	2	0	0	1		0	0	0							
325	R1	0	0	1	1	0	1	0	0	1		0	0	0							
326	R1	0	0	1	1	0	2	0	0	1		0	0	0							
327	R1	0	0	1	1	0	1	0	0	1		0	0	0							
328	R1	0	0	1	0	1	1	0	0	1		0	0	0	0		0	0		3331300000	*Flavobacterium indologenes*
329	R1	0	0	1	1	0	1	0	0	1											
330	R1	0	0	1	1	0	1	0	0	0		1	1	1							
331	R1	0	0	1	1	0	1	0	0	1											
332	R1	0	0	1	1	0	1	1	0	1											
333	R1	0	0	1	1	0	2	0	0	1		0	0	0							
334	R1	0	0	1	1	0	2	0	0	0		0	0	0							
335	R1	0	0	1	1	0	1	0	0	0		0	0	0							
336	R1	0	0	1	1	0	1	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
337	R1	0	0	1	1	0	2	0	0	0		0	0	0							
338	R1	0	0	1	0	1	2	0	0	0		0	0	0	0		0	0	0000000		no growth
339	R1	0	0	1	1	0	1	1	0	1											
340	R1	0	0	1	1	0	1	1	0	0		0	0	0							
341	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	4100044		*Listonella damsella*
342	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	300000		*Listonella damsella*
343	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	5147446		*Pseudomonas putida ?*
344	R1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	6210004		*Flavobacterium odoratum*
345	R1	0	0	1	0	1	1	0	0	0		0	0	0	0		0	1		11100000	*Acinetobacter lwoffii*
346	R1	0	1	0	1	0	1	0	0	0		1	0	1							
347	R1	0	1	0	1	0	1	0	0	0		1	0	1							
348	R1	0	1	1	1	0	1	0	0	0		0	0	0							
349	R1	0	1	0	1	0	0	0	0	0		0	0	0							
350	R1	0	1	1	1	0	1	0	0	0		0	0	0							
351	R2	1	1	0	1	0	0	1	1	0		0	0	0							
352	R2	1	1	0	1	0	0	1	1	0		0	0	0							
353	R2	1	1	0	1	0	0	0	0	0		0	0	0							
354	R2	1	1	0	1	0	0	0	0	0		0	0	0							
355	R2	1	1	0	1	0	0	0	0	0		0	0	0							
356	R2	1	1	0	1	0	0	0	0	0		0	0	0							
357	R2	1	1	0	1	0	0	0	0	0		0	0	0							
358	R2	1	1	0	1	0	0	0	0	0		0	0	0							
359	R2	1	1	0	1	0	1	0	0	0		0	0	0							
360	R2	1	0	0	1	0	0	0	0	0		0	0	0							
361	R2	1	0	0	1	0	1	1	1	0		0	0	0	0	0	0	0			
362	R2	1	0	0	1	0	1	1	1	0		0	0	0							
363	R2	1	0	0	1	0	1	0	0	0		1	0	0							
364	R2	1	0	0	0	1	0	0	0	1		0	0	0	1		0	1			
365	R2	1	0	0																	
366	R2	1	0	0																	
367	R2	1	0	0																	
368	R2	1	0	0	1	0	0	1	1	0											
369	R2	1	0	0	1	0	0	0	0	0											
370	R2	1	0	0	0	1	0	0	0	1		0	0	0	0		0	0	4100000		*Listonella damsella*
371	R2	1	0	0	0	1	2	0	0	1		0	0	0	0		0	1	4300000		*Listonella damsella*
372	R2	1	0	0	0	1	0	1	1	1		0	0	0	0		0	0			
373	R2	1	0	1	0	1	2	0	0	1	0	0	0	0	0		0	1	743004		*Flavobacterium indologenes*
374	M1	0	0	0	1	0	0	0	0	0		0	0	0	0		0	0			
375	M1	0	0	0	1	1	0	0	0	0		2	0	0							
376	M1	0	0	0	1	0	1	0	0	0		0	0	0							
377	M1	0	0	0	1	0	0	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
378	M1	0	0	0	1	0	0	0	0	0		0	0	0							
379	M1	0	0	0	1	0	1	0	0	0		0	0	0							
380	M1	0	0	0	1	0	1	0	0	0		2	0	0							
381	M1	0	0	0	1	0	0	1	1	0		2	0	0							
382	M1	0	0	0	0	1	1	0	0	0		1	0	0	0		0	0			
383	M1	0	0	0	0	1	0	0	0	0		2	0	0	0		0	0			
384	M1	0	0	0	0	1	1	1	1	0		0	0	0	1		0	0		0000000000	no growth
385	M1	0	0	0	0	1	0	1	0	0		0	0	0	0		0	0			
386	M1	0	0	0	1	0	0	0	0	0		0	0	0	0	0	0	0			
387	M1	0	0	0	2	0	1	0	0	0		0	0	0							
388	M1	0	0	0	1	0	1	0	0	0		2	0	0							
389	M1	0	0	0	1	0	0	1	1	0		0	0	0							
390	M1	0	0	0	0	1	1	1	1	1		0	0	0	0		0	0			
391	M1	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0			
392	M1	0	0	0	1	0	0	0	0	0		0	0	0							
393	M1	0	0	0	2	0	0	0	0	0		0	0	0							
394	M1	0	0	0	0	1	0	0	0	0		2	0	0	0		0	0			
395	M1	0	0	0	1	0	0	0	0	1		0	0	0							
396	M1	0	0	0	1	0	0	0	0	0		0	0	0							
397	M1	0	0	0	0	1	0	1	1	1		0	0	0							
398	M1	0	0	0																	
399	M1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
400	M1	0	0	0	1	0	0	0	0	0		2	0	0							
401	M1	0	0	0	0	1	1	0	0	0		2	0	0						0000000000	no growth
402	M1	0	0	0	1	0	1	0	0	0		1	0	0	0	0	0	0			
403	M1	0	0	0	1	0	0	0	0	0		0	0	0							
404	M1	0	0	1	0	1	1	1	1	0		0	0	0	0		0	0		0000000000	no growth
405	M1	0	1	1	0	1	1	0	0	1		0	0	0	0		0	1		0000000000	no growth
406	M1	0	1	1	0	1	0	0	0	1		0	0	0	0		0	0			
407	M1	0	1	1	1	0	1	0	0	1		0	0	0							
408	M1	0	1	1	0	1	1	0	0	1		0	0	0	0		0	0			
409	M1	0	1	1	0	1	0	0	0	1		0	0	0	0		0	0			
410	M1	0	1	0	0	1	0	1	0	1	0	0	0	0	0		1	0			
411	M1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
412	M1	0	1	0																	
413	M1	0	1	0																	
414	M1	0	1	0																	
415	M1	0	1	0	2	0	1	1	0	1		0	0	0							
416	M1	0	1	0																	
417	M1	0	1	0																	
418	M1	0	1	0																	
419	M1	0	1	0	0	1	1	0	0	1		0	0	0	0		0	0			

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
420	M1	0	1	0	1	0		0	0	0		0	0	0							
421	M1	0	1	0	2	0	0	1	0	0		0	0	0	1		0	0			
422	M1	0	1	0																	
423	M1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
424	M1	0	1	0	0	1	0	0	0	1		0	0	0	0	0	0	0			
425	M1	1	0	1	0	1	0	0	0	1		0	0	0	0		0	0			
426	M1	0	1	0																	
427	M1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0			
428	M1	0	1	0	1	0	0	0	0	0		0	0	0							
429	M1	1	0	1	0	1	0	0	0	0		0	0	0							
430	M1	0	1	0																	
431	M1	0	1	0	1	0	0	0	0	1		0	0	0							
432	M1	0	1	0																	
433	M1	0	1	0	1	0	0	0	0	0		0	0	0							
434	M1	0	1	0																	
435	M1	0	1	0																	
436	M1	0	1	0																	
439	M1	0	1	0																	
440	M1	0	0	1	0	1	0	0	0	1		0	0	0	0		0	0			
441	M1	0	0	1																	
442	M1	0	0	1	0	1	0	0	0	1		0	0	0	0		0	0			
443	M1	0	0	1	1	0	1	0	0	0		0	0	0							
444	M1	0	0	1																	
445	M1	0	0	1																	
446	M1	0	0	1	0	1	0	0	0	1											
447	M1	0	0	1	1	0	0	0	0	0		0	0	0							
448	M1	0	0	1	1	0	0	0	0	0		0	0	0							
449	M1	0	0	1	1	0	0	0	0	0		0	0	0							
450	M2	1	1	1	1	0	1	0	0	0		0	0								
451	M2	1	1	0	1	0	0	0	0	1		0	0	0							
452	M2	1	1	0	1	0	1	0	0	0		0	0	0							
453	M2	1	1	0			1	0	0	1		0	0	0							
454	M2	1	1	0	1	0	1	0	0	0											
455	M2	1	1	0	1	0	0	0	0	0		0	0	0							
456	M2	1	1	0	1	0	1	0	0	0		0	0	0							
457	M2	1	1	1	1	0	1	0	0	0		0	0	0							
458	M2	1	1	0	1	0	1	0	0	1		0	0	0							
459	M2	1	1	0	1	0	1	0	0	0		0	0	0							
460	M2	1	1	0	1	0	1	0	0	0		0	0	0							
461	M2	1	1	0	1	0	0	0	0	0		0	0	0							
462	M2	1	1	0	1	0	0	0	0	1		0	0	0							
463	M2	1	1	0	1	0	0	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
464	M2	1	1	0	1	0	0	0	0	0		0	0	0							
465	M2	1	1	0	1	0	0	0	0	1		0	0	0							
466	M2	1	1	0	1	0	0	0	0	1		0	0	0							
467	M2	1	1	0	1	0	0	0	0	1		0	0	0							
468	M2	1	1	0	1	0	0	0	0	0		0	0	0							
469	M2	1	1	0	0	1	0	0	0	1											
470	M2	1	1	0																	
471	M2	1	1	0																	
472	M2	1	1	0	1	0	0	0	0	0		0	0	0							
473	M2	1	1	0	1	0	2	0	0	1		0	0	0							
474	M2	1	1	0	1	0	1	0	0	0		0	0	0							
475	M2	1	1	0	1	0	0	0	0	0		0	0	0							
476	M2	1	1	0	1	0	0	0	0	0		0	0	0							
477	M2	1	1	0	1	0	0	0	0	1		0	0	0							
478	M2	1	1	0	1	0	0	0	0	1		0	0	0							
479	M2	1	1	0	1	0	1	0	0	0		0	0	0							
480	M2	1	0	1	1	0	1	0	0	0		0	0	0							
481	M2	1	0	1	1	0	1	0	0	0		0	0	0							
482	M2	1	0	1	1	0	1	1	1	0		0	0	0							
483	M2	1	0	1	1	0	0	1	1	1		0	0	0							
484	M2	1	0	1																	
485	M2	1	0	1																	
486	M2	1	0	1																	
487	M2	1	0	1																	
488	M2	1	0	1	1	0	1	0	0	0		0	0	0							
489	M2	1	0	1																	
490	M2	1	0	0	1	0	1	0	0	0		0	0	0							
491	M2	1	0	0	1	0	1	0	0	0		0	0	0							
492	M2	1	0	0	1	0	1	0	0	1		0	0	0							
493	M2	1	0	0	1	1	1	0	0	0		0	0	0							
494	M2	1	0	0	1	0	0	0	0	1		0	0	0							
495	M2	1	0	0	1	0	1	0	0	1		0	0	0							
496	M2	1	0	0					0												
497	M2	1	0	0	1	1	1	0	0	1		0	0	0							
498	M2	1	0	0																	
499	M2	1	0	0																	
500	M2	1	0	0																	
501	M2	1	0	0	1	0	1	0	0	0		0	0	0		0					
502	M2	1	0	0	1	0	0	1	1	0		0	0	0							
503	M2	1	0	0	1	0	0	0	0	0		0	0	0							
504	M2	1	0	0	1	0	0	0	0	0		0	0	0							
505	M2	1	0	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
506	M2	1	0	0	1	0	0	0	0	1		0	0	0							
507	M2	1	0	0	1	0	0	0	0	1		0	0	0							
508	M2	1	0	0																	
509	M2	1	0	0																	
510	M2	1	0	0	0	1	0	0	0												
511	M2	1	0	0	1	0	0	0	0												
512	M2	1	0	0			0	0	0												
513	M2	1	0	0	1	0	0	0	0	0		0	0	0							
514	M2	1	0	0	1	0	0	0	0	0		0	0	0							
515	M2	1	0	0	1	0	0	0	0	0											
516	M2	1	0	0																	
517	M2	1	0	0																	
518	M2	1	0	0	1	0	0	0	0	0											
519	M2	1	0	0	1	0	0	0	0	0											
520	N1	0	0	0	0	1	2	0	0	1		0	0	0	0		0	0	4110004		unacceptable
521	N1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
522	N1	0	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
523	N1	0	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
524	N1	0	0	0	1	0	0	0	0	1		0	0	0	0	00	0	0			
525	N1	0	0	0	2	0	0	0	0	1		0	0	0	0	0	0	0	1200004		*Moraxella phenylpyruvica*
526	N1	0	0	0	0	1	1	0	0	0		1	1	1	0	0	0	0		1201100000	*Ps.stutzer/A.lwoffii*
527	N1	0	0	0	1	0	2	0	0	1		1	1	1			0	0	0000000		no growth
528	N1	0	0	0	1	0	0	0	0	1		1	1	1							
529	N1	0	0	0	1	0	1	0	0	1		1	1	1						0000000000	no growth
530	N1	0	0	0	0	1	2	0	0	1		0	0	0	0	00	0	0	4110004		unacceptable
531	N1	0	0	0	1	0	0	0	0	1		0	0	0	0	0	0	0			
532	N1	0	0	0	0	1	2	0	0	1		0	0	0	0	0	0	0	10004		*Weeksella virosa*
533	N1	0	0	0	0	1	2	0	0	1		1	0	0	0	0	0	0	110004		*Weeksella virosa*
534	N1	0	0	0	2	0	1	0	0	1		2	0	0	0	00	0	0		2301100000	unacceptable
535	N1	0	0	0	1	0	2	0	0	1		2	0	0		0					
536	N1	0	0	0	0	1	2	0	0	1		2	0	0	0		0	0	4210004		*Weeksella zoohelicum*
537	N1	0	0	0	2	0	2	0	0	1		2	0	0	0		0	0	4320004		*Listonella damsella*
538	N1	0	0	0	0	1	0	0	0	1		1	0	0	0		0	0	4100004		*Listonella damsella*
539	N1	0	0	0	1	0	0	0	0	1		0	0	0	0		0	1			
540	N1	0	0	0	1	0	0	0	0	0											
541	N1	0	0	0	0	1	2	0	0	1		0	0	0	0		0	0	4310004		*Listonella damsella*
542	N1	0	0	0	0	1	2	0	0	0									4300000		*Listonella damsella*
543	N1	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
544	N1	0	0	0	0	1	0	0	0	1									4700004		*Listonella damsella*
545	N1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		0000000000	no growth
546	N1	0	0	0	2	0	0	0	0	0		0	0	0	0		0	0			

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
547	N1	0	0	0	1	0	0	0	0	0		1	0	0	0		0	0			
548	N1	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0	4010004		
551	R1	0	0	0	1	0	0	0	0	0		0	0	0							
552	R1	0	0	0	1	0	1	0	0	0											
553	R1	0	0	0	1	0	1	0	0	1		0	0	0							
554	R1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		3321120013	*Sphingomonas paucimobilis*
555	R1	0	0	0	2	0	1	0	0	0	0	0	0	0	0		0	0		7365520240	unacceptable
556	R1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		7310000000	unacceptable
557	R1	0	0	0	0	0	1	0	0	1		0	0	0	0		0	1		3302000000	*Pseudomonas vesicularis*
558	R1	0	0	0	1	0	0	0	0	1											
559	R1	0	0	0	0	0	1	0	0	1		0	0	0	0		0	0		3332022300	unacceptable
560	R1	0	0	0	2	0	1	0	0	1		0	0	0	1		0	0		2321100000	*Sphingobacterium multivorum*
561	R1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0	4100400		*Pasteurella sp.*
562	R1	0	0	0	1	0	0	0	0	1		0	0	0							
563	R1	0	0	0	1	0	1	0	0	1		0	0	0							
564	R1	0	0	0																	
565	R1	0	0	0																	
566	R1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		3221100000	unacceptable
567	R1	0	0	0	1	0	1	0	0	1		0	0	0							
568	R1	0	0	0	1	0	1	1	0	1		0	0	0							
569	R1	0	0	0	1	0	1	1	1	0		0	0	0							
570	R1	0	0	0	1	0	1	1	1	1		0	0	0							
571	R1	0	0	0	2	0	2	1	1	0		0	0	0	0		0	0	4000000		*Acinetobacter lwoffii*
572	R1	0	0	0	1	0	0	1	1	0		0	0	0							
573	R1	0	0	0	1	0	0	1	1	1		0	0	0							
574	R1	0	0	0	1	0	0	1	1	1		0	0	0							
575	R1	0	0	0	1	0	0	1	1	1		0	0	0	0		0	0			
576	R1	0	0	0	1	0	0	1	1	1		0	0	0							
577	R1	0	0	0	0	1	2	0	0	1		0	0	0	0		0	0	6340204		*Listonella damsella*
578	R1	0	0	0	1	0	0	1	1	1		0	0	0							
579	R1	0	0	0	1	0	1	0	0	1		0	0	0							
580	R1	0	0	0	1	0	0	0	0	1		1	0	1							
581	D1	0	0	0	0	1	2	0	0	0					0		0	0	0000000		no growth
582	D1	0	0	0	0	1	0	0	0	1					0		0	0			
583	D1	0	0	0	0	1	2	0	0	1											
584	D1	0	0	0	0	1	2	0	0	0											
585	D1	0	0	0	0	1	1	0	0	1					0		0	0		0000000000	no growth
586	D1	0	0	0	0	1	2	0	0	1					0		0	0	0000000		no growth
587	D1	0	0	0	0	1	0	0	0	1					0		0	0			
588	D1	0	0	0	0	1	0	0	0	1					0		0	0			
589	D1	0	0	0	0	1	0	0	0	1					0		0	0			

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
590	D1	0	0	0	0	1	0	0	0	1					0		0	0			
591	D1	0	0	0	0	1	0	0	0	1					0		0	0			
592	D1	0	0	0	0	1	0	0	0	1					0		0	0			
593	D1	0	0	0	1	0	0	0	0	0											
594	D1	0	0	0	0	1	0	0	0	0					0		0	0			
595	D1	0	0	0	0	1	0	0	0	1					0		0	0			
596	D1	0	0	0	0	0	0	0	0	1											
597	D1	0	0	0	0	0	0	0	0	1											
598	D1	0	0	0	0	1	0	0	0	1											
599	D1	0	0	0	0	0	0	0	0	1											
600	D1	0	0	0	0	0	0	0	0	1											
601	D1	0	0	0	0	0	0	0	0	0											
602	D1	0	0	0	0	1	0	0	0	1					0		0	0			
603	D1	0	0	0	0	1	0	0	0	0					0		0	0			
604	D1	0	0	0	0	1	0	0	0	1					0		0	0			
605	D1	0	0	0																	
606	D1	0	0	0																	
610	D1	0	0	1	0	1	2	0	0	1					0		0	0	0000000		no growth
611	D1	0	0	1	0	1	0	0	0	1					0		0	0			
612	D1	0	0	1	0	1	0	0	0	1					0		0	0			
613	D1	0	0	1	0	1	0	0	0	1					0		0	0			
614	D1	0	0	1	0	1	1	0	0	1					0		0	0		0000000000	no growth
615	D1	0	0	1	0	1	0	0	0	1					0		0	0			
616	D1	0	0	1	0	1	0	0	0	1					0		0	0			
617	D1	0	0	1	0	1	0	0	0	1					0		0	0			
618	D1	0	0	0	0	1	0	0	0	1					0		0	0			
620	D1	0	0	1	0	1	0	0	0	1					0		0	0			
621	D1	0	0	1	0	1	0	0	0	1					0		0	0			
623	D1	0	0	1	0	1	0	0	0	0					0		0	0			
624	D1	0	0	1	0	1	1	0	0	1					0		0	0			
626	D1	0	0	1	0	1	1	0	0	1					0		0	0		0000000000	no growth
627	D1	0	0	1	0	1	0	0	0	1					0		0	0			
629	D1	0	0	1	0	1	0	0	0	1					0		0	0			
630	D1	0	0	1	0	1	0	0	0	1					1		1	1			
631	D1	0	0	1	0	1	0	0	0	1					0		0	0			
637	D1	0	1	1	1	0	1	1	1	0											
638	D1	0	1	1	0	1	0	0	0	1					0		0	0			
639	D1	0	1	1	0	0	0	0	0	1											
640	D1	0	1	1	0	1	0	0	0	1					0		0	0			
641	D1	0	1	1	1	0	0	0	0	1											
642	D1	0	1	1	0	1	0	0	0	1					0		0	0			
643	D1	0	0	1	0	1	0	0	0	0					0		0	0			

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
644	D1	0	1	0	0	1	1	1	1	0					0		0	0		0000000000	no growth
664	D1	0	1	1																	
665	D1	0	1	0	0	1	0	0	0	0					0		0	0			
666	D1	0	1	0	0	1	0	0	0	1					0		0	0			
667	D1	0	1	0	0	0	0	0	0	0											
669	D1	0	1	0	0	1	0	0	0	1					0		0	0			
670	D1	0	1	0	1	1	0	0	0	1											
671	D1	0	1	0	0	1	0	0	0	1					0		0	0			
672	D1	0	1	0	0	1	0	0	0	1					0		0	0			
673	D1	0	1	0	1	0	0	0	0	1											
674	D1	0	1	0	0	1	0	0	0	1					0		0	0			
675	D1	0	1	0	0	1	0	0	0	1					0		0	0			
676	D1	0	1	0	0	1	0	0	0	1					0		0	0			
677	D1	0	1	0	1	1	0	0	0	1											
678	D1	0	1	0	0	1	0	0	0	1					0		0	0			
679	D1	0	1	0	0	1	0	0	0	1					0		0	0			
680	D1	0	1	0	0	1	0	0	0	1					0		0	0			
681	D1	0	1	0	0	1	0	0	0	1					0		0	0			
682	D1	0	1	0	0	1	0	0	0	1					0		0	0			
683	D1	0	1	0	0	1	0	0	0	1					0		0	0			
685	D1	0	1	0	0	1	1	0	0	0					0		0	0		0000000000	no growth
686	D1	0	1	0	0	1	0	0	0	1					0		0	0			
688	D1	0	1	0	0	1	0	0	0	1					0		0	0			
689	D1	0	1	0	0	1	0	0	0	1					0		0	0			
690	D1	0	1	0	0	1	0	0	0	0					0		0	0			
691	D1	0	1	0	0	1	0	0	0	0					0		0	0			
692	D1	0	1	0	0	1	0	0	0	1					0		0	0			
695	D2	1	0	0	0	1	0	0	0	0					0		0	0			
696	D2	1	0	0	0	1	0	0	0	0					0		0	0			
697	D2	0	1	0	0	1	0	0	0	0					0		0	0			
698	D2	1	0	0	0	0	0	0	0	0											
699	D2	1	0	0	0	0	0	0	0	0											
731	D2	1	0	1	0	1	1	0	0	0					0		0	0		0000000000	no growth
732	D2	1	0	1	0	1	0	0	0	0					0		0	0			
743	D2	1	0	1	1	0	1	0	0	0											
746	D2	1	0	1	0	1	0	0	0	0					0		0	0			
754	D2	1	0	1	0	1	0	0	0	0					0		0	0			
755	D2	1	1	0	0	1	1	0	0	0					0		0	0			
756	B1	0	0	0	1	0	0	0	0	1		0	0	0							
757	B1	0	0	0	1	0	0	0	0	0		0	0	0							
758	B1	0	0	0	1	0	0	0	0	0		0	0	0							
759	B1	0	0	0	1	0	0	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
760	B1	0	0	0	1	0	0	0	0	0		0	0	0							
761	B1	0	0	0	1	0	0	0	0	0		0	0	0							
762	B1	0	0	0	1	0	0	0	0	1		0	0	0							
763	B1	0	0	0	1	0	0	0	0	0		0	0	0							
764	B1	0	0	0	1	0	0	0	0	0		0	0	0							
765	B1	0	0	0	1	0	0	0	0	1		0	0	0							
766	B1	0	0	0	1	0	0	0	0	1		0	0	0							
767	B1	0	0	0	1	0	0	0	0	0		0	0	0							
768	B1	0	0	0	1	0	0	0	0	0		0	0	0							
769	B1	0	0	0	1	0	0	0	0	0		0	0	0							
770	B1	0	0	0	1	0	0	0	0	1		0	0	0							
771	B1	0	0	0	1	0	0	0	0	1		0	0	0							
772	B1	0	0	0	1	0	0	0	0	1		1	0	0							
773	B1	0	0	0	1	0	0	0	0	1		1	0	0							
774	B1	0	0	0	1	0	0	0	0	0		0	0	0							
775	B1	0	0	0	1	0	1	0	0	1		0	0	0							
776	B1	0	0	0	1	0	0	0	0	1		0	0	0							
777	B1	0	0	0	1	0	0	0	0	1		0	0	0							
778	B1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0			
779	B1	0	0	0	1	0	0	0	0	0		0	0	0							
780	B1	0	0	0	1	0	1	0	0	0		0	0	0							
781	B1	0	0	0	1	0	0	0	0	1		0	0	0							
782	B1	0	0	0	1	0	0	0	0	1		0	0	0							
783	B1	0	0	0	1	0	0	0	0	1		0	0	0							
784	B1	0	0	0	1	0	0	0	0	1		0	0	0		0					
785	B1	0	0	0	0	1	2	0	0	1		0	0	0	0	0	0	0	4240244		*Listonella damsella*
786	B2	1	1	1	1	0	1	1	1	1		1	1	0	0	11	0	0		6763306211	unacceptable
787	B2	1	1	1	1	0	1	1	1	0		1	0	0							
788	B2	1	1	1	1	0	1	1	1	0		1	0	1		0					
789	B2	1	1	1	2	0	1	1	1	1		0	1	1	0	0	0	0		771106440	unacceptable
790	B2	1	1	1	0	1	1	1	1	1		1	1	0	0	11	0	0			
791	B2	1	1	1	0	1	1	1	1	0		1	1	0	0	00	0	0			
792	B2	1	1	1	1	0	0	0	0	0		0	0	0							
793	B2	1	1	1	0	1	0	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
794	B2	1	0	1	0	1	1	1	1	1		1	1	1	0		0	0		3131102200	unacceptable
795	B2	1	0	1	0	1	2	0	0	1		0	0	0	0		0	0	4500004		*Listonella damsella*
796	B2	1	0	1	0	1	0	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
797	B2	1	0	1	1	0	1	1	1	0		1	1	1		1					
798	B2	1	0	1	1	0	2	0	0	0		0	0	0							
799	B2	1	0	1	1	0	0	0	0	0		0	0	0							
800	B2	1	0	1	1	0	0	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
801	B1	0	0	1	1	0	1	0	0	1		1	1	1		0					
802	B1	0	0	1	0	1	1	1	1	1		0	0	0	0		0	0		0000000000	no growth
803	B1	0	0	1	1	0	1	0	0	1		1	1	1							
804	B1	0	0	1	1	0	1	0	0	0		1	1	1							
805	B1	0	0	1	1	0	1	0	0	1		0	0	1							
806	B1	0	0	1	1	0	1	0	0	1		0	0	1							
807	B1	0	0	1	1	0	1	0	0	1		0	1	0							
808	B1	0	0	1	1	0	0	0	0	1		0	0	0							
809	B1	0	0	1	2	0	0	0	0	0		0	0	0							
810	B1	0	0	1	2	0	1	1	1	1		0	0	0							
811	B1	0	0	1	0	1	1	0	0	1		0	0	0	0		0	0		5601100024	unacceptable
812	B1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	5100004		*Listonella damsella*
813	B1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	4640044		*Listonella damsella*
814	B1	0	0	1	0	1	2	0	0	1		1	1	1	1	0	0	0	4440244		*Pseudomonas vesicularis*
815	B1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	4100044		*Listonella damsella*
816	B1	0	0	1	0	1	0	0	0	1		0	0	0	0		0	0	500044		*Pseudomonas vesicularis*
817	B1	0	0	1	0	1	0	0	0	1		0	0	0	0		0	0	500044		*Pseudomonas vesicularis*
818	B1	0	0	1	0	1	0	0	0	1		0	0	0	0		0	0	4500004		*Listonella damsella*
819	B1	0	0	1	0	1	2	0	0	1		1	0	0	0		0	0	4563204		*Sphingobacterium multivorum*
820	B1	0	0	1	0	1	0	0	0	1		1	0	0	0		0	0	4350204		*Listonella damsella*
821	B1	0	0	1																	
822	B1	0	0	1	1	0	0	0	0	1		2	2	2		0					
823	B1	0	0	1	1	1	0	0	0	1		2	2	2							
824	B1	0	0	1	1	1	1	0	0	1		0	0	0							
825	B1	0	0	1	1	0	1	1	1	1		0	2	2							
826	B1	0	0	1	0	1	2	0	0	1		0	0	0	0		0	0	4100004		*Listonella damsella*
827	B1	0	0	1	1	0	0	0	0	1		0	0	0							
828	B1	0	1	0	1	0	1	0	0	0		0	0	0							
829	B1	0	1	0	1	0	1	1	1	0		0	0	0	0	0	0	0			
830	B1	0	1	0	0	1	1	1	1	1		0	0	0	0		0	0		4000140000	unacceptable
831	B1	0	1	0																	
832	B1	0	1	0	1	0	0	0	0	0		0	0	0							
833	B1	0	1	0	1	0	0	0	0	1		0	0	0							
834	B1	0	1	0	1	0	0	0	0	1		0	0	0							
835	B1	0	1	0	0	1	0	0	0	1		0	0	0	0		0	0	4500004		*Listonella damsella*
836	B1	0	1	0	0	1	0	0	0	1		0	0	0	1						
837	B1	0	1	0	1	0	0	0	0	0		0	0	0							
839	B1	0	1	0	1	0	0	0	0	0											
840	B1	0	1	0	1	0	0	0	0	0											
841	B1	0	1	0	1	0	0	0	0	0											
842	B1	0	1	0	1	0	0	0	0	0		0	0	0							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
843	B1	0	1	0	1	0	0	0	0	0		0	0	0							
844	B1	0	1	0	1	0	1	0	0	1		0	0	0							
845	B1	0	1	0	1	0	1	0	0	0		0	0	0							
846	B1	0	1	0	1	0	1	0	0	0		2	2	2							
847	B1	1	0	0	1	0	0	0	0	1		0	0	0							
848	B2	1	0	0	1	0	1	0	0	0		2	2	2							
849	B2	1	0	0	1	0	1	0	0	1		2	2	2							
850	B2	1	0	0	1	0	1	0	0	1		0	0	0							
851	B2	1	0	0	1	0	0	0	0	1		0	0	0							
852	B2	1	0	0	1	0	0	0	0	1		0	0	0							
853	B2	1	0	0	1	0	0	0	0	0		0	2	0							
854	B2	1	0	0	1	0	0	0	0	0		0	2	0							
855	B2	1	0	0	1	0	0	0	0	0		0	0	0							
856	B2	1	0	0	2	0	0	0	0	0		0	0	0							
857	B2	1	0	0	2	0	0	0	0	0		0	2	0							
858	B2	1	0	0	2	0	0	0	0	1		0	0	0							
859	B2	1	0	0	2	0	0	0	0	1		0	0	0							
861	B2	1	0	0	2	0	1	0	0	0		2	2	2	0	0	0	0		3363300100	*Flavobacterium meningosepticum*
862	B2	1	0	0	1	0	1	1	0	0		0	2	0	0		0	0		0000000000	no growth
863	B2	1	0	0	1	0	0	0	0	1		0	0	0	0		0	0			
864	B2	1	0	0	1	0	0	0	0	1		0	0	0							
865	B2	1	0	0	1	0	0	0	0	0		0	0	0							
866	B2	1	0	0	1	0	1	0	0	1		0	0	0							
867	B2	1	0	0	1	0	0	0	0	0		0	0	0							
868	S2	1	0	0	1	0	1	1	1	0											
869	S2	1	0	0																	
870	S2	1	0	0	1	0	0	0	0	1											
871	S2	1	0	0	1	1	0	0	0	0											
872	S2	1	0	0	1	1	1	0	0	1											
873	S2	1	0	0	0	1	2	0	0	1					0		0	0	5577741		*Neisseria cinerea*
874	S2	1	0	0	0	1	1	1	1	0					0		0	0		0000000000	no growth
875	S2	1	0	0	0	1	1	1	1	1										0000000000	no growth
876	S2	1	1	0																	
877	S2	1	0	0																	
878	S2	1	0	0	1	0	1	0	0	1											
879	S2	1	0	0																	
880	S2	1	0	0																	
881	S2	1	0	0																	
882	S2	1	0	0																	
883	S2	1	0	0																	
884	S2	1	0	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
885	S2	1	0	0	1	0	1	0	0	1											
886	S2	1	0	0																	
887	S2	1	0	0	2	0	1	1	1	1											
888	S2	1	0	0	1	0	0	0	0	0											
889	S2	1	0	0																	
890	S2	1	0	0	1	0	1	0	0	0											
891	S2	0	0	0	1	0	1	0	0												
892	S2	1	0	0																	
893	S2	1	0	0																	
894	S2	1	0	0																	
895	S2	1	0	0																	
896	S2	1	0	1	0	1	1	1	1	1					0		0	0		221124002	*Agrobacterium tumefaciens*
897	S2	1	0	1	1	1	0	1	1	1											
898	S2	1	0	1																	
899	S2	1	0	1																	
900	S2	1	0	1																	
901	S2	1	0	1																	
902	S2	1	0	1																	
903	S2	1	0	1																	
904	S2	1	0	1	1	0	0	0	0	1											
905	S2	1	0	1					0												
906	S2	1	0	1					0												
907	S2	1	0	1	1	1	1	1	1	0											
908	S2	1	0	1																	
909	S2	1	0	1	1	0	1	0	0	0											
910	S2	1	0	1	1	1	0	0	0	0											
911	S2	1	0	1	1	0	0	0		0											
912	S2	1	0	1	1	0	0														
913	S2	1	0	1																	
914	S2	1	0	1	1	0	1	1	1	0											
915	S2	1	0	1																	
916	S2	1	0	1	1	0	1	0	0	0											
917	S2	1	0	1	0	1	0	0	0	0					0		0	0			
918	S2	1	0	1																	
919	S2	1	0	1																	
920	S2	1	0	1	1	0	1	0	0	0											
921	S2	1	0	1																	
922	S1	0	0	0	2	1	1	0	0	0					0		0	0		2301311012	*Xanthomonas maltophilia*
923	S1	0	0	0	0	1	2	1	1	0					0		0	0	477741		*Neisseria cinerea*
924	S1	0	0	0	0	1	1	0	0	0					0		0	0		1301310010	*Xanthomonas maltophilia*
925	S1	0	0	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
926	S1	0	0	0	0	1	1	0	0	1										1320000	*Acinetobacter lwoffii*
927	S1	0	0	0	1	0	0	0	0	0											
928	S1	0	0	0	1	0	1	1	1	0											
929	S1	0	0	0	1	0	1	0	0	1											
930	S1	0	0	0	1	0	1	0	0	0											
931	S1	0	0	0																	
932	S1	0	0	0	1	0	0	0	0	0											
933	S1	0	0	0	0	1	1	0	0	1					0		0	0			
934	S1	0	0	0	0	1	2	0	0	1					0		0	0	1000004		*Moraxella sp.*
935	S1	0	0	0																	
936	S1	0	0	0	1	0	1	0	0	0											
937	S1	0	0	0																	
938	S1	0	0	0	0	1	0	1	1	0					0		0	0		1100100	Misc Gram neg bacillus
939	S1	0	0	0	0	1	0	0	0	0											
940	S1	0	0	0	0	1	0	0	0	1											
942	S1	0	0	0	0	1	0	0	0	1											
943	S1	0	0	0	1	0	1	0	0	0											
944	S1	0	0	0				0	0	0											
945	S1	0	0	0				0	0	0											
946	S1	0	0	0				0	0	0											
947	S1	0	0	0				0	0	0											
948	S1	0	0	0				0	0	0											
949	S1	0	0	0				0	0	0											
950	S1	0	0	1	1	0	1	0	0	0											
951	S1	0	0	1	1	0	1	0	0	0											
952	S1	0	0	1	0	0	0	0	0	1											
953	S1	0	0	1	1	0	1	1	1	1											
954	S2	1	1	1	1	0	1	0	0	1											
955	S2	1	1	1	1	0	1	0	0	1											
956	S2	1	1	1																	
957	S2	1	1	1																	
958	S2	1	1	1																	
959	S2	1	1	1																	
960	S2	1	1	1																	
961	S2	1	1	1																	
962	S2	1	1	1																	
963	S2	1	1	1																	
964	S2	1	1	1																	
965	S2	1	1	0	1	1	1	0	0	1											
966	S2	1	1	0																	
967	S2	1	1	0	1	0	1	0	0	1											

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
968	S2	1	1	0																	
969	S2	1	1	0																	
970	S2	1	1	0																	
971	S2	1	1	0																	
972	S2	1	1	0	1	0	1	0	0	1											
973	S2	1	1	0																	
974	S2	1	1	0	1	0	1	0	0	1											
975	S2	1	1	0	1	0	0	0	0	0											
976	S2	1	1	0	1	0	1	1	1	1											
977	S2	1	1	0																	
978	S2	1	1	0																	
979	S2	1	1	0																	
980	S2	1	1	0	1	1		0	0	0											
981	S2	1	1	0																	
982	S2	1	1	0																	
983	S2	1	1	0	1	0	1	0	0	1											
984	S2	1	1	0	1	0	1	0	0	1											
985	S2	1	1	0																	
986	S2	1	1	0																	
987	S2	1	1	0	1	0	1	0	0	0											
988	S2	1	1	0																	
989	S2	1	1	0																	
990	S2	1	1	0																	
991	S2	1	1	0																	
992	S2	1	1	0																	
993	S2	1	1	0	1	0															
994	S2	1	1	0																	
995	S1	0	1	1	0	1	2	0	0	1					0		0	0	0000000		no growth
996	S1	0	1	1																	
997	S1	0	1	1	0	1	1	1	1	0					0		0	0		0000000000	no growth
998	S1	0	1	1	0	1	1	0	0												
999	S1	0	1	1	0	1	1	0	0						0		0	0		0000000000	no growth
1000	S1	0	1	0	1	0	1	0	0	0											
1001	S1	0	1	0	1	0	0	0	0	0											
1002	S1	0	1	0																	
1003	S1	0	1	0																	
1004	S1	0	1	0																	
1005	S1	0	1	0																	
1007	S1	0	1	0																	
1008	S1	0	1	0	1	0	1	0	0	0											
1009	S1	0	1	0	1	0	1	0	0	0											
1010	S1	0	1	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1011	S1	0	1	0																	
1012	S1	0	1	0	1	0	0	0	0	0											
1013	S1	0	1	0																	
1014	S1	0	1	0	0	1	0	0	0	0					0		0	0			
1015	S1	0	1	0	1	0	0	0	0	1											
1016	S1	0	1	0	1	0	1	0	0	0											
1017	S1	0	1	0	1	1	1	0	0	0											
1018	S1	0	1	0																	
1019	S1	0	1	0																	
1020	S1	0	1	0																	
1021	S1	0	1	0	1	0	0	0	0	0											
1022	G2	1	0	0	1	0	1	0	0	1											
1023	G2	1	0	0	1	0	0	0	0	0											
1024	G2	1	0	0	0	1	0	0	0	1		0	0	0	1	0	0	0		320311010	*Pseudomonas stutzeri*
1025	G2	1	0	0	1	0	0	0	0	0											
1026	G2	1	0	0	1	0	0	0	0	0											
1027	G2	1	0	0	1	0	0	0	0	0											
1028	G2	1	0	0	1	0	0	0	0	0											
1029	G2	1	0	0	1	0	0	0	0	0											
1030	G2	1	0	0	1	0	0	0	0	1											
1031	G2	1	0	0	1	0	0	0	0	0											
1032	G2	1	0	0	1	0	1	0	0	1											
1033	G2	1	0	0	1	0	0	0	0	1											
1034	G2	1	0	0	1	0	0	0	0	1											
1035	G2	1	0	0	1	0	1	0	0	0											
1036	G2	1	0	0	1	0	2	0	0	0											
1037	G2	1	0	0	1	0	0	0	0	1											
1038	G2	1	0	0	1	0	0	0	0	0											
1039	G2	1	0	0	1	0	0	0	0	0											
1040	G2	1	0	0	1	0	0	0	0	0											
1041	G2	1	0	0																	
1042	G2	1	0	0	1	0	0	0	0	1											
1043	G2	1	0	0	1	0	0	0	0												
1044	G2	1	0	0	1	0	0	0	0	0											
1045	G2	1	0	0	1	0	0	0	0	0											
1046	G2	1	0	0	1	0	1	0	0	1											
1047	G2	1	0	0			1	0	0	0											
1048	G2	1	0	0	0	1	0	0	0	1					0		0	0			
1049	G2	1	0	0	0	1	0	0	0	0					0		0	0			
1050	G2	1	0	0	0	1	1	0	0	0					0		0	0		0000000000	no growth
1051	G2	1	0	0	1	0	1	0	0	0											

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1052	G2	1	0	1	0	1	0	0	0	0					0		0	0		0000000000	no growth
1053	G2	1	0	1	1	0	1	0	0	0											
1054	G2	1	0	1	1	0	0	0	0	1											
1055	G2	1	0	1	1	0	1	0	0	0											
1056	G2	1	0	1	0	1	1	0	0	1		0	0	0	0		0	0		1003110010	*Pseudomonas flourescens*
1057	G2	1	0	1	0	1	1	0	0	1											
1058	G2	1	0	1	0	1	1	0	0	1		0	0	0	0		0	0		1203110010	*Pseudomonas stutzeri*
1059	G2	1	0	1	0	1	1	0	0	0		0	0	0	0		0	0		1003100000	Misc Gram neg bacillus
1060	G2	1	0	1	1	1	0	0	0	1					0		0	0			
1061	G2	1	0	1	1	0	1	0	0	1											
1062	G2	1	0	1	1	0	1	0	0	1											
1063	G2	1	0	1	1	0	1	0	0	1											
1064	G2	1	0	1	0	1	1	0	0	1					0		0	0		0000000000	no growth
1065	G2	1	0	1	1	0	0	0	0	1											
1066	G2	1	0	1	1	0	1	0	0	1											
1067	G2	1	0	1	1	0	1	0	0	1											
1068	G2	1	0	1	0	1	2	0	0	1					0		0	0	1047750		*Ochrobacter anthropi*
1069	G2	1	0	1	0	1	2	0	0	1					0		0	0	1247754		*Ochrobacter anthropi*
1070	G2	1	0	1	1	0	2	0	0	0											
1071	G2	1	0	1	1	0	1	0	0	1											
1072	G2	1	0	1	1	0	1	0	0	1											
1073	G2	1	0	1	1	0	1	0	0	0											
1074	G2	1	0	1	0	1	1	0	0	0		0	0	0	0		0	0		3203110010	*Xanthomonas maltophilia*
1075	G2	1	0	1	1	0	1	0	0	1											
1076	G2	1	0	1	0	1	1	0	0	1		0	0	0	0		0	0		1003000000	Misc Gram neg bacillus
1077	G2	1	0	1	0	1	1	0	0	1		0	0	0	0		0	0		2323300000	*Sphingobacterium multivorum*
1078	G2	1	0	1	0	1	1	0	0	1					0		0	0		2323120202	*Sphingobacterium multivorum*
1079	G2	1	0	1	1	0	1	0	0	1											
1080	G2	1	0	1	0	1	1	0	0	1					0		0	0		0000000000	no growth
1081	G2	1	0	1	0	1	1	0	0												
1082	G2	1	1	0	0	1	0	0	0	1											
1083	G2	1	1	0	0	1	0	0	0	1											
1084	G2	1	1	0	0	1	1	0	0	1											
1085	G2	1	1	0	0	1	0	0	0	1					0		0	0	440121		*Sphingomonas paucimobilis*
1086	G2	1	1	0																	
1087	G2	1	1	0																	
1088	G2	1	1	0																	
1089	G2	1	1	0																	
1090	G2	1	1	0	1	0	1	1	1	0											
1091	G2	1	1	0																	
1092	G2	1	1	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1093	G2	1	1	0	1	0	1	0	0	0											
1094	G2	1	0	1	1	0	1	0	0	0											
1095	G2	1	1	0	1	0	0	0	0	1											
1096	G2	1	1	0																	
1097	G2	1	1	0																	
1098	G2	1	1	0																	
1099	G2	1	1	0	1	0	1	0	0	0											
1100	G2	1	1	0																	
1101	G2	1	1	0																	
1102	G2	1	1	0																	
1103	G2	1	1	0																	
1104	G2	1	1	0																	
1105	G2	1	1	0																	
1106	G2	1	1	0																	
1107	G2	1	1	0																	
1108	G2	1	1	0																	
1109	G2	1	1	0																	
1110	G2	1	1	0	1	0	0	0	0	0											
1111	G2	1	1	0	1	0	0	0	0	0											
1112	G2	1	1	1	0	1	0	0	0	1					0		0	0			
1113	G1	0	1	0																	
1114	G1	0	1	0																	
1115	G1	0	1	0																	
1116	G1	0	1	0																	
1117	G1	0	1	0																	
1118	G1	0	1	0																	
1119	G1	0	1	0	0	1	0	0	0	1					0		0	0			
1120	G1	0	1	0	1	0	0	0	0	1											
1121	G1	0	1	0	0	1	0	0	0	1					0		0	0			
1122	G1	0	1	0	1	0	0	0	0	1											
1123	G1	0	1	0																	
1124	G1	0	1	0																	
1125	G1	0	1	0																	
1126	G1	0	1	0																	
1127	G1	0	1	0																	
1128	G1	0	1	0	1	0	0	0	0												
1129	G1	0	1	0																	
1130	G1	0	1	0	0	1	2	0	0	1					0		0	0	411001		*Flavobacterium indologenes*
1131	G1	0	1	0	1	0	2	0	0	0					0		0	0	10000		*Weeksella virosa*
1132	G1	0	1	0	1	0	0	0	0	0											
1133	G1	0	1	0	0	1	2	0	0	0					0		0	0	10000		*Weeksella virosa*

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1134	G1	0	1	0	1	0	0	0	0	0											
1135	G1	0	1	0																	
1136	G1	0	1	0	1	0	0	0	0	0											
1137	G1	0	1	0																	
1138	G1	0	1	0	0	1	0	0	0	0					0		0	0			
1139	G1	0	1	1	0	1															
1140	G1	0	1	1																	
1141	G1	0	1	1																	
1142	G1	0	1	1																	
1143	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1144	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1145	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1146	G1	0	0	1	1	0	0	0	0	0											
1147	G1	0	0	1																	
1148	G1	0	0	1																	
1149	G1	0	0	1																	
1150	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1151	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1152	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1153	G1	0	0	1																	
1154	G1	0	0	1	1	0	1	0	0	1											
1155	G1	0	0	1	0	1		0	0						0		0	0			
1156	G1	0	0	1	0	1	1	0	0	1					0		0	0			
1157	G1	0	0	1	1	0	0	0	0	1											
1158	G1	0	0	1	1	0	0	0	0	1											
1159	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1160	G1	0	0	1	0	1	2	0	0	1					0		0	0			
1161	G1	0	0	1	0	1	0	0	0	1					0		0	0			
1162	G1	0	0	1	1	0	0	0	0	0											
1163	G1	0	0	1																	
1164	G1	0	0	1	0	1	2	0	0	1					0		0	0			
1165	G1	0	0	1	0	1	0	0	0	0					0		0	0			
1166	G1	0	0	1																	
1167	G1	0	0	1	0	1	1	0	0	1					0		0			0000000000	no growth
1168	G1	0	0	1	1	0	0	0	0	0											
1169	G1	0	0	1																	
1170	G1	0	0	1	1	0	0	0	0	0											
1171	G1	0	0	1	1	0	0	0	0	0											
1172	G1	0	0	1																	
1173	G1	0	0	0																	
1174	G1	0	0	0																	
1175	G1	0	0	0																	

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1176	G1	0	0	0	0	1	2	0	0	0					0		0	0	450240		*Pseudomonas vesicularis*
1177	G1	0	0	0																	
1178	G1	0	0	0	0	1	0	0	0	0					1		0	0			
1179	G1	0	0	0	1	0	1	0	0	1											
1180	G1	0	0	0																	
1181	G1	0	0	0																	
1182	G1	0	0	0	1	0	1	0	0	1											
1183	G1	0	0	0																	
1184	G1	0	0	0																	
1185	G1	0	0	0																	
1186	G1	0	0	0	0	1	1	0	0	1		0	0	0	0		0	0		2331100000	*Sphingobacterium multivorum*
1187	G1	0	0	0	0	1	0	0	0	1											
1188	G1	0	0	0	0	1	0	0	0	0											
1189	G1	0	0	0	0	1	0	0	0	0		0	0	0	0		0	0		3301120000	*Flavobacterium gleum*
1190	G1	0	0	0	0	1	0	0	0	1					0		0	0		3201120000	*Flavobacterium breve*
1191	G1	0	0	0																	
1192	G1	0	0	0	0	1	0	0	0	1											
1193	G1	0	0	0	0	1	0	0	0	1											
1194	G1	0	0	0																	
1195	G1	0	0	0																	
1196	G1	0	0	0	1	0	1	0	0	0											
1197	G1	0	0	0	1	0	0	0	0	1											
1198	G1	0	0	0	0	1	0	0	0	1		0	0	0	0		0	0		2323100010	*Flavobacterium indologenes*
1199	G1	0	0	0																	
1200	G1	0	0	0	1	0	1	0	0	1											
1201	G1	0	0	0																	
1202	G1	0	0	0	1	0				0											
1203	B2	1	1	0	1	0	0	0	0	1		0	2	0							
1204	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	0	4100000		*Listonella damsella*
1205	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	0	100000		*Listonella damsella*
1206	B2	1	1	0	1	0	0	0	0	0		0	1	0							
1207	B2	1	1	0	1	0	0	0	0	0		0	1	0							
1208	B2	1	1	0	1	0	0	0	0	0		0	2	0							
1209	B2	1	1	0	2	0	2	0	0	0		1	1	1	0		0	0	4100000		*Listonella damsella*
1210	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1211	B2	1	1	0	2	0	0	0	0	0		0	0	0	0		0	0			
1212	B2	1	1	0	2	0	0	0	0	0		0	2	0	0		0	0			
1213	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1214	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1215	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1216	B2	1	1	0	1	0	0	1	0	0		1	1	1							

APPENDIX 4 (CONTINUED)

SERIAL	LABCODE	OCC	FLUSH	°C	GRAM	RODS	O/F	GAS	ACID	OXID	CAT	HS	HH	HM	VT	LT	ADHES	INV	API	BBL	IDENTIFICATION
1217	B2	1	1	0	1	0	1	0	0	0		1	1	1							
1218	B2	1	1	0	1	0	0	0	0	0		1	1	1							
1219	B2	1	1	0	1	0	0	1	0	0		1	1	1							
1220	B2	1	1	0	0	1	0	0	0	0		0	0	0	0		0	0	4500004		*Listonella damsella*
1221	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	0	4563204		*Sphingobacterium multivorum*
1222	B2	1	1	0	0	1	2	1	0	0		0	0	0	0		0	0	4350204		*Listonella damsella*
1223	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1224	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	1	4541260		*Chryseomonas luteola*
1225	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	0	4100004		*Listonella damsella*
1226	B2	1	1	0	2	0	0	0	0	0		0	0	0	0		0	0			
1227	B2	1	1	0	1	0	0	0	0	0		0	0	0	0		0	0			
1228	B2	1	1	0	1	0	0	1	0	0		2	2	2							
1229	B2	1	1	0	1	0	0	0	0	0		0	0	0							
1230	B2	1	1	0	0	1	2	0	0	0		0	0	0	0		0	0	4100004		*Listonella damsella*