

The University of Hull

Waterfowl, faecal indicators, and pathogenic bacteria in amenity ponds

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Summary of thesis submitted for the Degree of Doctor of Philosophy

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Waterfowl, faecal indicators, and pathogenic bacteria in amenity ponds

This study assessed the influence of waterfowl on the microbiological quality of village amenity ponds and the distribution of pathogenic bacteria in these ponds using both conventional and molecular genomic techniques.

To investigate the influence of waterfowl on the bacteriological quality of amenity ponds, two village ponds in East Yorkshire, one with abundant waterfowl and one without waterfowl, were sampled at monthly intervals from June 2001 to January 2002. A further five ponds (two with waterfowl and three without) were also sampled on three occasions between August and November 2001. *Escherichia coli*, faecal streptococci and to a degree *Clostridium perfringens*, were more abundant in the water and sediment of ponds with waterfowl. *Salmonella* was detected, during summer, in the sediment of a pond with waterfowl. There was a significant correlation between *E. coli* and the number of waterfowl. These results suggested that the microbiological quality of amenity ponds might be adversely affected by waterfowl. All water samples from ponds with waterfowl had faecal indicators at higher concentrations than EU requirements for bathing waters. Although these ponds are not used for bathing, skin contact and accidental ingestion of water should be avoided.

Problems associated with the detection of *Campylobacter* from amenity ponds with waterfowl were investigated. Water and sediment samples were collected from three ponds between August and December 2003. The analysis of three different volumes of water (10, 100 and 1000 ml) and three volumes of sediment (0.1, 1.0 and 5.0

ml) by enrichment culture showed that the filtration of a large volume of turbid pond water or the use of a large volume of sediment to assess the presence of campylobacters may be counterproductive and may not yield presumptive isolates due to competition by background microflora during enrichment culture. Thus pilot studies to establish appropriate volumes of environmental water or sediment samples should be undertaken before routine detection of *Campylobacter* is begun.

To test the hypothesis that waterfowl are a significant source of *Campylobacter* and *Salmonella* in amenity ponds, fresh duck faeces and samples of run-off water that flowed into the ponds were screened by conventional methods for the presence of these bacteria between August 2003 and January 2004. Both biochemical and morphological methods and PCR were used to confirm presumptive isolates. DNA sequencing of PCR products was used to type confirmed isolates. *Campylobacter* was isolated from both faeces and run-off water (as well as from water and sediment). Thus the faeces of waterfowl and rain-related run-off are both potential sources of campylobacters to the ponds. Different environmental *Campylobacter* species were, however, found to be of different origin: *Campylobacter jejuni* from duck faeces and *C. coli* from run-off. Furthermore, different ponds apparently had a different principal source of *Campylobacter* – faeces or run-off. *Salmonella*, in contrast, was not reliably isolated from duck faeces or run-off, nor this organism found in pond water, and only occasionally was it found in sediment.

The direct application of PCR to turbid pond water to detect naturally-occurring campylobacters was evaluated. The presence of the organism in small numbers with the presence of humic material and other PCR inhibitors, may have led to the negative results that were obtained. However, the use of a selective enrichment step followed by PCR facilitated the rapid detection of *Campylobacter*.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ
 أَقْرَأْ بِاسْمِ رَبِّكَ الَّذِي خَلَقَ ۝١ خَلَقَ الْإِنْسَانَ مِنْ عَلَقٍ ۝٢ أَقْرَأْ وَرَبُّكَ
 الْأَكْرَمُ ۝٣ الَّذِي عَلَّمَ بِالْقَلَمِ ۝٤ عَلَّمَ الْإِنْسَانَ مَا لَمْ يَعْلَمْ ۝٥

In the name of Allah the Most Gracious, the Most Merciful

1. Read! In the Name of your Lord Who has created (that all exists).
2. He has created man from a clot (a piece of thick coagulated blood).
3. Read! And your Lord is the Most Generous.
4. Who has taught (the writing) by the pen.
5. He has taught man which he knew not.

The Glorious Qura'an (Part 30, Chapter 96, Pp. 597)

TO MY PARENTS

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CHAPTER 1

GENERAL INTRODUCTION

Water pollution is a global concern. Fresh water in particular, which is essential to human life and well being, is becoming a limited resource due to the influence of population growth, pollution and global warming (Mason, 1996). Holdgate (1979) defined pollution as any substance or energy introduced to the environment by human activities that could have hazardous effects on human health, damage living resources and ecosystems, destroy the structure of, or interfere with the genuine use of the environment. Organic pollutants, particularly faeces, can cause severe consequences for the aquatic natural biota and may also be rich in disease-causing microorganisms (Mason, 1996). Faecal contamination of surface water can introduce a variety of pathogenic bacteria, viruses and protozoa into both open and closed aquatic environments. Water-related diseases remain a major hazard in many parts of the world. The World Health Organization (WHO) estimates suggested that 1.8 million people die every year from diarrhoeal diseases, 88 % of these diseases are attributed to unsafe water, inadequate sanitation and hygiene (WHO, 2004). The scale of the problem dramatically increases at times of crisis, especially in developing countries, for example between 28 February and 6 March 2005 there have been 304 reported cases of acute watery diarrhoea and 21 reported cases of bloody diarrhoea, in Banda Aceh, Indonesia, one of the areas that was greatly affected by the recent Indian Ocean earthquake-tsunami (WHO, 2005).

1.1 Water and Public Health

Microbiologically safe drinking and recreational water is a matter of major public health importance. Estimates indicate that 90 % of illnesses associated with the consumption of water are related to microbial agents, only 10 % are due to chemical agents (Theron and Cloete, 2002). Infections related to water may be classified into four groups (Gleeson and Gray, 1997; Hurst, 1997).

A - Waterborne diseases: this is where a pathogen is transmitted by ingestion of contaminated water. Cholera (*Vibrio cholerae*), typhoid fever (*Salmonella typhi*) and campylobacteriosis (*Campylobacter jejuni*) are important examples of waterborne diseases.

B - Water-washed diseases (i.e. transmitted during washing, sharing of washing water and towels): these include faecal-orally spread disease or disease spread from person to person by lack of adequate supply of water for washing. Many diarrhoeal diseases as well as diseases of the eye and skin, are transmitted in this way.

C - Water-based infection: these diseases are caused by pathogenic organisms that spend part of their life cycle in aquatic organisms. These include schistosomes and other trematode parasites which parasitise snails, and guinea worms.

D - Water-related diseases: caused by insect vectors which breed in water, such as mosquitoes which spread malaria and arthropods which carry yellow fever virus.

Amongst these groups, waterborne diseases are responsible for the majority of diarrhoea cases world-wide. The aetiology of waterborne diseases, however, varies between countries. In the United Kingdom, the most frequent aetiologies of diarrhoea cases that are attributed to contaminated water are viruses and *Campylobacter* (Stanwell-Smith, 1994). In the United States, however, *Giardia* and *Shigella* are the most identified aetiologies of waterborne diarrhoea (Gleeson and Gray, 1997). Although waterborne diseases such as cholera (*Vibrio cholerae*) and typhoid fever (*Salmonella*

typhi) have largely disappeared from developed countries (i.e. Europe and North America), they are still the major causes of waterborne diarrhoea in developing countries in Asia and Africa (Jones, 1994). The World Health Organization estimated that in poor countries in Asia and Africa there have been 5.5 million new cases of cholera per year with over 1.2×10^5 deaths annually (Jones, 1994). The most recognisable source of waterborne diarrhoea is contaminated water supplies. Diarrhoea infections can also be attributed to faecally-contaminated recreational waters.

1.1.1 Drinking water

In England and Wales during a period of 75 years (1911-1986) there were around 34 outbreaks of waterborne diseases associated with contaminated public water supplies (Galbraith, 1994). During this period, there was a decrease in the reported cases of waterborne typhoid fever (*S. typhi*) which totally disappeared after 1942. The number of waterborne outbreaks increased again in the 1980s when outbreaks associated with the newly recognised pathogens such as *Campylobacter* and *Cryptosporidium* began to be reported (Galbraith, 1994).

Campylobacter enteritis has lately been the main cause of waterborne outbreaks related to private water supplies in England and Wales, where water was not adequately disinfected (Furtado *et al.*, 1998; Said *et al.*, 2003). In Scandinavian countries, campylobacters are considered to be the most frequent bacterial agent in diarrhoea associated with drinking water supplies, for example, in Sweden there were six waterborne *Campylobacter* outbreaks between 1992-1996, with over 6000 people involved (Szewzyk *et al.*, 2000).

1.1.2 Recreational water

Various skin, ear and eye infections attributed to opportunistic bacterial pathogens that are native aquatic flora (e.g. *Pseudomonas* spp., *Aeromonas* spp. and *Mycobacterium avium*) are commonly related to the recreational use of surface waters.

Other pathogens such as *Staphylococcus* and *Acinetobacter*, which are part of the normal flora of the skin can be involved in skin, ear and eye infections associated with recreational waters (Rusin *et al.*, 2000). In addition, bathing in faecally-contaminated recreational waters poses a health risk if accidental ingestion of water occurs. This is not surprising since most diarrhoea infections require a small dose to cause infection. Specific threshold infective doses cannot be quoted because liability to infection is related to diverse factors as well as to dose (Blaser and Newman, 1982). However, as few as between 10^1 - 10^3 *Salmonella* cells can cause infection (Blaser and Newman, 1982), and less than 5×10^2 *Campylobacter* cells are required to cause enteritis (Schroeder and Wuertz, 2003). By and large, available evidence suggests that the most frequent adverse health outcome associated with exposure to faecally-polluted recreational water is gastrointestinal infection (WHO, 2003).

In the UK, typhoid fever (*S. typhi*) was the most common enteric illness associated with contaminated recreational waters, between 1939 and 1958 there were around 61 cases (out of 75 cases of typhoid) attributed to the ingestion of polluted recreational fresh water. Similarly, cases of typhoid fever were also reported following accidental drinking of contaminated sea water (Galbraith, 1994). Although waterborne typhoid fever associated with recreational waters has disappeared in the UK since 1958, cases of *Campylobacter* and viral enteritis attributed to ingestion of contaminated recreational waters have been frequently reported (Galbraith, 1994; Hunter, 1997). In the USA, most of the diarrhoea outbreaks associated with recreational water were attributed to *Shigella*, *E. coli* O157: H7, *Giardia* and viral agents (Hunter, 1997)

Medema *et al.* (1995) conducted a study on the relationship between the health of triathletes and microbiological quality of freshwater and found that 7.7 % of the participants in an Olympic distance triathlon (1-1.5 km swimming, 40 km cycling, 10 km running) (n = 314) reported gastrointestinal symptoms during or shortly after the

events as they accidentally ingested water from the swimming course. Microbiological analysis of water samples that were collected 17 days prior to the competition, from the swimming course (River Lek, the Netherlands) revealed the presence of *Campylobacter*, *Salmonella*, enteroviruses and other pathogenic microorganisms that are responsible for skin, eye, ear and respiratory infections.

1.2 Microbiological assessment of safe drinking and recreational water

The detection of bacterial, parasitic and viral intestinal pathogens in water is complex and not easily incorporated into routine monitoring (Geldreich, 1996). Since these pathogens are shed in the faeces of infected individuals and/or animals, the most logical alternative to pathogen analysis are indicators of proved correlation with faecal contamination in water (Geldreich, 1996). Many indicators of faecal contamination in water have been proposed: heterotrophic plate counts; total coliforms; faecal coliforms; *Escherichia coli*; faecal streptococci; *Bifidobacteria* and bacteriophages (Geldreich, 1996). Of these indicators, *E. coli* and faecal streptococci were found to be most predictive of the presence of diarrhoea agents in drinking water and in marine and fresh bathing waters (Dufour, 1984a; Cheung *et al.*, 1990; Moe *et al.*, 1991; Kay *et al.*, 1994; EPA, 2000).

In Cebu City, Philippines, the prevalence of diarrhoeal diseases associated with contaminated drinking water was shown to increase when *E. coli* count per 100 ml was greater than 10^3 (Moe *et al.*, 1991). In Hong Kong, a linear relationship was found between *E. coli* densities at marine bathing beaches and gastrointestinal illnesses in swimmers (Cheung *et al.*, 1990). Similarly, a good correlation was observed between swimming-associated gastrointestinal symptoms and either *E. coli* or faecal streptococci densities in various recreational freshwater sites in the USA and in the UK (Dufour, 1984a; Kay *et al.*, 1994). Medema *et al.* (1997) found a correlation between the

geometric mean density of *E. coli* and thermotolerant coliforms and risk of gastrointestinal illnesses in swimmers using seven freshwater courses in the Netherlands. These results have led to the conclusion that *E. coli* and faecal streptococci are good predictors of the risk of waterborne diarrhoeal diseases (Dufour, 1984a; Cheung *et al.*, 1990; Moe *et al.*, 1991; Kay *et al.*, 1994; EPA, 2000). The use of bacterial faecal indicators to assess the microbiological quality of recreational waters and the current guidelines for safe recreational waters will be further discussed in Chapter 4.

1.3 Bacterial enteropathogens associated with waterborne diseases

The commonly recognised bacterial waterborne pathogens consist of two groups, native aquatic bacteria and enteric bacteria. *Vibrio cholerae* and *Salmonella typhi* were the first enteric pathogens causing waterborne disease to be recognised, in the 19th century (Moe, 1997). In recent years, *Campylobacter* species have been considered as an important emerging aetiology of waterborne diseases world-wide, also traditional waterborne enteric bacteria such as *Salmonella* species (other than *S. typhi*) remain serious threats to water quality and public health.

1.3.1 *Campylobacter* species

1.3.1.1 Historical perspective

In 1886, Escherich described a spiral bacterium that was found in the faeces of children with diarrhoea, which could not be isolated by means of culture media (Griffiths and Park, 1990). A member of the genus *Campylobacter* was first described in 1913 by McFadyean and Stockman as the cause of reproductive tract infections in animals. It was classified within the genus *Vibrio* in 1919 and was known as *Vibrio fetus* (Ketley, 1997; Thomas *et al.*, 1999a; Percival *et al.*, 2004). In 1927, *Vibrio jejuni* was isolated from the jejunum of calves with diarrhoea, later *Vibrio coli* was isolated

from pigs with swine dysentery in 1944 (Percival *et al.*, 2004). Further taxonomic investigations of the genus *Vibrio* showed that *V. fetus*, *V. jejuni* and *V. coli* fundamentally differ from other vibrios, this led to the re-classification of the organisms as members of the newly proposed genus, *Campylobacter* (Ketley, 1997; Thomas *et al.*, 1999a). Despite being known as important veterinary pathogens for many years, *Campylobacter* was not recognised as a cause of diarrhoea in humans until the mid 1970s, when Skirrow (1977) successfully isolated the bacterium from stool samples of diarrhoea patients using solid culture media. Since then it has been recognised as the most common bacterial cause of human enteritis world-wide (Hunter, 1997).

1.3.1.2 Taxonomy and basic microbiology

The genus *Campylobacter* falls into Group Two of Bergey's Manual of Determinative Bacteriology, the aerobic/microaerophilic, motile, helical/vibrioid in shape Gram-negative bacteria (Holt *et al.*, 1994). The organism is slender, vibrioid in shape, 0.2-0.4 μm wide by 0.5-5 μm long. Cells appear as S-shape or gull-wing shaped; in old cultures they appear spherical. Cells are motile with a single, unsheathed polar flagellum at one or both ends of the cell. The organism is Gram negative, and for optimum growth it requires a microaerophilic atmosphere (O_2 3-15 %, CO_2 3-5 % and N_2 85 %) and a temperature of 42 °C. The organism is oxidase and catalase positive and urease negative. Members of the genus that are associated with human intestinal disease are *Campylobacter jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*. These are characterised by growth at 42 °C but not below 30 °C and are known as thermophilic campylobacters (Holt *et al.*, 1994; Ketley, 1997; Thomas *et al.*, 1999a; Percival *et al.*, 2004). *Campylobacter* species of medical importance and their characteristics are listed in Table 1.1.

1.3.1.3 Clinical features

Gastroenteritis or campylobacteriosis is characterised by watery diarrhoea, usually accompanied by abdominal cramping (Percival *et al.*, 2004). The disease varies from complete absence of symptoms to full acute colitis (Thomas *et al.*, 1999a). Diarrhoea and abdominal pain are the most common symptoms (Park *et al.*, 1991). *Campylobacter* gastritis usually starts with fever of 40 °C that lasts for up to two days (Griffiths and Park, 1990). This is followed by nausea, severe abdominal cramp, malaise and vomiting (Griffiths and Park, 1990; Koenraad *et al.*, 1997; Percival *et al.*, 2004). Diarrhoea is characterised by being watery or slimy and sometimes contains blood (Griffiths and Park, 1990; Park *et al.*, 1991). With cases of *Campylobacter* diarrhoea stools are often culture negative after three weeks (Percival *et al.*, 2004).

The incubation period is around 24 to 72 hours after ingestion, the duration of the infection is between 2 to 7 days (Griffiths and Park, 1990; Koenraad *et al.*, 1997). Infective dose is believed to be low, at less than 500 cells (Bitton, 1998; Schroeder and Wuertz, 2003). For up to two weeks, up to 10^6 to 10^8 campylobacters can be isolated from one gram of the faeces of an infected person (Koenraad *et al.*, 1997). Further complications can follow the infection. These may include meningitis, inflammation of the gall bladder, urinary tract infection, bacterimia, Reiter's syndrome (urethritis and arthritis) and Guillain-Barre' syndrome (reversible paralysis); fatalities are not likely to occur (Griffiths and Park, 1990; Park *et al.*, 1991; Koenraad *et al.*, 1997; Percival *et al.*, 2004).

1.3.1.4 Epidemiology

The genus *Campylobacter* includes 16 species (Vandamme, 2000), all of which are potential human or animal pathogens (Thomas *et al.*, 1999a). *Campylobacter jejuni* and *C. coli* are the most frequent disease-causing species in humans (Percival *et al.*, 2004). *Campylobacter jejuni* is responsible for up to 90 % of human campylobacteriosis

(Ketley, 1997). Other *Campylobacter* species, such as *C. upsaliensis*, *C. hyointestinalis* and *C. lari*, have been isolated from faeces of diarrhoea patients (Griffiths and Park, 1990; Vandamme, 2000), the pathogenic role of these species remains unclear (Ketley, 1997).

Sporadic *Campylobacter* infections usually take the faecal-oral route. Raw milk and eating undercooked meat, particularly poultry are important sources of infection (Griffiths and Park, 1990). Handling poultry, domestic pets and wild animals are secondary sources of infection (Koenraad *et al.*, 1997; Baker *et al.*, 1999). In contrast, large outbreaks of *Campylobacter*-associated enteritis are believed to occur through contaminated drinking water (Frost, 2001).

Table 1.1. *Campylobacter* species and features that differentiate them

Species	Oxidase	Catalase	Growth at			Hippurate hydrolysis	Aerobic growth	Nitrate reduction	H ₂ S	Sensitive to		TSI	Growth 1 % glycine
			25 °C	37 °C	42 °C					Cephalothin	NA		
<i>C. jejuni</i>	+	+	-	+	+	+	-	+	+	R	S	-	+
<i>C. coli</i>	+	+	-	+	+	-	-	+	+	R	S	-	+
<i>C. fetus</i>	+	+	+	+	(V)	-	-	+	V	S	R	-	+
<i>C. upsaliensis</i>	+	-/W	-	+	V	-	-	+	U	S	S	-	-
<i>C. cinaedi</i>	+	-	-	+	-	-	-	+	+	S	S	-	+
<i>C. lari</i>	+	+	-	+	+	-	-	+	+	R	R	-	+
<i>C. fennellia</i>	+	-	-	+	-	-	-	-	+	S	S	-	+
<i>C. hyointestinalis</i>	+	+	V	+	V	-	-	+	+	S	S	+	-

NA, Nalidixic acid; TSI, triple sugar iron agar

R, resistant; S, sensitive; V, variable; W, weak; U, unknown; (V), a few strains +

Source: modified from Park, *et al.* (1991) and Holt, *et al.* (1994)

1.3.1.5 Ecology

Campylobacter is believed to be widely distributed in the environment and has been reported as normal inhabitant in the intestinal tract of a wide range of birds and other animals (Stelzer *et al.*, 1991; Hunter, 1997; Jones, 2001; Percival *et al.*, 2004). Aquatic environments that receive faecal contamination should be regarded as potentially contaminated by *Campylobacter* (Leclerc *et al.*, 2004). Indeed, *Campylobacter* has been isolated from faecally-contaminated drinking water, rivers, lakes, ponds, drains, ground water and marine waters worldwide (Bolton *et al.*, 1987; Carter *et al.*, 1987; Mawer, 1988; Alnosno and Alnosno, 1993; Arvanitidou *et al.*, 1995; Stanley *et al.*, 1998; Obiri-Danso and Jones, 1999a; Obiri-Danso and Jones, 1999b; Rosef *et al.*, 2001; Eyles *et al.*, 2003; Hänninen *et al.*, 2003; Hörman *et al.*, 2004). *Campylobacter* species have also been isolated from contaminated freshwater and marine sediments (Obiri-Danso and Jones, 1999a; Obiri-Danso and Jones, 2000). Results from several studies showed that campylobacters are ubiquitous in sewage, both human and animal, and in waste from abattoirs and animal processing plants (Jones *et al.*, 1990a; Jones *et al.*, 1990b; Betaieb and Jones, 1990; Koenraad *et al.*, 1994; Baffone *et al.*, 1995).

The natural habitat of *Campylobacter* is the intestinal tract of mammals and wild birds, which are regarded as the main environmental reservoir of campylobacters (Koenraad *et al.*, 1997; Jones, 2001). *Campylobacter* has been isolated from the faeces and/or rectal swabs of cats and dogs (Baker *et al.*, 1999), poultry (Pearson *et al.*, 1996), livestock, e.g. cattle and sheep (Manser and Dalziel, 1985; Atabay and Corry, 1998), wild birds such as gulls, pigeons and crows (Kapperud and Rosef, 1983), waterfowl, i.e. ducks and geese (Luechtefeld *et al.*, 1980; Pacha *et al.*, 1987; Fallacara *et al.*, 2001; Aydin *et al.*, 2001), and from the general farm environment (Stanley and Jones, 2003). Birds seem to carry *Campylobacter* without obvious symptoms of diarrhoea

(Luechtefeld *et al.*, 1980; Kapperud and Rosef, 1983), this suggests that campylobacters inhabiting avian intestines are commensals (Park *et al.*, 1991; Waldenström *et al.*, 2002). This may be due to the high internal temperature of wild birds (42 °C), which is the optimum growth temperature of thermophilic campylobacters (i.e. *C. jejuni*, *C. coli*, *C. lari* and *C. upsaliensis*) (Waldenström *et al.*, 2002).

1.3.1.6 Environmental aspects

Campylobacter is exogenous to aquatic environments, thus its presence suggests recent faecal contamination (Jones, 2001). The abundance of campylobacters in aquatic environments is potentially strongly dependent on rainfall-related run-off and on the presence of waterfowl (Stelzer *et al.*, 1991). The high carriage rates of *Campylobacter* by domestic and wild animals and birds provide a continuous flow of large numbers of *Campylobacter* into the environment (Koenraad *et al.*, 1997). So far, there is no available evidence that thermophilic campylobacters are capable of growing in the environment, i.e. other than in the warm-blooded host or laboratory media (Park *et al.*, 1991; Thomas *et al.*, 1999a).

The presence of campylobacters in polluted environmental waters is marked with a strong seasonal pattern. The organism is frequently found in high number during late autumn and winter, while it is found in lower number or is absent during spring and summer; this phenomenon was reported from studies conducted on river water (Bolton *et al.*, 1987; Pianetti *et al.*, 1998; Obiri-Danso and Jones, 1999a), lake water (Carter *et al.*, 1987; Martikainen *et al.*, 1990), pond water (Mawer, 1988), and sea water (Alonso and Alonso, 1993; Obiri-Danso and Jones, 1999b). This seasonal variation is probably explained by the fact that campylobacters survive for long periods in water at low temperature (Thomas *et al.*, 1999b; Obiri-Danso *et al.*, 2001). In addition, it was proved that UV radiation has a lethal effect on *Campylobacter* in aquatic environments, thus a combination of high temperature and exposure to sunlight for prolonged periods may

explain, in part, the absence of campylobacters during the summer months (Obiri-Danso *et al.*, 2001).

Seasonal trends of *Campylobacter* have also been observed in sewage effluents. Interestingly, unlike in natural waters, May and June was the major peak time for the presence of campylobacters in sewers whereas there was a minor peak in September and October in NW England (Jones *et al.*, 1990a; Jones *et al.*, 1990b). This seasonality precisely coincided with the seasonal pattern of infection by *Campylobacter* in the human community, which was strongly linked to zoonotic infection (Jones *et al.*, 1990a). Also, in the UK, Louis, *et al.* (2005) observed a correlation between the seasonal pattern of *Campylobacter* infection and environmental factors, especially the presence of the organism in livestock. Recent studies on human campylobacteriosis in the UK confirmed that the annual seasonal peak of reported cases is in late June to early July (Miller *et al.*, 2004; Meldrum *et al.*, 2005; Louis *et al.*, 2005).

In a laboratory microcosm experiment, *Campylobacter* was shown to survive in water for up to four months at low temperature (4 °C) (Rollins and Colwell, 1986). High temperature, predation, lack of nutrients and UV radiation were found to influence adversely the survival of campylobacters in water microcosms (Rollins and Colwell, 1986; Korhonen and Martikainen, 1991; Thomas *et al.*, 1999b; Obiri-Danso *et al.*, 2001).

In order for *Campylobacter* to survive for long periods in the environment, it enters a viable but non-culturable stage (VBNC). This means that the organism remains viable by retaining basal metabolic activities, yet is unable to grow or multiply in microbiological culture media. This phenomenon is believed to be a survival strategy as a result of prolonged exposure to environmental stressors, such as nutrient deprivation, sub-optimal temperature, UV radiation, and biological interactions. When *Campylobacter* enters the VBNC stage, it was found to undergo physiological and

morphological changes (e.g. transforming from spiral to coccoid shape). Non-culturable coccal forms of *Campylobacter* were shown to cause infection in mice, and possibly humans, this property, however, may differ between strains (Xu *et al.*, 1982; Rollins and Colwell, 1986; Jones *et al.*, 1991; Thomas *et al.*, 1999a; Thomas *et al.*, 2002).

1.3.2 *Salmonella* species

1.3.2.1 *Historical perspective*

Diarrhoea due to *Salmonella* infections has been recognised since the late 19th century. Typhoid diseases were, in the early part of the 20th century, the commonest known waterborne diseases in both the UK and the USA (Hunter, 1997). In addition non-typhoid salmonellae have been recognised as a leading cause of bacterial enteritis in the UK and worldwide (Timbury *et al.*, 2002). *Salmonella* infections in animals are common and have been well documented in the UK since 1958, with around 10,000 recorded incidences of bovine salmonellosis per year (Linton and Hinton, 1988).

1.3.2.2 *Taxonomy and basic microbiology*

Salmonella species are members of the family *Enterobacteriaceae*, being facultatively anaerobic, non-spore forming, Gram-negative rods (Group five of Bergey's Manual of Determinative Bacteriology) (Holt *et al.*, 1994). Generally they are 2-5 µm long and 0.8-1.5 µm wide, straight rods, being motile by peritrichous flagella. As they are facultatively anaerobic, they have both respiratory and fermentative metabolism. Optimal growth temperature is 37 °C. D-Glucose and other carbohydrates are catabolised with the production of acid and usually gas. They are oxidase negative, catalase positive, indole and Voges-Proskauer negative, and methyl red and Simmons citrate positive. H₂S is produced; urea is not hydrolysed (Holt *et al.*, 1994; Lightfoot, 2004; Percival *et al.*, 2004).

The genus *Salmonella* consists of two species: (1) *Salmonella enterica*, which is divided into six subspecies – *S. enterica* subsp. *enterica* (I), *S. enterica* subsp. *salamae*

(II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI); and (2) *Salmonella bongori* (formerly subsp. V). There are around 2501 serovars/serotypes in the genus *Salmonella* (Table 1.2). This new nomenclature reflects recent advances in *Salmonella* taxonomy which are based on DNA-hybridization studies. For simplicity, serotypes can be abbreviated, for example *S. enterica* subsp. *enterica* serovar Enteritidis to *S. enteritidis* (Bopp *et al.*, 1999; Timbury *et al.*, 2002; Lightfoot, 2004; Percival *et al.*, 2004).

Table 1.2. Number of serovars in each species and subspecies of *Salmonella*

Species / subspecies	Number of serovars
<i>Salmonella enterica</i> subsp.	
<i>Enterica</i>	1478
<i>Salamae</i>	498
<i>Arizonae</i>	94
<i>diarizonae</i>	327
<i>Houtenae</i>	71
<i>Indica</i>	12
<i>Salmonella bongori</i>	
	21
Total	2501

Adapted from Lightfoot (2004)

Most of the serotypes pathogenic to humans and animals belong to *Salmonella enterica* subsp. *enterica* (i.e. subsp. I). Some serovars have a habitat limited to a particular host species, such as humans (serovars Typhi, Paratyphi A), sheep (serovars Abortusovis), or fowls (serovar Gallinarum). In general, subspecies I strains are usually isolated from humans and warm-blooded animals, whereas subspecies II, IIIa, IIIb, IV, VI and *S. bongori* are usually isolated from cold-blooded animals and the environment (rarely from humans). Biochemical reactions of *S. enterica* serovars and differential characteristics of *Salmonella* species and subspecies are given in Tables 1.3 and 1.4.

1.3.2.3 Clinical features

There are three clinically distinguishable forms of *Salmonella* infection in humans. These are gastroenteritis, enteric fever and septicaemia (Hunter, 1997; Percival *et al.*, 2004). Gastroenteritis is caused by *Salmonella enteritidis*, symptoms include watery, sometimes bloody diarrhoea, fever and abdominal pain. Symptoms usually occur 18-48 hours after ingestion of the bacterium. The infection generally lasts 2-5 days. After recovery, faecal carriage may persist for up to 12 weeks. Less than 10 % of patients are reported as carriers for a longer period (Hunter, 1997; Percival *et al.*, 2004).

Table 1.3. Biochemical reactions of *Salmonella enterica* serovars

	Typical <i>Salmonella</i>	<i>S. choleraesuis</i>	<i>S. pullorum</i>	<i>S. gallinarum</i>	<i>S. typhi</i>	<i>S. typhisuis</i>	<i>S. paratyphi A</i>
H ₂ S	+	-	±	±	+(weak)	+	-
Citrate	+	+	+	+	-	-	-
Gas from glucose	+	+	+	±	-	+	+
Dulcitol	+	-	-	+	-	+	+
Mucate	+	-	-	+	-	-	-
Maltose	+	+	-	+	+	+	+
Trehalose	+	-	+	+	+	+	+

From Jones, *et al.* (2000)

* Simmon's citrate-negative, Christensen citrate-positive.

Enteric fever is most often caused by *Salmonella typhi* (typhoid fever) and *S. paratyphi A, B and C* (paratyphoid fever). Enteric fever from *S. typhi* is more prolonged and has a higher mortality rate than paratyphoid fever. Symptoms for typhoid fever include sustained fever, diarrhoea, abdominal pain and may involve fatal liver, spleen, respiratory and neurological damage. Paratyphoid fever has similar, but less severe symptoms. The incubation period for typhoid fever is 7-14 days and for paratyphoid fever 1-10 days. Between 1 and 3 % of patients become chronic carriers (Hunter, 1997; Percival *et al.*, 2004).

Salmonella septicaemia is characterised by chills, high remittent fever, anorexia and bacteraemia. The bacterium may localise in any organ in the body and produce focal lesions resulting in meningitis, endocarditis and pneumonia (Percival *et al.*, 2004).

Studies aimed at the determination of the infectious dose for salmonellosis suggests that infectious doses are certainly below 10^3 and can be <10 organisms (Blaser and Newman, 1982; Hunter, 1997).

1.3.2.4 Epidemiology

Cases of typhoid (*Salmonella typhi*) and paratyphoid fevers (*S. paratyphi* A and B) have been reported since 1897. In England and Wales between 1911 and 1960 there were about 17 waterborne outbreaks of typhoid and paratyphoid fevers causing about 155 deaths (Galbraith, 1994). In the United States, more than 30 people out of every 100,000 died of typhoid in 1890 (Rusin *et al.*, 2000). Although infections attributed to typhoid and paratyphoid salmonellae have declined in the UK and USA since 1960 (Galbraith, 1994; Leclerc *et al.*, 2004), cases of waterborne typhoid and paratyphoid are still reported regularly from other parts of the world, mainly undeveloped and poor countries in Asia and Africa, affecting 12.5 million people every year (Hunter, 1997). Waterborne and foodborne salmonellosis (non-typhoidal species) are now the second leading cause of gastroenteritis around the world, and according to the US Centre for Disease Control and Prevention, 1.4 million cases of salmonellosis occur annually in the USA (Hunter, 1997; Lightfoot, 2004; Percival *et al.*, 2004). Global surveillance data has suggested that increased salmonellosis is associated with the consumption of raw or undercooked eggs, poultry meat or dairy products, and salads prepared with mayonnaise (Khakhria *et al.*, 1997; Guard-Petter, 2001; Costalunga and Tondo, 2002).

Table 1.4. Differential characteristics of *Salmonella* species and subspecies

Character	<i>S. enterica</i>							<i>S. bongori</i>
	Subsp. <i>enterica</i>	Subsp. <i>salamae</i>	Subsp. <i>arizonae</i>	Subsp. <i>diarizonae</i>	Subsp. <i>houtenae</i>	Subsp. <i>indica</i>		
Dulcitol	+	+	-	-	-	d	+	
OPNG (2h)	-	-	+	+	-	d	+	
Malonate	-	+	+	+	-	-	-	
Gelatinase	-	+	+	+	+	+	-	
Sorbitol	+	+	+	+	+	-	+	
Culture with KCN	-	-	-	-	+	-	+	
L(+)-tatrane	+	-	-	-	-	-	-	
Galacturonate	-	+	-	+	+	+	+	
γ -Glutamyltransferase	+	+	-	+	+	+	+	
β -Glucuronidase	-	d	-	+	-	d	-	
Mucate	+	+	+	- (70 %)	-	+	+	
Salicin	-	-	-	-	+	-	-	
Lactose	-	-	- (75 %)	+	-	d	-	
Lysis by phage 01	+	+	-	+	-	+	d	
Natural habitat	Warm blooded animals			Cold-blooded animals and the environment				

OPNG, *o*-nitrophenyl- β -D-galactopyranoside; KCN, potassium cyanide, d, different reactions given by different serovars.

Adapted from: Bopp, *et al.* (1999); Jones, *et al.* (2000).

Contaminated drinking water is also an important vehicle of *Salmonella* infection (Hunter, 1997; Percival *et al.*, 2004). Handling of pets, such as snakes and lizards, may also lead to infection (Schröter *et al.*, 2004). By and large, salmonellosis is associated with poor hygiene and sanitation during food production (Lightfoot, 2004).

1.3.2.5 Ecology

Salmonella species are primary pathogens of the intestinal tract of humans and animals, thus their presence in the environment mirrors the distribution of human and animal faecal contamination (Hunter, 1997; Winfield and Groisman, 2003).

The presence of *Salmonella* species in surface water contaminated with human or animal waste is well documented. *Salmonella* serotypes have been frequently isolated from rivers and lakes (Cherry *et al.*, 1972; Dondero *et al.*, 1977; Arvanitidou *et al.*, 1995; Pianetti *et al.*, 1998; Johnson *et al.*, 2003; Lemarchand and Lebaron, 2003), estuaries and marine water (Polo *et al.*, 1999; Baudart *et al.*, 2000; Dionisio *et al.*, 2000). Bottom sediments of polluted rivers and sands of contaminated bathing beaches, and soil of agricultural areas were also found to be a reservoir for *Salmonella* (Hendricks, 1971; Abdel-Monem and Dowidar, 1990; Bolton *et al.*, 1999). *Salmonella* spp. are prevalent in solid and slurry wastes of human and animal origin (Murray, 2000; Koivunen *et al.*, 2001).

The zoonotic origin of *Salmonella* infection means that a wide range of warm and cold-blooded animals are important reservoirs of the organism. These include faecal dropping and/or rectal swabs derived from poultry (Al-Nakhli *et al.*, 1999), livestock (Lightfoot, 2004), wild birds, i.e. gulls pigeons and waterfowl (Mitchell and Ridgwell, 1971; Quessy and Messier, 1992; Casanovas *et al.*, 1995; Feare *et al.*, 1999; Refsum *et al.*, 2002a), pet snakes and lizards (de Sa and Solari, 2001; Schröter *et al.*, 2004), animal feeds and the farm environment (Murray, 2000).

1.3.2.6 Environmental aspects

The most important factor in the dissemination of *Salmonella* species is their ability to grow readily and survive in the environment (Rhodes and Kator, 1988; Winfield and Groisman, 2003; Lightfoot, 2004). Laboratory studies on the survival of *Salmonella* in aquatic environments showed that the organism can survive for up to two weeks in water at temperature below 10 °C (Rhodes and Kator, 1988). In a microcosm study, *Salmonella* survived in freshwater sediment for around two months (Fish and Pettibone, 1995). When compared to other enteric bacteria (e.g. *E.coli*), *Salmonella* has high survival rates in aquatic environments (Rhodes and Kator, 1988; Mezrioui *et al.*, 1995). These findings suggest that *Salmonella* can withstand a wider range of environmental stressors (i.e. high temperature, UV radiation, predation and competition for nutrients) and may persist in water environments for long periods (Burton *et al.*, 1987; Rhodes and Kator, 1988; Fish and Pettibone, 1995; Mezrioui *et al.*, 1995).

Under prolonged exposure to stressful conditions in aquatic environments, *Salmonella* cells were found to enter a dormant stage where they are viable but cannot be cultured by artificial media, i.e. the VBNC stage (Roszak *et al.*, 1984; Chmielewski and Frank, 1995; Caro *et al.*, 1999). Viable but non-culturable *Salmonella* cells can be resuscitated and this may suggest that they retain pathogenicity and virulence (Roszak *et al.*, 1984).

The abundance of *Salmonella* in marine environments has not been shown to be seasonally dependant (Alonso *et al.*, 1992). In freshwaters, however, *Salmonella* showed a marked seasonal pattern, being more readily isolated during the summer and autumn (Alcaide *et al.*, 1984; Pianetti *et al.*, 1998).

1.4 Aims and outline of the work described in this thesis

The present study investigated the microbiology of amenity ponds. It aimed primarily at assessing the bacteriological quality of amenity ponds and at investigating the presence of enteric pathogens in relation to waterfowl. Both conventional and molecular genomic methods were applied. A description of the sites is given in *Chapter 2* while methods are described in *Chapter 3*. To achieve the aims of the study the work outlined below was carried out.

- *Chapter 4*

Amenity ponds are man-made ecosystems. As in other aquatic habitats, some bacteria are native to the ponds, while others are exogenous and enter the water from soil and run-off and from human and/or animal excreta. Ponds in villages are regarded as important amenity sites. For example, in Bangladesh, ponds in villages are used for recreation and sometimes for other purposes (e.g. washing, swimming) and were found to be heavily contaminated by faecal material of human and animal origin (Islam *et al.*, 2000). The water and sediments of these ponds yielded faecal indicator counts exceeding the guidelines of the WHO (Islam *et al.*, 1994; Islam *et al.*, 2000). Pathogenic bacteria (i.e. *Vibrio cholerae* and *Aeromonas*) were also isolated from these ponds (Islam *et al.*, 1992; Islam *et al.*, 1995). Thus it was concluded that they have a high potential for transmission of diarrhoeal and other waterborne diseases (Islam *et al.*, 2000).

In the United Kingdom, village amenity ponds are not used for washing nor generally bathing. However, they are used for picnicking, dog walking and feeding ducks and contact between people and water may still occur. Studies that address the microbiological quality of such ponds are rare. The present study investigated whether or not the presence of waterfowl adversely influences the microbiological

quality of village amenity ponds. In order to achieve this, counts of faecal indicators, heterotrophic bacteria, and direct microscopic counts of total bacteria in water and/or sediment in ponds with waterfowl were compared to ponds without waterfowl. Also the presence of bacterial pathogens (*Campylobacter* and *Salmonella*) in ponds with and without waterfowl was investigated.

- *Chapter 5*

The work described in this chapter aimed to address problems associated with the conventional detection of campylobacters in turbid pond water and sediment. The work investigated the recovery of the organism by enrichment culture using different volumes of pond water or sediment. In addition counts of heterotrophic bacteria, coliforms and microscopic counts of total bacteria were made in *Campylobacter* enrichment cultures to investigate potential competition.

The conventional confirmation of *Campylobacter* relies on a set of morphological and biochemical tests. The work carried out in Chapter 5 also evaluated PCR as a rapid confirmatory tool.

- *Chapter 6*

The work described in Chapter 6 aimed at investigating the sources and distribution of enteric bacteria in amenity ponds. Fresh faecal samples of waterfowl and run-off water from the catchments were screened for *Campylobacter* and *Salmonella*. Conventional methods were used for the detection of the organisms. Both conventional and molecular-genomic methods (PCR) were used for confirmation of presumptive isolates. DNA sequencing of PCR products was used as a tool for the typing of confirmed isolates to enable the investigation of the distribution and sources of different species within the ponds' environment.

- Chapter 7

In view of the problems associated with conventional enrichment culture for the detection of campylobacters in turbid pond waters, the use of direct PCR assay was evaluated. Four different methods of bacterial DNA extraction were compared and the detection limit of direct PCR assay for the detection of campylobacters was determined by seeding water samples with known concentrations of *Campylobacter*. The applicability of PCR assay for the rapid detection of campylobacters in turbid pond water after a selective enrichment step was also described.

The main findings of the work are discussed and conclusions are drawn in Chapter 8. In addition suggestions for future work are made in that chapter.

AIMS

In summary the aims of the work described in this thesis were to:

- test the hypothesis that the presence of waterfowl adversely influences the microbiological quality of amenity ponds.
- address methodological problems associated with the conventional detection of campylobacters in turbid pond water and sediment.
- determine the importance of faeces from waterfowl and run-off water as sources of the different species of *Campylobacter* and *Salmonella* in village ponds.
- investigate the appropriateness of PCR for the direct detection of campylobacters in turbid pond water

OBJECTIVES

The aims were achieved through objectives which were:

- count faecal indicators, heterotrophic bacteria and total bacteria by direct microscopic counting in water and sediment in ponds with waterfowl and without waterfowl;
- assess the presence of bacterial pathogens (*Campylobacter* and *Salmonella*) in ponds with and without waterfowl;
- to solve problems associated with recovery of campylobacters in pond water by enrichment culture using different volumes of pond water and sediment;
- To count heterotrophic bacteria, coliforms and total bacteria in *Campylobacter* enrichment cultures to test the hypothesis of competitive exclusion of campylobacters;
- To screen fresh faecal samples and run-off water from catchments for *Campylobacter* and *Salmonella* by conventional methods;
- To use conventional and molecular-genomic methods (PCR) to confirm presumptive isolates and to use DNA sequencing of PCR products as a tool for typing confirmed isolates;
- To evaluate direct PCR assay for detection of *Campylobacter* in turbid pond water;
- To compare four different methods of bacterial DNA extraction and to assess the detection limit of direct PCR assay for the detection of campylobacters by seeding water samples with known concentrations of *Campylobacter*;

- To describe the applicability of PCR assay for the rapid detection of campylobacters in turbid pond water after a selective enrichment step.

CHAPTER 2

DESCRIPTION OF SITES

This Chapter aims to introduce the village ponds that feature in this study. It also provides information about potential sources of contamination to these ponds.

The history of village ponds goes back to the Victorian times and earlier. Through the years, the ponds were essential elements in farming and other rural economic activities. As the practices of modern agriculture have developed the need for ponds has decreased (Hall, 1997). Now, the ponds and their environs serve as amenity sites for village residents and visitors. Degradation of the aesthetic qualities of village ponds may, however, reduce their amenity value. Degraded village ponds may be neglected by village communities and this may result in their loss. Conservation and restoration of degraded and neglected village ponds has recently received considerable attention. For instance, projects initiated by the Pond Conservation Group that are aimed at restoring and enhancing the amenity value and the flora and fauna of village ponds (Drake and Pickering, 1997). While the aesthetic and macrobiology qualities of village ponds have been widely considered, the microbiological qualities of village ponds and their environs need attention. Village ponds may be recipients of faecal pollution from non-point sources and/or from the wild fauna that live in, on or around the ponds. Faecally contaminated village ponds may have public health implications as they may serve as a vehicle for the dissemination of enteric bacterial pathogens.

2.1 East Yorkshire Village Ponds

Many villages in East Yorkshire have ponds, these are usually adjacent to roads and are often ancient in origin; those with abundant waterfowl were found to have sparse aquatic vegetation (Linton and Goulder, 1997; 2000). Little or nothing is known about the microbiology of these ponds. The microbiology of seven village ponds in East Yorkshire, North-East of England (Fig. 2.1) was studied during the work that is described in the present thesis. All seven ponds are close to village centres and are adjacent to roads from which they receive run-off. They are all amenity sites. Some ponds have populations of semi-tame ducks and geese that have sometimes been introduced to the ponds (e.g the goose at Little Weighton) and are often fed by the public. Descriptions of these ponds and their environs are given below.

2.1.1 The Pond at Bentley

Bentley is about 6 km northwest of Hull (National Grid Reference TA 019 359) (Fig. 2.1). The pond area is about 220 m². The water is clear with a hard underlying bottom (Plate 2.1). There is extensive summer cover by aquatic plants (Table 2.1). A pig farm is across the road from the pond, but there is no obvious run-off from this. The waterfowl population on this pond consisted of only a few (2-4) coot (Table 2.2).

2.1.2 The Pond at Brantingham

Brantingham is about 7.5 km northwest of Hull (National Grid Reference SE 941 296) (Fig. 2.1). The size of the pond is around 240 m². The pond has concrete margins and bottom (Plate 2.2). There are three drainage pipes that carry run-off from the road. The water is turbid and aquatic vegetation is absent (Table 2.1). A large population of waterfowl is present on and in the vicinity of the pond (Table 2.2)

2.1.3 The pond at Garton-on-the-wolds

Garton-on-the-Wolds is approximately 40 km north of Hull (National Grid Reference SE 983 594) (Fig. 2.1). The pond is of large size, area around 1800 m².

Chalky gravel margins slope to a muddy bottom. The water is turbid (Plate 2.3). Aquatic vegetation is sparse (Table 2.1). Ducks were abundant (Table 2.2). Allison (1974) described the presence of the pond as “The Mere” in the 18th century, which strongly indicates its ancient origin.

2.1.4 The pond at Holme-on-Spalding-Moor

Holme-on-Spalding-Moor is about 25 km to the northwest of Hull (National Grid Reference SE 827 390) (Fig. 2.1). The pond area is about 1530 m². The water is clear; the bottom is soft mud (Plate 2.4). There is abundant aquatic vegetation (Table 2.1). There is a drainage pipe from the road. The catchment is in part grazing land with horses. Waterfowl on this pond are a small and transient population of coot and moorhen (Table 2.1). The pond completely dried during summer of drought years (2003). This was also observed by Linton (1999).

2.1.5 The pond at Little Weighton

Little Weighton is about 7.5 km to the northwest of Hull (National Grid Reference SE 988 338) (Fig. 2.1). The pond area is about 640 m². The margin on the side with the road is concrete, falling to a soft muddy bottom. Aquatic vegetation is sparse (Table 2.1). The water is turbid and there are three drainage pipes from the road (Plate 2.5). There is a large number of permanently resident waterfowl on and in the vicinity of the pond (Table 2.2).

2.1.6 The pond at Sancton

Sancton is a small village about 17.5 km to the northwest of Hull (National Grid Reference SE 901 392) (Fig. 2.1). The pond is small area, about 85 m²; it receives some inflow from a small spring and there is an outlet. The water is very clear (Plate 2.6). There is abundant submerged and emergent aquatic vegetation (Table 2.1). Waterfowl are absent from this pond (Table 2.2).

2.1.7 The pond at South Dalton

South Dalton is a small village about 30 km to the northwest of Hull (National Grid Reference 969 454) (Fig. 2.1). The pond area is about 2410 m². It has a concrete margin adjacent to the road, otherwise it has mud margins and a silty and soft bottom and there is an island in the middle (Plate 2.7). The water is turbid and aquatic vegetation is absent (Table 2.1). There is a dense population of waterfowl (Table 2.2). Marked on the 1827 ordnance 1:2500 survey map as “the East Mere”, the pond may have ancient origin (Allison, 1979)



Figure 2.1 A map of East Yorkshire; sampling sites, in alphabetical order, are numbered (amended from Anon., 2004).



Plate 2.1 Bentley Pond, August 2001. Note the luxurious marginal vegetation (*Iris pseudacorus*) and submerged aquatic vegetation (largely *Ceratophyllum demersum*) and the absence of waterfowl.



Plate 2.2 Brantingham pond, August 2001. Note the concrete margins, the lack of aquatic vegetation and the large number of waterfowl.



Plate 2.3 Garton-on-the-wolds pond, August 2001. Note the sparcity of aquatic vegetation and the large population of waterfowl.



Plate 2.4 Holme-on-Spalding-Moor pond, August 2001. The abundant vegetation is largely *Sparganium erectum* and *Typha latifolia*; floating-leaves *Persicaria amphibia* is also abundant. Note the absence of waterfowl.



Plate 2.5 Little Weighton pond, August 2001. Note the lack of aquatic vegetation and the turbid water. Waterfowl were sitting adjacent to the pond when this photograph was taken.



Plate 2.6 Sancton pond, August 2001. Note the abundant emergent vegetation (largely *Glyceria maxima*) and the absence of waterfowl.



Plate 2.7 South Dalton pond, August 2001. Note the absence of aquatic vegetation and the presence of waterfowl.

Table 2.1 Aquatic macrophytes in East Yorkshire village ponds, June-August 2001 (data from R. Goulder, personal communication)

	Bentley	Brantingham*	Garton-on-the-wolds*	Holme-on-Spalding-Moor	Little Weighton*	Sancton	South Dalton*
<i>Agrostis stolonifera</i>	2	-	-	2	-	1	1
<i>Alisma plantago-aquatica</i>	-	-	-	2	-	-	-
<i>Apium nodiflorum</i>	-	-	-	-	-	3	-
<i>Callitriche</i> sp.	-	-	-	-	-	2	-
<i>Caltha palustris</i>	-	-	-	-	-	2	-
<i>Ceratophyllum demersum</i>	3	-	-	-	-	-	-
<i>Crassula helmsii</i>	-	-	-	1	-	-	-
<i>Eleocharis palustris</i>	-	-	-	1	-	-	-
<i>Galium palustre</i>	-	-	-	1	-	-	-
<i>Glyceria maxima</i>	-	-	-	-	-	3	-
<i>Iris pseudacorus</i>	2	-	1	-	-	2	-
<i>Juncus articulatus</i>	-	-	-	1	-	-	-
<i>Juncus effusus</i>	-	-	-	2	-	-	-
<i>Juncus inflexus</i>	-	-	-	2	-	-	1
<i>Lemna minor</i>	2	-	-	3	-	3	-
<i>Lemna trisulca</i>	-	-	-	2	-	-	-
<i>Mentha aquatica</i>	-	-	-	2	-	-	-
<i>Myosotis scorpioides</i>	-	-	-	1	-	-	-
<i>Oenanthe fistulosa</i>	-	-	-	1	-	-	-
<i>Persicaria amphibia</i>	-	-	-	3	-	-	-
<i>Potamogeton crispus</i>	2	-	-	-	-	-	-
<i>Ranunculus aquatilis</i>	-	-	-	2	-	-	-
<i>Ranunculus sceleratus</i>	-	-	-	1	-	-	-
<i>Rorippa nasturtium-aquaticum</i>	-	-	-	2	-	-	-
<i>Solanum dulcamara</i>	-	-	-	2	-	-	-
<i>Sparganium erectum</i>	-	-	-	3	-	-	-
<i>Typha latifolia</i>	3	-	-	3	-	-	-
<i>Veronica beccabunga</i>	-	-	-	2	-	-	-
<i>Veronica catenata</i>	-	-	-	1	-	-	-
Total number of species	6	0	1	22	0	7	1

* Indicates the presence of a substantial waterfowl population.
1 = <0.1% cover, 2 = 0.1-5% cover, 3 = >5% cover, - = not recorded.

Table 2.2 Waterfowl of East Yorkshire village ponds (June 2001-January 2002)

	Bentley [†]	Brantingham [†]	Garton-on-the-wolds [‡]	Holme-on-Spalding-Moor [†]	Little Weighton [‡]	Sancton [†]	South Dalton [†]
Coot (<i>Fulica atra</i>)	3 (1-4)	1 (0-1)	-	2 (0-2)	-	-	-
Duck (<i>Anas platyrhynchos</i>)	-	34 (13-75)	39 (35-45)	-	23 (21-24)	-	30 (13-41)
Goose (<i>Anser anser</i>)	-	-	-	-	1 (0-1)	-	6 (0-6)
Moorhen (<i>Gallinula chloropus</i>)	-	-	-	4 (2-5)	-	-	3 (2-4)

Values are mean (with range).

- Indicates not present.

† Ponds visited seven times.

‡ Ponds visited three times.

CHAPTER 3

MATERIALS AND METHODS

3.1 Sampling

3.1.1 Water samples

Surface water samples were collected in the morning, at the margin of each pond, near the roadside, about two meters away from the edge, into sterile one-litre, wide-mouth polypropylene bottles (BDH, Dorset, UK). Two or three samples were taken and processed separately.

3.1.2 Sediment samples

Sterile toughened glass tubes with an internal diameter of 4.3 cm and length of 29 cm were used to collect cores of bottom sediment by wading about 2 meters from the edge (Carr and Goulder, 1990). Sediment tubes were taken by pushing the glass core into the mud at the bottom of the pond and then placing a bung into the top end of the tube. The core was then lifted till the bottom end was just above the sediment and another bung was placed in the bottom end of the tube. This yielded a core of undisturbed sediment and overlying water. Water and sediment samples were brought to the laboratory on ice in darkness. Cores were transported in an upright position away from direct sunlight.

The top 1 cm stratum of sediment was removed from the cores as follows. The overlying water was siphoned off and the top 1 cm sediment layer was transferred to a sterile polythene stomacher bag after easing the bung at the bottom end of the glass core to discard the bottom layers of the sediment core and keep the top 1 cm. A volume of 130 ml of sterile pond water was added to the sample. This gave a $\times 10$ dilution. This

sediment slurry was treated in a stomacher (Colworth 400; A.J. Seward Ltd, London) for five minutes to homogenise the slurry.

3.1.3 Dilutions

Diluent (i.e. sterile pond water), for making dilutions of pond water samples and sediment slurries, was prepared by filtering water, from the pond at South Dalton through GF/C glass microfiber filters (Labsales, Cambridge, UK) with normal pore size 1.2 μm . Volumes of 50 ml of the filtered water were dispensed into 100 ml glass bottles and sterilised by autoclaving at 121 $^{\circ}\text{C}$, 1.1 kg/cm^2 , for 15 minutes.

3.2 Abiotic Variables

3.2.1 Temperature, pH, conductivity, turbidity, dry weight of sediment

Temperature, pH, conductivity and turbidity of water samples were measured. Temperature and pH were measured in the field using a mercury-in-glass thermometer and a portable pH meter (pH/mV meter 42D, pHox Systems Ltd). Conductivity was measured in the laboratory at room temperature (20 $^{\circ}\text{C}$) (HMSO, 1972) using a Jenway model 4070 conductivity meter (Felstead, Essex, UK). Conductivity values were compensated to 25 $^{\circ}\text{C}$ by the meter. A Unicam 8625 UV/VIS spectrophotometer was used to measure the absorbance at wavelength 580 nm (A_{580}) in a 4 cm path length cuvette as suggested by HMSO (1972). The results were divided by four so that they were expressed as absorbance by a 1 cm pathway; the values reflected turbidity. Sediment dry weight was also determined. An empty crucible was weighed and then; 50 ml of the $\times 10$ diluted sediment slurry were transferred to the empty crucible. The slurry was dried overnight in an oven at 100 $^{\circ}\text{C}$, then left overnight to cool in a desiccator. The

crucible was then weighed; the difference between the initial and final weight equalled dry weight of sediment in 50 ml of the slurry.

3.3 Microbiological examination

3.3.1 Enumeration of faecal indicator bacteria

Membrane filtration techniques were employed for the enumeration of faecal indicator bacteria from water and sediment (PHLS, 1998a). *Escherichia coli* and faecal streptococci were enumerated following the standard methods for the examination of water as used by the Public Health Laboratory Service (PHLS, 1998b-c). *Clostridium perfringens* was assayed by a membrane filtration technique as recommended by HMSO (1984). All samples were filtered through 0.45 µm, gridded, 47 mm diameter, cellulose nitrate membrane filters (Labsales, Cambridge, UK). At least three replicates filtrations were made from each sample. Further details of the processing of water and sediment samples are as follows:

3.3.1.1 Enumeration and confirmation of *Escherichia coli*

Volumes of 0.1 ml (with a 10 ml carrier of sterile pond water), 10 ml and 100 ml of water samples were filtered. Sterile absorbent pads, 47 mm in diameter, were placed in sterile Petri dishes and 2.5 ml of sterile membrane lauryl sulphate broth (Oxoid, Basingstoke, UK) was added to saturate the pads. Pads were left to soak for at least five minutes. Post-filtration membranes were then placed, face-up, on the saturated absorbent pads and incubated at 30 °C for four hours in an airtight container, incubation was then continued at 44 °C for 14 hours. Yellow colonies (lactose fermentors), were counted (PHLS, 1998b). For sediment slurries, 0.1 ml was filtered with a 10 ml carrier of sterile pond water, otherwise the same procedure as for water analysis was applied.

Presumptive *E. coli* (yellow colonies) were confirmed by using Fluorocult[®] LMX broth (BDH, Dorset, UK) (Ossmer, 1993). Aliquots of 0.1 ml of the broth were transferred into a U-bottom sterile polystyrene well cluster (Costar[®], Corning Inc., NY, USA). Each well was inoculated with a single presumptive *E. coli* colony using an inoculation loop. The wells were then incubated at 37 °C for 24 hours. The formation of blue-green colour together with the observation of fluorescence under UV illumination confirmed that an isolate was *E. coli*. The test relies upon the production of extracellular β -D-galactosidase and β -D-glucuronidase. *E. coli* is characterised by the production of both enzymes (Ossmer, 1993). At least five colonies were examined from each plate.

3.3.1.2 Enumeration and confirmation of faecal streptococci

For enumerating faecal streptococci, 10 ml and 100 ml volumes of water samples were separately filtered. Membranes were placed on plates of Slanetz and Bartley agar (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for four hours, in an airtight container, followed by 40 hours at 44 °C. After incubation, red, maroon, pink and colourless colonies were counted (PHLS, 1998c). The preliminary incubation at 37 °C was to encourage the growth of environmentally stressed organisms (Oxoid, 1998). With slurry samples, a volume of 0.1 ml was filtered with 10 ml of carrier sterile pond water. Otherwise the technique was the same as with the water samples.

Presumptive faecal streptococci were confirmed by subculturing suspected colonies on bile aesculin agar (Oxoid, Basingstoke, UK). Plates were incubated at 44 °C for 16 hours. The formation of black or brown colour confirmed the isolates as faecal streptococci. The test demonstrates the ability to hydrolyse aesculin, which is characteristic of Group D streptococci (Oxoid, 1998). At least five colonies from each plate were examined.

3.3.1.3 Enumeration and confirmation of *Clostridium perfringens*

Water and sediment samples were heat-treated prior to filtration. For this, samples were incubated at 75 °C in a water bath for 10 minutes. This was to ensure that all vegetative cells were killed and only *Clostridium perfringens* spores remained in the samples (HMSO, 1984).

After heat treatment, 10 ml and 100 ml of water samples, or 0.1 ml of sediment slurry with 10 ml of carrier sterile pond water, were filtered. Filters were placed on reinforced clostridial agar (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 24 hours in anaerobic conditions (AnaeroGen™, Oxoid, Basingstoke, UK), using an anaerobic jar (Oxoid, Basingstoke, UK). Light brown colonies were counted.

Suspected *Clostridium perfringens* colonies were confirmed by using Crossley milk medium (Oxoid, Basingstoke, UK). This medium was designed to replace litmus milk medium as a confirmatory medium for *Clostridium perfringens* (Oxoid, 1998). At least three tubes containing 10 ml of sterile medium were inoculated with one suspected colony from each plate. At least five colonies were examined from each plate. All tubes were incubated in an anaerobic atmosphere (AnaeroGen™, Oxoid, Basingstoke, UK) at 37 °C for 24 hours. The formation of acid (bright yellow colour) and a stormy clot confirms *Clostridium perfringens* (Oxoid, 1998).

3.3.2 Detection of enteric bacterial pathogens

Membrane filtration methods were employed for the detection of enteric bacterial pathogens (*Campylobacter* spp. and *Salmonella* spp.) in water by applying the standard methods for the examination of water as used by the Public Health Laboratory Service (PHLS, 1998d-e). Sediment samples were subjected directly to selective

enrichment. Detection of *Campylobacter* and *Salmonella* utilised a presence/absence assay, thus, absolute numbers were not determined.

3.3.2.1 Detection and confirmation of *Campylobacter* species

One litre of water samples was filtered. Each filter was transferred to a screw-capped sterile glass bottle. 150 ml of Preston selective enrichment broth (Oxoid, Basingstoke, UK), containing 5 % lysed horse blood (Oxoid Basingstoke, UK), and growth supplement consisting of ferrous sulphate (0.125 g l^{-1}), sodium metabisulphite (0.125 g l^{-1}) and sodium pyruvate (0.125 g l^{-1}) (FBP) (Oxoid, Basingstoke, UK), was added to the bottle. A microaerobic atmosphere was achieved by leaving little headspace and by tightly closing the bottle tops (PHLS, 1998d). Bottles were incubated at $37 \text{ }^\circ\text{C}$ for 22 hours, followed by $42 \text{ }^\circ\text{C}$ for 22 hours. After incubation broth cultures were subcultured onto *Campylobacter*-selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid, Basingstoke, UK). Plates were incubated under microaerobic conditions (N_2 85 %, O_2 5 %, CO_2 10 %) in an anaerobic jar (CampyGen™, Oxoid, Basingstoke, UK), at $37 \text{ }^\circ\text{C}$ for 48 hours (PHLS, 1998d). For sediment samples, 5.0 ml of undiluted sediment samples were transferred to a 30-ml sterile universal bottle using a sterile porcelain crucible as a measuring cup. A volume of 25 ml of Preston selective enrichment broth was added to the universal bottle. The procedure was then continued as described above.

Campylobacter jejuni strains on mCCDA may sometimes appear as grey, moist, flat and spreading colonies whereas *Campylobacter coli* strains produce creamy-grey, moist, slightly-raised colonies (Oxoid, 1998). These criteria were not used in the present study.

The confirmation criterion for presumptive *Campylobacter* isolates was a positive oxidase test together with growth on blood agar under microaerobic but not aerobic conditions (PHLS, 1998d). Oxidase reagent (BDH, Dorset, UK) was prepared

by adding 0.1 ml of tetramethyl-p-phenylenediamine hydrochloride to 10 ml of distilled water (HSMO, 1984). The reagent was freshly prepared at the time of use (HSMO, 1984). The oxidase test was performed by adding two or three drops of the reagent to a filter paper (Whatman grade A, Labsales, Cambridge, UK). One suspected *Campylobacter* colony was transferred, using a cocktail stick, to the moistened paper. The appearance of a dark purple colour within 10 seconds indicated a positive reaction. Oxidase negative colonies did not require further testing because *Campylobacter* species are oxidase positive (PHLS, 1998d).

Oxidase positive colonies were subcultured to two blood agar plates. One of these plates was placed in a microaerobic atmosphere (CampyGen™, Oxoid, Basingstoke, UK) in an anaerobic jar (Oxoid, Basingstoke, UK) and incubated at 37 °C for 24 hours. The other plate was incubated in aerobic conditions at 37 °C for 24 hours. *Campylobacter* species are microaerophilic, thus, growth under microaerobic but not aerobic conditions confirms that isolates are *Campylobacter* (PHLS, 1999d).

3.3.2.2 Detection and confirmation of *Salmonella* species

One litre of water samples was filtered. Prior to filtration, 15 ml of a well-mixed sterile filter aid were added to the samples and mixed thoroughly. The filter aid stock solutions were prepared by adding 1.0 g of Hyflo-supercel (BDH, Dorset, UK) to 15 ml of distilled water in 20-ml universal bottles. The universal bottles were sterilised by autoclaving at 121 °C, 1.1 kg/cm², for 15 minutes. The use of filter aid is recommended by HMSO (1984) and PHLS (1998e). It is suggested that more than 90 % of the bacteria present in a sample will be retained as the filter aid will form an initial retaining layer on the membrane. After filtration, membranes were transferred to bottles containing 50 ml of sterile buffered peptone water (Oxoid, Basingstoke, UK). Bottles were incubated at 37 °C for 18 hours. This was a pre-enrichment stage.

After incubation, 10 ml of the pre-enrichment culture were transferred to 10 ml sterile double strength Rappaport Vassiliadis soya peptone broth (Oxoid, Basingstoke, UK) in universal bottles. The bottles were then incubated at 41 °C for 20 hours. A further 10 ml of the pre-enrichment cultures were transferred to 10 ml of sterile double strength selenite cysteine broth (Oxoid, Basingstoke, UK) in a universal bottle. These were incubated at 37 °C for 20 hours. These two latter processes represented selective enrichment stages.

After incubation, all selective enrichment broths were subcultured to xylose desoxycholate agar (XLD) (Oxoid, Basingstoke, UK) and to brilliant green agar (BG) (Oxoid, Basingstoke, UK). Plates were incubated at 37 °C for 24 hours. *Salmonella* appears on XLD agar as red colonies with black centres. On BG agar, however, *Salmonella* appears as red colonies surrounded by a bright red halo (PHLS, 1998e).

Sediment samples were analysed as follows. Five milliliters of the undiluted original sediment sample were transferred to a bottle containing 45 ml of sterile buffered peptone water. A sterile porcelain crucible was used as a measuring cup. Further processing followed the post-filtration procedure used with water samples.

Red colonies with black centres on XLD agar might also be produced by *Edwardsiella* species, some strains of *Proteus* and *Pseudomonas*. Moreover, H₂S-negative salmonellae such as *S. paratyphi* A, *S. senftenberg* and *S. pullorum* produce colonies with no black centres on XLD (Oxoid, 1998). Likewise, false-positive results might occur through the growth on BG agar of red colonies produced by some strains of *Proteus* and *Pseudomonas* (PHLS, 1998e).

Because of the possibility of false-positive results, biochemical confirmatory test were performed. At least three presumed *Salmonella* colonies from each XLD and BG agar plates were subcultured to cystine lactose electrolyte deficient (CLED) agar. These

plates were incubated at 37 °C for 18 to 24 hours. *Salmonella* grew on CLED as flat blue (non-lactose fermenting) colonies (PHLS, 1998e). Presumed colonies on CLED were subcultured to urea broth (Oxoid, Basingstoke, UK) and to lysine iron agar slopes (Oxoid, Basingstoke, UK). Urea broth cultures were incubated at 37 °C for 4-6 hours, and were further incubated for 14-16 hours if negative. The lysine iron agar slopes were incubated at 37 °C for 20-24 hours. *Salmonella* lack urease, therefore a negative reaction with urea broth (no pink colouration), indicates *Salmonella*. Lysine iron agar was used to detect the presence of lysine decarboxylase and the production of H₂S, thus *Salmonella* gave an alkaline (purple) reaction throughout the medium (i.e. at both butt and slope ends) with intense blackening, which indicates the production of hydrogen sulphide (PHLS, 1998e). Different reactions that might occur on lysine iron agar due to diverse Gram negative bacteria are listed in Table 3.1.

Table 3.1 Gram negative bacteria and their reaction with lysine iron agar (Oxoid, 1998)

Genus	Slope	Butt	H ₂ S
<i>Salmonella</i>	Alkaline (purple)	Alkaline	+
<i>Proteus</i>	Red	Acid (yellow)	-
<i>Providencia</i>	Red	Acid	-
<i>Citrobacter</i>	Alkaline	Acid	+
<i>Escherichia</i>	Alkaline	Acid or neutral	-
<i>Shigella</i>	Alkaline	Acid	-
<i>Klebsiella</i>	Alkaline	Acid	-
<i>Enterobacter</i>	Alkaline	Acid	-

3.3.3. Quality Control

Preliminary trials with positive cultures (positive controls) and with sterile pure water (negative controls) confirmed the reliability of the methods. Controls were run with *E. coli*, faecal streptococci and *Salmonella*.

3.3.4 Heterotrophic Plate Count (HPC)

Heterotrophic plate counts from both water and sediment-slurry samples were made using casein-peptone-starch agar (CPS) (Collins and Willoughby, 1962; Jones, 1970). A volume of 0.5 ml of the samples was added to, and mixed thoroughly with, 50 ml of sterile pond water. Sub-samples (0.1 ml) of the diluted samples were pipetted onto the surface of dried CPS agar plates. The 0.1 ml sub-samples were spread with the aid of a sterile glass spreader. At least 10 replicates plates were inoculated from each sample. Plates were incubated at 20 °C for two weeks and colonies were then counted.

3.3.5 Count of total bacteria (DAPI counts)

Abundance of total bacteria in water samples was determined by the 4'-6'-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, USA) direct count method (Yu *et al.*, 1995). 10 ml sub-samples of pond water were preserved for up to two weeks at 4 °C with 0.5 ml of 0.2 µm filtered formalin (final concentration 2 % formaldehyde). DAPI stock solution was prepared by dissolving 0.01 g of the dye in 10 ml of sterile 0.2 µm filtered water. The prepared solution was stored in aluminium-foil-wrapped vials at -20 °C. An aliquot of 0.1 ml of DAPI stock solution was added to the preserved samples which were kept for 40 minutes in darkness at room temperature to allow staining of bacteria to occur. After staining with DAPI, 0.1 ml of the sample, with 5.0 ml carrier of 0.2 µm filtered sterile water, was filtered through 0.2 µm polycarbonate membrane filters (Labsales, Cambridge, UK). Irgalan Black solution (2.0 g l⁻¹ acetic acid in 2.5 % glutaraldehyde) was used to dye the filters for 10 minutes prior to filtrations (Jones and Simon, 1975); this improved contrast during counting. A Nikon Alphaphot epifluorescence microscope, with UV illumination, at a total magnification of × 1250, was then used to count bacteria on the filters. Bacteria were easily observed as blue

fluorescent cells against a black background. For each sample preparation, more than 600 cells were counted in n eyepiece squares of side 0.078 mm. Sterile 0.2 μm filtered water was used for a blank count. Concentration was calculated as follows:

$$\text{Concentration (cells/ml)} = \frac{\text{Count (cells)}}{n} \times \frac{\text{Area of filter (mm}^2\text{)}}{\text{Area of eyepiece square (mm}^2\text{)}} \times \frac{1}{\text{Volume filtered (ml)}}$$

3.3.6 Molecular methods

3.3.6.1 PCR assays

PCR assays were performed on presumptive *Campylobacter* and *Salmonella* isolates as a method of confirmation.

3.3.6.1.1 DNA extraction

Bacterial DNA was extracted by suspending a loop of a suspected *Campylobacter* or *Salmonella* colony in 100 μl of sterile, pure water and boiling for 5 minutes. The suspension was then centrifuged for 5 minutes at 1260 RCF and 10 μl of the supernatant were used as target DNA.

3.3.6.1.2 Oligonucleotides and quality control

Primers CF03 and CF04 (Invitrogen, Paisley, UK) from the *Campylobacter jejuni* *flaA* and *flaB* gene sequence were used (Wegmüller *et al.*, 1993). Their sequences are as follows:

CF03 5'-GCT CAA AGT GGT TCT TAT GCN ATG G-3' (forward)

CF04 5'-GCT GCG GAG TTC ATT CTA AGA CC-3' (reverse)

For *Salmonella*, primers S18 and S19 (Invitrogen, Paisley, UK) from the outer membrane protein C (*omp C*) gene of *Salmonella* were used (Kwang *et al.*, 1996). Their sequences are as follows:

S18 5'-ACC GCT AAC GCT CGC CTG TAT-3' (forward)

S19 5'-AGA GGT GGA CGG GTT GCT GCC GTT-3' (reverse)

In order to confirm the specificity of the primers CF03; CF04 and S18; S19, each set was tested against DNA extracted from wide range of Gram positive and Gram negative bacterial strains including *Campylobacter* and *Salmonella*. The results are shown in Table 3.2. CF03; CF04 amplified only *Campylobacter* isolates, while S18; S19 amplified only *Salmonella*.

Table 3.2 Bacterial strains used to determine the specificity of *Campylobacter* and *Salmonella* primers set

Primer code	Bacterial strains	
	Positive PCR amplification	Negative PCR amplification
CF03; CF04	<i>Campylobacter</i> (four clinical isolates from PHLS Scunthorpe) [‡]	<i>E. coli</i> K12 [†] <i>E. coli</i> (pond water isolate from South Dalton) <i>Pseudomonas aeruginosa</i> [†] <i>Klebsiella aerogenes</i> [†] Faecal streptococci (pond water isolate from South Dalton)
S18; S19	<i>Salmonella enteritidis</i> NCTC 12694	<i>E. coli</i> K12 [†] <i>E. coli</i> (pond water isolate from South Dalton) <i>Pseudomonas aeruginosa</i> [†] <i>Klebsiella aerogenes</i> [†] Faecal streptococci (pond water isolate from South Dalton)

[†] These were cultures that are maintained in the Department of Biological Sciences, University of Hull.

[‡] Isolates were not identified beyond species level by PHLS.

3.3.6.1.3 PCR reaction mixture

The PCR reaction mixture (50 µl total volume) consisted of the following: 25 µl of 2 × PCR master mix (ABgene, Surry, UK) contains the following: 75 mmol l⁻¹ Tris-HCl; 20 mmol l⁻¹ (NH₄)₂SO₄; 2.0 mmol l⁻¹ MgCl₂; 0.01 % (v/v) Tween[®] 20; 0.2 mmol l⁻¹ each of dATP, dCTP, dGTP and dTTP; 1.25 units of Thermoprime Plus DNA Polymerase; 0.5 µl of each primer (0.25 µmol l⁻¹); 10 µl bacterial DNA extract and 14

µl sterile pure water. For DNA-free controls, 10 µl sterile pure water replaced the bacterial DNA extract.

3.3.6.1.4 Gel electrophoresis

The gel was prepared by dissolving 0.9 g of agarose (BioLine, London, UK) in 60 ml of $0.5 \times$ Tris-borate EDTA buffer (TBE), containing the following: 0.045 mol l^{-1} Tris-borate; 0.001 mol l^{-1} EDTA. The gels were stained with 2.5 µl of ethidium bromide (final concentration 0.5 µg ml^{-1}).

3.3.6.1.5 PCR temperature cycles

The amplification of *Campylobacter* DNA used the following temperature cycles: denaturation at 94 °C for 4 minutes, 30 cycles at 95 °C for 1 minute, 53 °C for 1 minute, 72 °C for 1 minute, and final extension at 72 °C for 5 minutes.

Amplification of *Salmonella* DNA used the following temperature: denaturation at 95 °C for 5 minutes, 30 cycles at 95 °C for 30 seconds, 56 °C for 45 seconds, 72 °C for 60 seconds, and final extension at 72 °C for 5 minutes.

A total of 8 µl of PCR products mixed with 2 µl loading dye (BioLine, London, UK) was analysed by 1.5 % (w/v) agarose gel (BioLine, London, UK) electrophoresis (0.1 volt for 70 minutes) and made visible by ethidium bromide (0.5 µg ml^{-1}) staining and UV transillumination.

CHAPTER 4

WATERFOWL AND THE BACTERIOLOGICAL QUALITY OF AMENITY PONDS

4.1 INTRODUCTION

Water pollution is a key issue worldwide. Much concern has been expressed about sewage-derived pollutants in aquatic environments, particularly in fresh waters. Sewage waste is potentially a major source of water-borne disease and of causative agents of water-borne illness in the environment, since faecal solids of human origin carry up to 10^{12} bacteria per gram, around 10^9 are pathogens (Bitton, 1999). Pathogens of faecal origin may be stable in water, and bacteria in particular may have the capability to grow outside their host when the right environmental conditions exist (Rusin, *et al.* 2000). Thus, a health risk might occur through human consumption or contact with faecally-polluted water.

Faecally-derived bacteria reach aquatic environments in various ways. These include direct discharge of treated and raw sewage effluents (Mason, 1996); agricultural run-off (Fernández-Alvarez, *et al.* 1991) and direct deposit of faecal matter from wild fauna (Jones, 2002). Bacteria of faecal origin may settle down in bottom sediment once they reach watercourses, where they might die or persist and perhaps be resuspended. The results of Burton, *et al.* (1987) on survival of enteric bacterial pathogens in a laboratory study of fresh water sediments supported the findings of earlier studies that concentrations of faecal bacteria may be much higher in bottom sediments than in overlying water. Crabill, *et al.* (1999) found that the average counts of faecally-derived bacteria in two sediment samples were 2200 times higher than the counts of the same bacteria in water samples in Oak Creek, Arizona. The high concentrations of bacteria in

bottom sediment may be explained, in part, by the protection that bottom sediment provides from some of the adverse environmental factors that might stress bacteria, such as adverse light conditions and limited availability of nutrients. Thus, it was suggested that sedimentation allows pathogenic bacteria to survive for several months (Laliberte and Grims, 1982; Burton, *et al.* 1987; Sherer, *et al.* 1992). These results were supported by the findings of Davies, *et al.* (1995) that suggested that bottom sediments provide non-starvation environments for faecal bacteria. These findings led to the conclusion drawn by Obiri-Danso and Jones (2000) and Alm, *et al.* (2003) that bottom sediments act as a reservoir for enteric bacteria. The presence of enteric bacteria in high concentrations, and their ability to survive for long periods, in bottom sediments has been recognised as a possible health hazard (Hendricks, 1971; Van Donzel and Geldreich, 1971; Laliberte and Grimes, 1982; Burton, *et al.* 1987).

The presence of faecal bacteria in surface water might be at times attributed, in part, to resuspension of contaminated bottom sediments. Irvine and Pettibone (1993) found that resuspension of bottom sediment of the Buffalo River, New York might be caused by high flow velocity associated with storm events. In Oak Creek, Arizona, storm events were found to play a part in resuspending contaminated bottom sediment and, therefore, caused the release of sediment-bound bacteria to the overlying water column (Crabill, *et al.* 1999). Likewise, in Morecambe Bay, rough weather, together with recreational activities, were also thought to contribute to increase in bacterial densities in surface waters by resuspending contaminated bottom sediments (Obiri-Danso and Jones, 2000).

Between 1985 and 1994 a total of 55 outbreaks of disease associated with recreational contact with surface waters were reported in the USA, resulting in a total of 3713 cases (Hunter, 1997). *Shigella* and *E. coli* O157:H7 were responsible for 18 outbreaks, while 13 outbreaks of acute gastroenteritis with 1005 cases were associated

with unknown aetiology (Hunter, 1997). Similarly, in the period between 1986 to 1996, a total number of 710 waterborne disease outbreaks associated with drinking and recreational water were reported in 19 European countries (Anderson and Bohan, 2001). Bacterial agents such as *Campylobacter*, *Salmonella*, *Vibrio* and *Aeromonas* species were found to be the cause of the majority of these outbreaks (Anderson and Bohan, 2001). As a consequence, there is a demand for further information about the sources, distribution and persistence of pathogenic bacteria in aquatic environments, particularly in fresh waters. Considerable numbers of pathogens have been found in fresh waters; including waters used for human consumption. Pathogenic agents included bacteria, enteric viruses and protozoa (Geldreich, 1996). Bacterial agents in particular, were found to fall into two groups according to their origin: faecal-origin pathogens and native aquatic pathogens. *Campylobacter* spp., *Salmonella* spp., *Shigella* spp. and pathogenic strains of *E. coli* were found to originate from human and/or animal faeces, whereas *Aeromonas* spp., *Pseudomonas* spp. and some species of *Mycobacterium* were found to be ubiquitous and indigenous in aquatic environments (Hunter, 1997).

4.1.1 Faecal indicator bacteria

The detection of waterborne pathogenic bacteria in routine monitoring of microbiological water quality has been considered as an expensive, laborious, impractical and time-consuming task. Thus, a surrogate was needed. The presence of a surrogate organism or group of organisms in water samples should give indication of faecal pollution and, therefore, of the possible presence of waterborne pathogens. The faecal indicator organisms should be present in large numbers in faeces; absent or less abundant in other sources; easy to enumerate and unable to grow in aquatic environments (HMSO, 1984). In practice, there is no one organism that fulfils these criteria, however, there are groups of bacteria that have been used extensively by public

health authorities worldwide and have proved reliable; these groups include coliforms, faecal streptococci, and sulphite-reducing clostridia.

4.1.1.1 The coliform group

The coliform group comprises aerobic and facultatively anaerobic, Gram negative, non-spore forming, short-rod bacteria that ferment lactose with production of acid and gas at 35-37 °C, within 24-48 hours. They also possess the enzyme β -D-galactosidase (Gleeson and Gray, 1997). The group comprises the following genera: *Klebsiella*, *Enterobacter*, *Serratia*, *Citrobacter* and *Escherichia* (Edberg, *et al.* 2000). The coliform group has been widely used to assess the bacteriological quality of drinking and recreational water for many years. Among the coliform group, *Escherichia coli*, a thermotolerant coliform, was found to be a more specific indicator of faecal contamination. It is suggested that *E. coli* is the only member of the coliform group that is exclusively of faecal origin. It is abundant in faeces (Gleeson and Gray, 1997; Edberg, *et al.* 2000; Leclerc *et al.*, 2001). *E. coli* can ferment lactose with acid and gas at 44 °C within 24 hours, and can be differentiated from other coliforms by displaying β -glucuronidase activity and lack of urease activity (Toranzos and McFeters, 1997; Gerba, 2000). Unlike other coliform bacteria, *E. coli* does not normally grow in temperate aquatic environments (Edberg, *et al.* 2000). Baudišová (1997) suggested that the use of *E. coli* as an indicator of faecal pollution has more advantages than the use of total coliforms. In addition, *E. coli* was found to survive as long as, or longer than, *Salmonella* species in freshwater sediments, and to show less sensitivity to chlorination in drinking water when compared with *Campylobacter* species; these findings support the use of the organism as an adequate indicator for faecal pollution (Burton, *et al.* 1987; Fish and Pettibone, 1995; Lund, 1996). Furthermore, a direct linear correlation was found between swimming-associated gastrointestinal illness and density of *E. coli*

in recreational waters (Dufour, 1984a; Van Asperen *et al.*, 1998). In the light of these findings, it may be concluded that *E. coli* is the best indicator of faecal contamination of water (Edberg, *et al.* 2000).

4.1.1.2 Faecal streptococci

The faecal streptococci group represents all Gram positive, non-motile and non-spore forming cocci that occur in chains and when tested with Lancefield antisera, they give a positive reaction with group-D. They will grow at 44 °C in a medium that contains bile salts and sodium azide (Gleeson and Gray, 1997). The group comprises species in two genera; *Enterococcus* and *Streptococcus*. These genera were found to be natural inhabitants of the intestinal tract of humans and many animals; Table 4.1 (Godfree, *et al.* 1997).

Table 4.1 Species of the genera *Enterococcus* and *Streptococcus* of faecal origin

Species	Intestinal origin
<i>Enterococcus</i>	
<i>faecium</i>	Man, cattle, pigs, birds
<i>faecalis</i>	Man, cattles, pigs, birds
<i>durans</i>	Man, pigs, birds
<i>Hirae</i>	Man, pigs, birds
<i>Avium</i>	Man, cattle, pigs, birds
<i>gallinarum</i>	Man, birds
<i>cecorum</i>	Cattle, pigs, birds
<i>columbae</i>	Cattle, pigs, birds
<i>Streptococcus</i>	
<i>Bovis</i>	Man, cattle, pigs
<i>equines</i>	Man, cattle, pigs
<i>alactolyticus</i>	Pigs, birds
<i>hyointestinalis</i>	Pigs
<i>intestinalis</i>	Pigs
<i>Suis</i>	Pigs

Adapted from Godfree, *et al.* (1997)

Faecal streptococci are now recognised as a good indicator of faecal pollution because they are present in the faeces of humans and animals (Godfree, *et al.* 1997). They are also a good faecal indicator because they (i) rarely grow in water, and, (ii) are resistant to environmental adverse conditions and chlorination and (iii) generally persist

for long periods in aquatic environments (Gleeson and Gray, 1997). The use of faecal streptococci as indicators may lead to the tracing of a source of faecal contamination by distinguishing between sewage-derived pollution (human) and run-off related pollution (animal) (Godfree, *et al.* 1997). The recommendation that faecal streptococci are a good indicator is also based on the finding of a strong relationship between swimming-related gastrointestinal diseases and number of faecal streptococci in natural bathing waters (Dufour, 1984a; Kay *et al.*, 1994).

4.1.1.3 Faecal coliform:Faecal streptococci ratio

The use of faecal coliforms in conjunction with faecal streptococci has been found to give substantial information about the pollution history of natural waters (Feachem, 1974). Geldreich and Kenner (1969) proposed the use of the faecal coliform:faecal streptococci ratio to locate the source of faecal pollution, i.e. to differentiate between human and animal faecal contamination. They found that a ratio of 4 or greater indicates a human source, while a ratio of 0.7 or less indicates an animal source. According to Geldreich and Kenner (1969), the FC:FS ratio is only valid for the first 24 hours after the bacteria have been released into the water. The difference between faecal coliform and faecal streptococci die off rates was, however, found to give strength to the FC:FS ratio concept, whereby a fall in an initially high FC:FS ratio indicates a human source, whereas a rise in an initially low FC:FS ratio indicates non-human contamination (Feachem, 1975); Table 4.2.

Table 4.2 Faecal source related to FC:FS ratio

FC:FS ratio	Source of pollution
>4.0	Strong evidence that pollution is of human origin
>2.0 <4.0	Good evidence of the predominance of human wastes in mixed pollution
>0.7 <2.0	Good evidence of the predominance of animal wastes in mixed pollution
<0.7	Strong evidence that pollution is of animal origin

Adapted from Gleeson and Gray (1997)

Recent studies showed various limitations to the application of the FC:FS ratio. For instance, the need for sampling within the first 24 hours after discharge; the difference in die-away rates between faecal coliforms and faecal streptococci; differences in die-away rates between individual faecal streptococci; the impact of the methods that are used to detect faecal streptococci. Thus the reliability of the FC:FS ratio has been questioned (Howell, *et al.* 1996; Gleeson and Gray, 1997; Toranzos and McFeters, 1997; Ashbolt, *et al.* 2001; Horan, 2003).

4.1.1.4 *E. coli* and faecal streptococci, the ideal faecal indicators?

The use of *E. coli* and/or faecal streptococci as indicators of faecal pollution is based on their fulfilling many of the criteria that should be found in indicator organisms. It has been suggested that the indicator of choice should meet a number of criteria, which according to Dufour (1984b) are the ideal characteristics for an indicator of faecal pollution. These criteria are that the indicator should:

- 1- be present where pathogens are;
- 2- be unable to grow in aquatic environments;
- 3- be more resistant to disinfectant than pathogens;
- 4- be easy to isolate and enumerate;
- 5- be applicable to all types of water;
- 6- not be subject to antibiosis;
- 7- be absent from sources other than faeces or be exclusively associated with faeces;
- 8- occur in greater number than pathogens;
- 9- vary in density in direct relationship to the degree of faecal contamination;
- 10- show a correlation between indicator density and the health hazard posed by a given type of pollution.

Unfortunately, neither *E. coli* nor faecal streptococci meet all the above criteria. Faecal streptococci fulfil more of the criteria than *E. coli*. By and large, it is suggested

that the use of both indicators is valuable because it allows the assessment of the significance of doubtful results from one method or the other. For example, if coliform bacteria are abundant in the absence of *E. coli*, the presence of faecal streptococci confirms faecal pollution of the water under examination (HMSO, 1984). Table 4.3 shows which of the suggested criteria of an ideal indicator are met by *E. coli* and faecal streptococci (Dufour, 1984b).

Table 4.3 Ideal characteristics of indicators of faecal contamination met by *E. coli* and faecal streptococci

Ideal characteristics	<i>E. coli</i>	Faecal streptococci
Present when pathogens are	Yes	Yes
Unable to grow in aquatic environments	Yes	Yes
More resistant to disinfectant than pathogens	No	Yes
Applicable to all types of water	No	Yes
Easy to isolate and enumerate	Yes	Yes
Not subject to antibiosis	?	?
Occurs in greater numbers than pathogens	Yes	Yes
Density of indicator has a direct relationship to degree of faecal contamination	Yes	Yes
Indicator density correlates with health hazard from given type of pollution	Yes	Yes

Adapted with modification from: Dufour (1984b)

4.1.1.5 Sulphite-reducing clostridia

Sulphite-reducing clostridia are Gram positive, spore-forming, strictly anaerobic, non-motile rods that are largely faecal in origin (Gleeson and Gray, 1997). *Clostridium perfringens* (formerly *Cl. welchii*) is the most important member of the group. It is directly associated with gas gangrene and wound infection and it also causes food poisoning (Timbury, *et al.* 2002) and it is the species of the clostridia that is most present in the faeces of human and warm-blooded animals although, in lower numbers than *E. coli* and faecal streptococci (HMSO, 1984). The spores of *Cl. perfringens* are

heat resistant (75 °C for 15 minutes), persist for long periods in the environments and are very resistant to disinfectant (Glesson and Gray, 1997). The use of *Cl. perfringens* as an indicator of faecal pollution was suggested for the first time by Hudson in the late 1800s (Toranzos and McFeters, 1997). A number of studies have been conducted to ascertain the efficacy of this organism as an indicator of faecal contamination. Consequently, *Cl. perfringens* has been suggested as a very sensitive indicator of the sedimentation and movement of sewage solids, and as an indicator of distant sources of faecal contamination (Emerson and Cabelli, 1982; Skanavis and Yanko, 2001). This suggestion was supported by Sorensen, *et al.* (1989), who detected *Cl. perfringens* spores at a distance of 10 km from a wastewater treatment plant. Furthermore, the presence of enteric viruses and parasite cysts, such as *Giardia* and *Cryptosporidium*, was significantly correlated with the presence of *Cl. perfringens* spores in surface water. This confirms the suitability of *Cl. perfringens* as an indicator of the presence of pathogens of faecal origin. In this context, the spores of *Cl. perfringens* were suggested as the indicator of choice in investigation of virus and cyst removal and the overall efficiency of wastewater treatment (Payment and Franco, 1993). The spores of *Cl. perfringens* were also suggested as the most useful indicator for the presence of *Giardia* by Ferguson, *et al.* (1996). In general, if the spores of *Cl. perfringens* are detected in a water sample, where *E. coli* and faecal streptococci are not, its presence may imply a remote or intermittent faecal pollution. That is, of pollution that has occurred in the past, since when *E. coli* and faecal streptococci have died, but not the spores of *Cl. perfringens* (HMSO, 1984; Toranzos and McFeters, 1997; Horan, 2003). Such distant pollution might be of less significance in the sense of direct risks to public health (HMSO, 1984), and might explain the limited use of this organism in setting safe recreational water quality standards (e.g. HMSO, 1969).

4.1.2 Recreational Water Quality

The term 'recreational water' refers to marine and freshwater bodies that are used for public recreational activities. These activities are varied; some involve a total contact of the water with the body, such as swimming and diving, while some activities involve lesser contact, such as fishing, power-boating and water skiing. According to the U.S. Environmental Protection Agency (U.S EPA), the former activities are defined as 'primary contact activities', whilst the latter are defined as 'secondary contact activities' (EPA, 2000). The microbiological quality of recreational waters is of concern. This is due to the increased number of swimming-associated outbreaks that are being reported (Dufour, 1984b). The assessment of recreational water quality may be based on four approaches: (i) a sanitary survey, (ii) epidemiological studies, (iii) faecal coliform limits, and (iv) the presence of pathogens (Tobin and Ward, 1984). These factors have been taken into consideration in several studies, which have determined the hazards of faecally-polluted recreational waters. Consequently, governmental bodies and public health authorities around the world have been able to set guidelines for recreational water quality to ensure that people who have primary contact with water are safe from contracting diseases. These guidelines are summarised in Table 4.4.

Table 4.4 Guidelines for recreational water quality standards

Country/Agency	Criterion/Standard	Regime ^e
World Health Organisation (WHO)	Faecal streptococci ^f 200/100 ml	
U.S Environmental Protection Agency (U.S. EPA)	<i>E. coli</i> ^a 126/100 ml <i>Enterococci</i> ^a 33/100 ml <i>Enterococci</i> ^b 35/100 ml	5 samples/30 days
European Union (EU)	<i>E. coli</i> 100/100 ml ^c Faecal streptococci 100/100 ml ^d <i>Salmonella</i> 0/100 ml Enteroviruses pfu 0/10000 ml	2 samples/fortnightly
Ontario, Canada	Coliforms ≤1000/100 ml <i>E. coli</i> ≤100/100 ml	5 samples/30 days

Sources: Tobin and Ward (1984); EU (1994); El-Shaarawi and Marsalek (1999); Gerba (2000); EPA (2000); WHO (2003).

^aFresh waters only.

^bMarine waters only.

^cValues are Guidelines (Imperative value 2000/100 ml).

^dValues are Guidelines (Imperative value 400/100 ml).

^eGeometric mean.

^f95th percentile approach.

As can be seen from Table 4.4, there is no universal agreement on standards. The EU directive, however, is comprehensive because specified pathogens as well as indicators are taken into consideration. The detection of specific pathogens is thought to be important, particularly when they become predominant in a recreational area (Tobin and Ward, 1984). The EU directive has been considered to be more restrictive than other schemes, this conclusion was realised by Marino, *et al.* (1995), who compared the directives proposed by EU and those of the WHO. Recently, the European Parliament has proposed new tightened guidelines for safe bathing waters and these are still under evaluation (EU, 2004).

According to the U.S. EPA, the guidelines are for application to recreational sites where primary contact occurs. No risk-based water quality criteria for protection from secondary contact have been published. However, it was recommended that

guidelines for primary contact waters should be applied too for secondary contact waters, unless new criteria with less firm restrictions are adopted (EPA, 2000).

4.1.3 Waterfowl and recreational water quality

Waterfowl have been reported to excrete large numbers of faecal indicator bacteria (Ashbolt, *et al.* 2001). The average density of faecal coliforms, and faecal streptococci in one gram dry weight duck faeces may be around 4.9×10^{11} and 6.3×10^8 respectively (Obiri-Danso and Jones, 1999a), whereas the average number of *E. coli* in a gram dry weight of mallard faeces was suggested to be around 3.3×10^7 (Taylor, 2003). The association of wild fowl particularly ducks and geese, with the contamination of water bodies that are used for recreation has been described. In 1978, Vilas Park Beach on Lake Vingra, Madison, Wisconsin, USA, was closed after high concentrations of faecal coliforms were found. These high concentrations were attributed to approximately 100 to 200 mallard ducks, which were residing permanently in the beach area (Standridge, *et al.* 1979). Likewise, in the UK, mallard ducks together with other wild birds were found to be, in part, responsible for high densities of faecal coliforms at Morecambe Bay, which failed to comply with EU guidelines for safe bathing water (Jones, 2002).

Waterfowl were also reported to harbour bacteria in their intestinal tract that are potential human pathogens. *Campylobacter* and *Salmonella* are major causative agents of bacterial gastroenteritis in both the UK and the USA (Rusin, *et al.* 2000; Timbury, *et al.* 2002). Both organisms have been successfully isolated from the faeces of ducks and geese (Luechtefeld, *et al.* 1980; Pacha, *et al.* 1988; Risdale, *et al.* 1998; Feare, *et al.* 1999; Aydin, *et al.* 2001; Dieter, *et al.* 2001; Kassa, *et al.* 2001; Refsum, *et al.* 2002a). The carriage of these pathogens by ducks and geese suggests that waterfowl may act as a reservoir for their transmission through the contamination of water. Faecally

contaminated water is a potential source of *Campylobacter* and *Salmonella* and may be a vehicle for their transmission to domestic animals and humans (Bolton, *et al.* 1987; Melloul and Hassani, 1999; Thomas, *et al.* 1999a).

4.1.4 Aims

Although the impact of ducks and geese on the microbiological water quality of bathing sites has been described, little is known about the impact of ducks and geese on non-bathing recreational water, particularly village ponds. High numbers of waterfowl are often found on village ponds in England. These ponds and their environs are typically used as recreational and amenity sites. Visitors and residents frequently encourage waterfowl by feeding them, and direct contact with faecal material or contaminated water is likely to occur. In response to an increased public concern about transmission of infectious diseases through human contact with faecally contaminated water, this chapter aimed to test the hypothesis that waterfowl have a negative impact on the bacteriological quality of some amenity village ponds in East Yorkshire, north-east England.

4.2 MATERIALS AND METHODS

4.2.1 Sites and sampling

The study sites were seven roadside village ponds in East Yorkshire, NE England. Two ponds were used as the main sampling stations, from which water and sediment samples were collected at monthly intervals from June 2001 to January 2002. One of these ponds, at South Dalton, had dense populations of waterfowl, while the other at Holme-on-Spalding-Moor had no waterfowl. The five other ponds were used as supplementary sampling stations. Water samples, but not sediments, were collected from these five ponds on three sampling occasions, one in August, one in October and

one in November 2001. Three of these five ponds had dense populations of ducks and geese, these were at Brantingham, Garton-on-the-wolds and Little Weighton; while the other two ponds had no ducks and geese, these were at Bentley and Sancton. Detailed descriptions of these sites and their environs were given in Chapter 2.1. Water and sediment samples were collected from sampling sites using the procedures described in Chapter 3.1.

4.2.2 Abiotic variables

Temperature, pH, conductivity and turbidity of pond water were measured in all seven ponds as described in Chapter 3.2.

4.2.3 Bacteriological assays

Protocols for faecal indicators (*E. coli*; faecal streptococci and *Clostridium perfringens*) and human pathogens (*Campylobacter* and *Salmonella*) from water and sediments were based on those used by the Public Health Laboratory Service as described in Chapter 3.3.1 and 3.3.2. Counts of culturable heterotrophic bacteria from water and sediment were made using the spread plate method as described in Chapter 3.3.3. Counting of total bacteria in water samples was performed using epifluorescence microscopy, bacteria were stained with DAPI as described in Chapter 3.3.4.

4.2.4 Statistical analysis

The Chi-square test (χ^2) was used to test agreement between observations and the null hypothesis that when ponds are compared pair-wise the numerical abundance of bacteria should equally often be lower as greater in the pond with waterfowl. Spearman's correlation coefficient was used to test the relationship between faecal indicators and waterfowl numbers. Non-parametric statistical methods were used because the data were mostly non-normally distributed. Median values were used because extreme values in some cases skewed the means, hence the mean values poorly

represented the whole set of observations. Statistical analysis was done using SPSS for Windows (version 10).

4.3 RESULTS

Results for abiotic variables and waterfowl numbers for all seven ponds are shown in Table 4.5. Absorbance values show that ponds without waterfowl were much less turbid than those with waterfowl. Otherwise there were no obvious differences between the ponds with water fowl and those without them.

Counts of faecal indicators, heterotrophic plate counts and DAPI counts for water samples from the ponds at South Dalton and Holme-on-Spalding-Moor are given in Table 4.6. Values are mostly expressed as cfu 100 ml⁻¹ for the sake of conformity with EU guidelines for safe recreational water. Faecal indicators, particularly *E. coli* and faecal streptococci, were more abundant at South Dalton than at Holme-on-Spalding-Moor. Heterotrophic plate counts and DAPI counts were also significantly higher at South Dalton than at Holme-on-Spalding-Moor.

Results of faecal indicator counts and heterotrophic plate counts on sediments from the ponds at South Dalton and Holme-on-Spalding-Moor are shown in Table 4.7. Values are given per gram dry weight of sediment. *E. coli* and faecal streptococci were mostly higher at South Dalton than at Holme-on-Spalding-moor although this was not necessarily shown by the median values. Values for heterotrophic plate counts showed no difference between the ponds.

Values for sediments expressed per unit volume of sediment are shown in Table 4.8. *E. coli* was significantly more abundant at South Dalton, faecal streptococci were also more abundant at South Dalton but less markedly so than *E. coli*. Heterotrophic plate counts showed similar results as when expressed per gram dry weight of sediment, i. e. there was no significant difference between the ponds.

Counts of faecal indicators in water from the five supplementary ponds are given in Table 4.9. Abundances were significantly higher in ponds with dense populations of waterfowl.

Table 4.5 Abiotic variables and waterfowl numbers for all ponds, June 2001-January 2002

	Bentley [†]	Brantingham [‡]	Garton-on-the-wolds [‡]	Holme-on-Spalding-Moor [†]	Little Weighton [‡]	Sancton [‡]	South Dalton [†]
Temperature (°C)	12 (7-15)	14 (8-20)	13 (8-18)	12 (4-19)	13 (7-18)	13 (11-13)	12 (4-19)
pH	6.7 (6.5-6.9)	7.1 (6.5-8.0)	6.9 (6.4-7.7)	7.0 (6.2-7.6)	6.8 (6.7-6.9)	6.6 (6.4-6.8)	7.2 (6.3-8.2)
Absorbance (580 nm) cm ⁻¹	0.017 (0.008-0.024)	0.237 (0.034-0.58)	0.149 (0.067-0.31)	0.07 (0.0197-0.23)	0.235 (0.048-0.58)	0.002 (0.001-0.004)	0.14 (0.08-0.39)
Conductivity (µS cm ⁻¹)	533 (415-744)	641 (410-913)	788 (648-866)	815 (630-917)	594 (400-922)	1084 (1002-1135)	480 (440-539)
Waterfowl*	0 (0-0)	34 (13-75)	39 (35-45)	0 (0-0)	24 (22-25)	0 (0-0)	31 (14-41)

Values are mean (with range), [†] n = 7 samples, [‡] n = 3 samples, * refers to ducks and geese only.

Table 4.6 Faecal indicators, heterotrophic plate counts and total bacterial counts (DAPI) for water samples from South Dalton and Holme-on-Spalding-Moor, June 2001-January 2002

	Median (range) n		<i>P</i>
	South Dalton	Holme-on-Spalding-Moor	
<i>E. coli</i> ($\times 10^3$ cfu 100 ml ⁻¹)	81 (3-180) 7	4 (0.0066-11) 7	< 0.01
Faecal streptococci ($\times 10^3$ cfu 100 ml ⁻¹)	34 (1.6-490) 6	11 (0-24) 7	< 0.1
<i>Cl. perfringens</i> ($\times 10^3$ cfu 100 ml ⁻¹)	6.4 (0.11-100) 6	3.9 (0.01-60) 6	NS
Heterotrophic plate count ($\times 10^5$ cfu ml ⁻¹)	2.5 (1.6-3.3) 5	0.83 (0.32-2.4) 5	< 0.05
Total bacteria ($\times 10^7$ ml ⁻¹)	4.7 (3.4-7.4) 6	1.2 (1.1-3.0) 6	< 0.05

n = number of samples, *P* values are from the χ^2 test; NS = *P* > 0.1.

The null hypothesis is that there is no difference in bacterial abundance between the two ponds. If this is so we expect that for each bacterial variable, the higher count will be at South Dalton as often as at Holme-on-Spalding Moor. The χ^2 test is used to test whether the expected observations are significantly different from the observed.

It is also possible to do a χ^2 test that pools all the observations, i.e. total observations = 30, expected observations = 15 and 15; observed = 25 and 5, $\chi^2 = 13.2$, *P* < 0.01.

Table 4.7 Faecal indicators and heterotrophic plate counts for sediment samples from South Dalton and Holme-on-Spalding-Moor, per gram dry weight of sediment, June 2001-January 2002

	Median (range) n		<i>P</i>
	South Dalton	Holme-on-Spalding-Moor	
<i>E. coli</i> ($\times 10^3$ cfu g ⁻¹)	124 (0.90-350) 7	32 (0-331) 7	< 0.1
Faecal streptococci ($\times 10^3$ cfu g ⁻¹)	33 (0.28-640) 7	33 (0-1470) 7	< 0.1
<i>Cl. perfringens</i> ($\times 10^3$ cfu g ⁻¹)	218 (2.84-640) 6	130 (1.41-2581) 6	NS
Heterotrophic plate count ($\times 10^6$ cfu g ⁻¹)	11 (5.94-14.5) 5	12 (7.2-18.0) 5	NS

n = number of samples, *P* values from χ^2 test, NS = *P* > 0.1.

Table 4.8 Faecal indicators and heterotrophic plate counts for sediment samples from South Dalton and Holme-on-Spalding-Moor, per unit volume of sediment, June 2001-January 2002

	Median (range) n		<i>P</i>
	South Dalton	Holme-on-Spalding-Moor	
<i>E. coli</i> ($\times 10^3$ cfu ml ⁻¹)	24 (0.14-105) 7	4.6 (0-9.3) 7	< 0.01
Faecal streptococci ($\times 10^3$ cfu ml ⁻¹)	9.9 (0.3-113) 7	0.65 (0-49.6) 7	< 0.1
<i>Cl. perfringens</i> ($\times 10^3$ cfu ml ⁻¹)	38 (0.65-113) 6	17.3 (0.6-87.1) 6	< 0.05
Heterotrophic plate count ($\times 10^6$ cfu ml ⁻¹)	1.61 (1.14-2.32) 5	1.71 (1.3-2.75) 5	NS

n = number of samples, *P* values from χ^2 test, NS = *P* > 0.1.

Table 4.9 Faecal indicator bacteria in water from five additional ponds, August-November 2001

	Median (range) n		<i>P</i>
	Ponds with waterfowl [†]	Ponds without waterfowl [‡]	
<i>E. coli</i> ($\times 10^3$ 100 ml ⁻¹)	21 (8.8-300) 7	0.009 (0.003-1.5) 6	< 0.01
Faecal streptococci ($\times 10^3$ 100 ml ⁻¹)	1.6 (1.3-300) 9	0.009 (0.001-0.36) 6	< 0.01
<i>Cl. perfringens</i> ($\times 10^3$ 100 ml ⁻¹)	80 (0.16-930) 9	0.01 (0.009-16.6) 6	< 0.01

P values from the Mann-Whitney U-test, n = number of samples.

[†] Ponds at Brantingham; Garton-on-the-Wolds and Little Weighton.

[‡] Ponds at Bentley and Sancton.

The null hypothesis is that the median values do not differ between the ponds with waterfowl and ponds without waterfowl.

The U-test clearly indicates that the null hypothesis can be rejected, hence more powerful statistical tests were not required.

Table 4.10 summarises the correlations between faecal indicators and waterfowl numbers in village ponds. Values of Spearman's correlation coefficient showed that there were significant relationships between faecal indicators in pond water and waterfowl number and waterfowl number per unit pond area. The closest relationship (i.e. with greatest significance) was between *E. coli* and number of waterfowl. The relationship between abundance of *E. coli* and bird numbers in all seven ponds is illustrated in Fig. 4.1.

Faecal indicators and heterotrophic bacterial abundance in water was compared with that in the sediment at South Dalton and Holme-on-Spalding-Moor. Values were expressed as cfu ml⁻¹ for all samples. Tables 4.11 and 4.12 summarise the results. Faecal indicators and heterotrophic bacteria were significantly higher in sediment than in water at both ponds.

All presumptive colonies of *E. coli* and faecal streptococci that were tested confirmed as positive, but only about 50 % of presumptive *Cl. perfringens* were positive, hence these results for *Cl. perfringens* represent presumptive isolates.

Table 4.13 shows the results for the detection of *Campylobacter* and *Salmonella* in water and sediment from all ponds. *Campylobacter* and *Salmonella* assays were performed on water from all seven ponds and on sediment from South Dalton and Holme-on-Spalding-Moor. *Salmonella* was not recovered from water samples from any pond on any sampling occasion. However, it was recovered from sediment samples at South Dalton during summer (June, July, August). *Campylobacter* was not recovered from water or sediment on any sampling occasion.

Table 4.10 Relationship between faecal indicators and waterfowl number and waterfowl per unit pond area, June 2001-January 2002

	Relationship with number of waterfowl		Relationship with waterfowl per unit pond area	
	r_s	P	r_s	P
<i>E. coli</i> *	0.84	< 0.01	0.75	< 0.01
Faecal streptococci [†]	0.63	< 0.01	0.49	< 0.01
<i>Cl. perfringens</i> *	0.42	< 0.05	0.50	< 0.01

Values are Spearman's correlation coefficients, a two-tailed test was used.

*n = 27 samples from 7 ponds; [†]n = 29 samples from 7 ponds.

Note that this analysis allowed the pooling of data from all ponds (i.e. data from Tables 4.6 and 4.9).

Pooled data for *E. coli* in pond water is also used in Fig. 4.1.

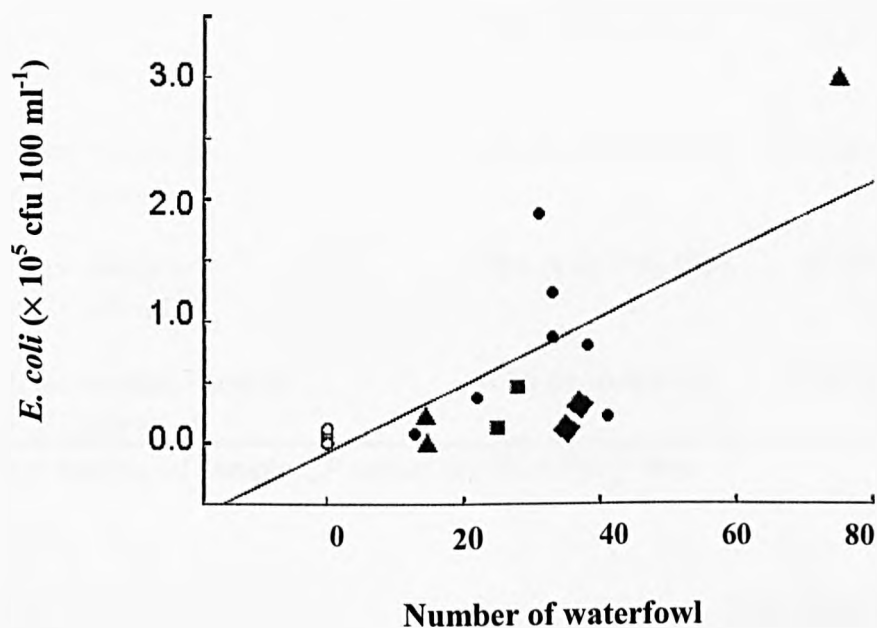


Fig. 4.1 Relationship between *E. coli* and abundance of waterfowl (Spearman's rank correlation coefficient $r_s = 0.84$, $n = 27$, $p < 0.01$) in seven amenity village ponds in East Yorkshire, England, June 2001 to January 2002. Ponds with waterfowl at; (▲) Brantingham (pond area 240 m²), (■) Garton-on-the-wolds (1,800 m²), (◆) Little Weighton (640 m²), (●) South Dalton (2,410 m²). Ponds without waterfowl at; (○) Bentley (220 m²), (○) Holme-on-Spalding-Moor (1,530 m²) and (○) Sancton (85 m²). A total of 13 samples represented ponds without waterfowl: most of the symbols are superimposed.

Table 4.11 Comparison between faecal indicators and heterotrophic bacterial abundance in water and sediment at South Dalton pond, June 2001-January 2002

	Median (range) n		<i>P</i>
	Water	Sediment	
<i>E. coli</i> ($\times 10^3$ cfu ml ⁻¹)	0.81 (0.003-1.8) 7	24 (0.14-105) 7	< 0.01
Faecal streptococci ($\times 10^3$ cfu ml ⁻¹)	0.35 (0.0016-4.92) 6	9.9 (0.0043-113) 7	< 0.01
<i>Cl. perfringens</i> ($\times 10^3$ cfu ml ⁻¹)	0.064 (0.00114-10) 6	38 (0.65-113) 6	< 0.05
Heterotrophic bacteria ($\times 10^6$ cfu ml ⁻¹)	0.25 (0.16-0.33) 5	1.69 (1.14-2.32) 5	< 0.05

n = number of samples, *P* values are from the χ^2 test.

Table 4.12 Comparison between faecal indicators and heterotrophic bacterial abundance in water and sediment at Holme-on-Spalding-Moor pond, June 2001-January 2002

	Median (range) n		<i>P</i>
	Water	Sediment	
<i>E. coli</i> ($\times 10^3$ cfu ml ⁻¹)	0.049 (0.000066-0.115) 7	4.6 (0-9.25) 7	< 0.1
Faecal streptococci ($\times 10^3$ cfu ml ⁻¹)	0.011 (0-0.24) 7	0.65 (0-49.6) 6	< 0.1
<i>Cl. perfringens</i> ($\times 10^3$ cfu ml ⁻¹)	0.039 (0.0009-0.60) 6	17.3 (0.24-87.1) 6	< 0.05
Heterotrophic bacteria ($\times 10^6$ cfu ml ⁻¹)	0.083 (0.032-0.244) 5	1.71 (1.3-2.75) 5	< 0.05

n = number of samples, *P* values from χ^2 test.

Table 4.13 Results of *Campylobacter* and *Salmonella* assays on water and sediment from all seven ponds, June 2001-January 2002

Location of pond	n:p (%)	
	<i>Campylobacter</i>	<i>Salmonella</i>
South Dalton (water [†])	7:0 (0)	7:0 (0)
South Dalton (sediment [*])	7:0 (0)	7:3 (43) [‡]
Hole-on-Spalding-Moor (water [†])	7:0 (0)	7:0 (0)
Holme-on-Spalding-Moor (sediment [*])	7:0 (0)	7:0 (0)
Bentley (water [†])	3:0 (0)	3:0 (0)
Brantingham (water [†])	3:0 (0)	3:0 (0)
Garton-on-the-Wolds (water [†])	3:0 (0)	3:0 (0)
Little Weighton (water [†])	3:0 (0)	3:0 (0)
Sancton (water [†])	3:0 (0)	3:0 (0)

n = total number of samples; p = total positive (percentage of positive samples is given in brackets).

[†] A volume of 1000 ml of water was used.

^{*} A volume of 5.0 ml of sediment was used.

[‡] Total number of *Salmonella* from all three positive samples were seven isolates

4.4 DISCUSSION

The principal aim of the study described in this chapter was to test the hypothesis that waterfowl, largely ducks and geese, have an adverse influence on the microbiological quality of village ponds, which are amenity sites, and to consider the possibility of these sites being vehicles for the dissemination of water-borne pathogens.

4.4.1 Abiotic variables

The abiotic variables that were measured did not reveal much information about the impact of waterfowl because few differences were observed between ponds with ducks and geese and those without. However, the A_{580} measurement did show that the water was more turbid in ponds with waterfowl (Table 4.5). This was probably due to the disturbance of bottom sediment by waterfowl. The abundance and diversity of submerged and marginal aquatic vegetation were also observed to be very much less in ponds with waterfowl (Chapter 2). This is probably because ducks and geese ate these plants. Thus, it can be concluded that waterfowl have a negative impact on the aesthetic qualities of village ponds.

4.4.2 Faecal indicators and water quality

Faecal indicators were used to measure the extent of microbiological contamination. The presence of abundant *E. coli* and faecal streptococci in water samples from South Dalton but not at Holme-on-Spalding-Moor (Table 4.6) suggests that waterfowl are a likely cause of contamination; duck faeces contain about 4.9×10^{11} , 6.3×10^8 faecal coliforms, and faecal streptococci respectively (Obiri-Danso and Jones, 1999a) and 3.3×10^7 *E. coli* per gram dry weight (Taylor, 2003). In the absence of ducks and geese at Holme-on-Spalding-Moor, the low and very variable background

numbers of faecal indicators that were recorded (Table 4.6) perhaps originated from small family groups of coot (*Fulica atra*) and moorhen (*Gallinula chloropus*) and/or from rainfall-related run-off from adjacent roads and agricultural land. Run-off, especially from land carrying livestock, may be an appreciable source of faecal indicators to natural waters (Fernández-Alvarez *et al.*, 1991; Jones, 2002). However, the pond at South Dalton, which had more faecal indicators was not more liable to contamination by run-off, hence the waterfowl were probably responsible for the high concentration of indicator bacteria.

Analysis of sediment samples gave similar results as for water. The abundant *E. coli* and faecal streptococci found in South Dalton sediment (Tables 4.7; 4.8), may have resulted from the settling of faecally-derived material in the bottom sediment. Although the median count of faecal streptococci was similar at South Dalton and Holme-on-Spalding-Moor, this was a feature of the use of median values. There was a statistically significant difference in the likelihood that samples from South Dalton had higher counts than in paired samples from Holme-on-Spalding-Moor.

Faecal indicators were also found to be abundant in other ponds that had a dense population of waterfowl (Table 4.9). The small number of faecal indicators that were recorded in ponds without waterfowl may again have been from small populations of coot and moorhens and/or from run-off water. These results strongly suggest that waterfowl were the leading cause of high counts of faecal indicators observed in some of the village ponds in East Yorkshire.

Correlation analysis showed that there was, sometimes, a closer relationship between faecal indicators in pond water and absolute number of waterfowl than with waterfowl per unit pond area (Table 4.10). This was probably due to non-uniform horizontal distribution of both waterfowl and bacteria. Waterfowl gather to be fed and will defecate at ponds margins adjacent to roadsides; i. e. where the samples were taken.

Although *Cl. perfringens* is suggested as a promising new faecal indicator in the context of water treatment (Payment and Franco, 1993), it seems not to convey much information about the impact of waterfowl on the microbiological quality of pond water. The lack of a significant difference between *Cl. perfringens* in the water at South Dalton and Holme-on-Spalding-Moor (Table 4.6) and its relatively weak correlation with waterfowl abundance (Table 4.10) suggests that waterfowl were not necessarily the principal source of presumptive *Cl. perfringens*. Since the spores of *Cl. perfringens* persist for long periods the presence of *Cl. perfringens* may indicate distant or past pollution (Horan, 2003). It is possible that contaminated soil might be a significant source.

4.4.3 Heterotrophic plate counts and counts of total bacteria in pond water

The greater abundance of culturable heterotrophic bacteria and of total bacteria by DAPI counts in the water at South Dalton than at Holme-on-Spalding-Moor (Table 4.6) was probably related to higher levels of organic matter. This is very likely related to enrichment caused by defecation by the dense population of waterfowl.

The greater abundance of culturable heterotrophic bacteria in sediment than in water at both South Dalton and Holme-on-Spalding-Moor (Tables 4.7; 4.8) probably reflected the accumulation of organic matter in sediment at both sites. Waterfowl faeces will contribute to this at South Dalton, but there will be other, diverse, both natural and artificial sources of organic matter at both ponds.

4.4.4 Comparison between bacterial concentrations in water and bottom

sediments of village ponds

The present study has highlighted the role of sediment as a reservoir for faecal indicators. A comparison of the faecal indicator and heterotrophic bacterial concentrations in water and sediment samples was made at South Dalton (Table 4.11) and Holme-on-Spalding-Moor (Table 4.12). At both sites, the abundance of faecal indicators and heterotrophic bacteria was observed to be more than hundred times greater in sediment than in water (Tables 4.11; 4.12). These results support the findings of other studies which have suggested that concentrations of faecal coliforms, heterotrophic bacteria and potential pathogens are many fold higher in aquatic sediments than in the overlying water, both fresh and marine (Tunncliffe and Brickler, 1984; Sherer *et al.*, 1992; Davies *et al.*, 1995; Buckley *et al.*, 1998; Crabill *et al.*, 1999; Niewolak and Opieka, 2000; An *et al.*, 2002; Alm *et al.*, 2003). The prevalence of faecal indicators at high densities in bottom sediments may be attributed, in part, to a combination of sedimentation and adsorption, which provides favourable, non-starvation, conditions to bacteria (Davies *et al.*, 1995). The bacteria may survive for long periods in sediments and so cumulatively become responsible for the occurrence of high densities of faecal bacteria in sediments (Burton *et al.*, 1987).

The impact of sediment-bound bacteria on the microbiological quality of overlying water has been investigated (Grimes, 1980; Crabill *et al.*, 1999; An *et al.*, 2002). The disturbance of bottom sediments that are heavily contaminated with faecal bacteria can result in their resuspension. The activity of waterfowl may do this, as is shown by the high turbidity of ponds with large waterfowl populations (Table 4.5). Consequently, bacterial numbers in overlying water may be reinforced, which creates a further potential health hazard. Thus, in the light of previous and present findings, it is

suggested that public health authorities might consider bottom sediment in their monitoring of recreational waters.

4.4.5 Presence or absence of enteric bacterial pathogens

4.4.5.1 *Campylobacter* species

The apparent absence of *Campylobacter* from the East Yorkshire ponds that had waterfowl (Table 4.13) was unexpected (the hypothesis being tested was that *Campylobacter* would more likely be present in ponds with waterfowl); three post-enrichment isolates on mCCDA from South Dalton, Little Weighton and Brantingham, were culturable on blood agar under both aerobic and microaerobic conditions and hence were not confirmed as *Campylobacter*. In contrast, clinical isolates of *Campylobacter*, supplied by the Public Health Laboratory Service (PHLS) at Hull, did confirm as *Campylobacter*. The absence of *Campylobacter* during summer months from water and sediments of ponds with dense populations of waterfowl, when the water temperature was up to 19 °C, was explicable since this organism shows strong seasonal trends in recovery rates, being present in large numbers only during winter months (Bolton *et al.*, 1987; Carter *et al.*, 1987; Mawer, 1988; Pianetti *et al.*, 1998; Obiri-Danso and Jones, 1999a). However, the absence of this bacterium from the water and sediments of ponds with waterfowl during winter months remains puzzling. The standard PHLS protocol used in the present study required the filtration of 1000 ml water samples. It is suggested that the seeding of enrichment cultures with the residue from a large volume of water, or with 5.0 ml of microbially-rich sediment, may have led to out-competition of *Campylobacter*, by non-specific heterotrophic bacteria, to the extent that it was unable to grow to detectable levels in the enrichment culture. This hypothesis is tested later in this thesis (Chapter 5).

Campylobacter species have been found in the intestinal tract of many species of waterfowl, including ducks and geese (Luechtefeld *et al.*, 1980; Pacha *et al.*, 1988; Aydin *et al.*, 2001; Kassa *et al.*, 2001). However, Hill and Grimes (1984) failed to isolate *Campylobacter* from water, sediment or from about 50 avian caecal-content samples from Lake Onalaska, a Mississippi River navigation pool that harboured approximately 6.1×10^4 ducks and geese. This finding suggested that *Campylobacter* may exhibit sporadic distribution in response to feeding habits, geographical distribution and mixing of waterfowl with other birds and animals (Hill and Grimes, 1984). Feare, *et al.* (1999) also did not isolate *Campylobacter* from 600 faecal droppings of ducks and geese that were collected from 12 parks located in London, south-east England, Yorkshire and northern England over a period of two years. This variation between studies is perhaps explained by differences in the methods used to detect *Campylobacter*. For this reason some of the literature should be viewed with caution.

4.4.5.2 *Salmonella* species

The recovery of *Salmonella* from South Dalton bottom sediment and its absence from the water (Table 4.13) might be attributed, in part, to concentration through sedimentation and also to greater survivability of *Salmonellae* in sediment than in water (Burton *et al.*, 1987; Fish and Pettibone, 1995; Winfield and Groisman, 2003). This finding agrees with Hendricks (1971) and Van Donzel and Geldreich (1971), who observed higher *Salmonella* recovery rates from bottom sediments than from surface water in diverse rivers. The *Salmonella* at South Dalton was found only in summer (June-August). This supported the findings of Pianetti, *et al.* (1998), who recorded that *Salmonella* is most readily found during summer and autumn.

The absence of *Salmonella* from the water of the East Yorkshire village ponds, particularly these with dense populations of ducks and geese may be because healthy waterfowl do not necessarily harbour enteric pathogens. Damaré, *et al.* (1979) and Hussong, *et al.* (1979) were unable to isolate *Salmonella* from waterfowl, and concluded that healthy birds, away from pollution, do not carry enteric pathogens. If these pathogens were present, they were probably in numbers below the level of detection. In contrast, however, isolation of *Salmonella* from a wide range of apparently healthy birds was reported by Kapperud and Rosef (1983) and Feare, *et al.* (1999).

The present study utilised a presence/absence assay for the enumeration of *Salmonella*, hence, absolute numbers were not determined. Infective doses for salmonellosis in humans are as little as between 10^1 and 10^3 bacterial cells (Blaser and Newman, 1982). Hence, the presence of this potential pathogen in the sediment of South Dalton pond might indicate a possible health threat.

4.5 CONCLUSIONS

Waterfowl are often regarded as a positive feature of amenity ponds in English villages; adults and children enjoy feeding them.

- Significant correlation, however, was found between abundance of *E. coli* in pond water and numbers of waterfowl (Table 4.10).
- Faecal indicators in the water of East Yorkshire ponds with ducks and geese (Tables 4.7; 4.9) always exceeded not only EU guidelines, but the absolute requirement for bathing waters (EU, 1994) of < 2000 *E. coli* per 100 ml and < 400 faecal streptococci per 100 ml.
- These ponds are not used for bathing, nevertheless with these high concentrations of faecal indicators there is a potential hazard from pathogens especially since *Salmonella* was found in some samples.

- Skin contact and accidental ingestion of water is probably best avoided.
- Ponds without waterfowl were better than EU requirements for bathing waters or only intermittently infringed them.

CHAPTER 5

THE RECOVERY OF THERMOPHILIC CAMPYLOBACTERS FROM POND WATER AND SEDIMENT AND THE PROBLEM OF INTERFERENCE BY BACKGROUND BACTERIA IN ENRICHMENT CULTURE

5.1 INTRODUCTION

The association of *Campylobacter* species with mammalian disease has been described since 1913 (Thomas *et al.*, 1999a). However, *Campylobacter* was not recognised as a common cause of diarrhoea in humans until the mid 1970s, when Skirrow (1977) successfully isolated the bacterium for the first time from stool samples of diarrhoea patients. Skirrow developed a selective solid medium that supports the growth of campylobacters and at the same time, suppresses the growth of other bacterial flora. This medium contained blood, vancomycin, polymixin and trimethoprim. Skirrow's success initiated further developments in the methods and culture media that are applied to the isolation of the organism from faecal and environmental samples. These developments have facilitated the elucidation of many problems related to the presence of *Campylobacter* in the environment and its epidemiology.

Epidemiological investigations have suggested that *Campylobacter* is possibly the leading world-wide cause of human enteritis (Thomas *et al.*, 1999a; Oberhelman and Taylor, 2000; Frost, 2001). *Campylobacter* species that are implicated in water-borne and food-borne human infections are *Campylobacter jejuni* and *C. coli* (Percival *et al.*, 2004; Nel and Markotter, 2004). Campylobacters have been found in almost all types of surface waters that are subject to faecal contamination, i. e. rivers (Stelzer *et*

al., 1989), lakes (Arvanitidou *et al.*, 1995), ponds and land drains (Mawer, 1988) as well as in marine waters (Alonso and Alonso, 1993). *Campylobacter* species are wide spread in the environment. Sewage effluents, livestock farming, and wild animals are established as potential sources of campylobacters to environmental waters (Stelzer *et al.*, 1991; Koenraad *et al.*, 1997; Jones, 2001). Contaminated surface water is believed to be a significant vehicle for the transmission of *Campylobacter* to animals and humans (Bolton *et al.*, 1987; Thomas *et al.*, 1999a; Frost, 2001).

5.1.1 Detection and confirmation of campylobacters from environmental waters

The detection of campylobacters from environmental waters poses some problems. The presence of stressed *Campylobacter* cells in small numbers, within a large native bacterial flora, necessitates the application of extremely sensitive recovery methods. The Public Health Laboratory Service (PHLS) standard protocol for the detection of presumptive campylobacters in all types of water (i.e. drinking and recreational) recommends the membrane filtration of 1000 ml samples followed by overnight incubation of the membranes in selective enrichment broth (PHLS, 1998d). The confirmation of presumptive isolates is conventionally based on a range of morphological and biochemical tests. In recent years, PCR has been extensively applied to the detection and identification of a wide range of pathogenic bacteria from environmental and clinical samples (Karch *et al.*, 1995; Toze, 1999; Olsen, 2000), including *Campylobacter* species (Waage *et al.*, 1999). PCR primers that amplify a conserved region of the *flaA* and *flaB* genes have been successfully applied to the detection of *Campylobacter jejuni* and *C. coli* (Oyofe *et al.*, 1992; Wegmüller *et al.*, 1993).

5.1.2 Aims

A previous Chapter (Chapter 4) describes difficulties that were encountered with the isolation of thermophilic campylobacters from water and sediment in a survey conducted on village ponds, which were subject to faecal pollution, primarily from waterfowl, over a period of seven months. Failure to detect campylobacters in environmental waters within which the organism is present can be attributed to many factors, including shortcomings of the isolation method. The aim of this Chapter is to address this problem and to describe a development of the United Kingdom PHLS (PHLS, 1998d) protocol for the detection of presumptive thermophilic campylobacters that allows its application to turbid pond water and bottom sediment, and to evaluate PCR for the rapid confirmation of campylobacters from these habitats.

5.2 MATERIALS AND METHODS

5.2.1 Sites and sampling

The study sites were five roadside village ponds in East Yorkshire, NE England. Three ponds have permanent populations of ducks and sometimes geese. These ponds are at Brantingham, Little Weighton and South Dalton. Waterfowl populations were not observed in the other two ponds. These were at Bentley and Sancton. The pond at Holme-on-Spalding-Moor was found to be completely dry when visited during (October 2003). All ponds receive run-off from adjacent roads, and are amenity sites. Detailed description of these five ponds and their environs is given in Chapter 2.1.

Water and sediment samples were collected in the morning in August, October, and December 2003 from Brantingham, Little Weighton and South Dalton. Water samples only were collected during October and November 2003 from Bentley and Sancton. Surface water samples were collected in sterile polypropylene bottles as

described in Chapter 3.1.1. Samples of the superficial sediment were scooped with the aid of a spoon, from shallow areas, with water depth of less than 10 cm, near the roadside, during September-November 2003, and transferred to sterile 30-ml universal bottles. All samples were kept on ice and in darkness during transportation; microbiological assays were begun on the same day as sampling.

5.2.2 Abiotic variables

Water temperature, pH, conductivity and turbidity of the water of these ponds were measured as described in Chapter 3.2.

5.2.3 Detection of thermophilic campylobacters from water and sediment

A membrane filtration technique was employed for the detection of thermophilic campylobacters in water samples, based on the standard methods for the examination of water as used by the Public Health Laboratory Service (PHLS, 1998d), but a range of volumes was used. Water samples, 10 ml, 100 ml and 1000 ml were membrane filtered and were processed as described in Chapter 3.3.2.1.

Different quantities of sediment were also used for enrichment. 1.0 ml of sediment was transferred to 9.0 ml of sterile, quarter strength Ringer solution. This made a $\times 10$ dilution. Aliquots of 1.0 ml of the ten times diluted sediment, and 1.0 ml and 5.0 ml of undiluted sediment samples were transferred to sterile universal bottles. Preston enrichment broth (32 ml) was added and the procedure was then continued as described in Chapter 3.3.2.1.

5.2.4 Confirmation of presumptive thermophilic campylobacters

Presumptive colonies were subjected to the following confirmatory tests: Gram stain; cell shape; presence of oxidase and catalase; growth on blood agar at 37 °C under microaerobic but not aerobic conditions and PCR.

Gram stain was performed as follows: a loopful of sterile deionised water was placed on a clean slide. A small amount of a presumptive *Campylobacter* colony was mixed with the water drop and spread over a small area of the slide. The smear was left to air-dry at room temperature and was heat-fixed by passing the slide two or three times over the hot top of a Bunsen flame. The heat-fixed smear was covered with crystal violet stain for 60 seconds. The crystal violet (85 % dye content) was gently washed off with deionised water and the smear was then covered with Gram's iodine solution for 60 s. The Gram's iodine was gently washed off with deionised water and the stained smear was decolourised with 59 % ethyl alcohol. The slide was held in a slant position, alcohol was applied drop by drop for 10 to 20 s, or until the solvent flowed colourless. The decolourised smear was finally covered with 0.85 % carbol fuchsin as counter stain for 30 s. The use of diluted carbol fuchsin as counter stain instead of safranin was recommended by the American Public Health Association (APHA, 1998) and other authors (Pacha *et al.*, 1988; Baffone *et al.*, 1995; Stanley *et al.*, 1998), who suggested that some *Campylobacter* strains may not be stained by safranin. The stained smear was then air-dried and examined using oil-immersion, light microscopy at a magnification of $\times 1000$.

The oxidase test was performed as described in Chapter 3.3.2.1. The catalase test was done by adding several drops of 3 % hydrogen peroxide (H₂O₂) to *Campylobacter* colonies growing on mCCDA plates. The observation of bubbles was regarded as a positive reaction.

Growth on blood agar at 37 °C under a microaerobic but not aerobic atmosphere was performed as described in Chapter 3.3.2.1, while PCR was done as detailed in Chapter 3.3.5.1.

5.2.5 Determination of heterotrophic plate counts and coliform counts

Plate counts of culturable heterotrophic bacteria and coliforms in *Campylobacter* enrichment cultures were made on Nutrient Agar (Oxoid, Basingstoke, UK) and MacConkey Agar (Oxoid, Basingstoke, UK) respectively. A volume of 1.0 ml of post enrichment broth was added to 9.0 ml of sterile, quarter strength Ringers' solution. This made a 10^{-1} dilution. This was further diluted to achieve 10^{-2} and 10^{-3} dilutions. Sub-samples (0.1 ml) of 10^{-3} and 10^{-2} dilutions were pipetted onto the surface of dried Nutrient Agar and MacConkey Agar plates respectively. The 0.1 ml sub-samples were spread with the aid of a sterile glass spreader. At least ten replicates plates (Nutrient Agar and MacConkey Agar) were inoculated from each post enrichment broth. Nutrient Agar plates were incubated at 25 °C for 48 hours, whereas MacConkey Agar plates were incubated at 37 °C for 24 hours. Colonies were then counted.

5.3.6 Direct counts of total bacteria

Counts of total bacteria in *Campylobacter* post enrichment cultures were performed by the acridine-orange direct count method (Hobbie *et al.*, 1977). Acridine-orange stock solution was prepared by dissolving 10 mg of the dye in 20 ml of sterile 0.2 µm filtered water. An aliquot of 0.1 ml of acridine-orange stock solution was added to 5.0 ml sub-samples of the enrichment broth which were kept for 40 minutes in darkness at room temperature. Preparation and staining of the filters was carried out as described in Chapter 3.3.4. Counting of bacterial abundances was performed under the

blue light of an Olympus epifluorescence microscope at a total magnification of $\times 1250$. For each sample preparation, more than 600 cells were counted. Sterile 0.2 μm filtered water was used for a blank count.

5.2.7 Statistical analysis

To test the null hypothesis that enrichments from different volumes filtered did not have different concentrations of background bacteria the Kruskal-Wallis non-parametric test for differences among means was used (3 treatments \times 4 samples).

5.3 RESULTS

5.3.1 Abiotic variables

Results for abiotic variables from all five ponds are shown in Table 5.1. It is notable that the ponds with waterfowl had more turbid water (A_{580}); Brantingham pond was especially turbid. Other abiotic variables were not notably different in the ponds with waterfowl, except for temperature which was a reflection of sampling dates.

5.3.2 Detection of thermophilic campylobacters in pond water and sediment

In all twenty six sets of three samples of pond water (volumes 10 ml, 100 ml, 1000 ml) and twelve sets of three samples of sediment (0.1 ml, 1.0 ml, 5.0 ml) were examined for the presence of thermophilic campylobacters. Table 5.2 shows that presumptive thermophilic campylobacters were recovered only from the filtration of 10 ml and 100 ml samples of pond water. Presumptive *Campylobacter* isolates were never recovered following the filtration of 1000 ml samples of pond water. Furthermore, campylobacters were never recovered from the ponds without waterfowl, at Bentley and Sancton, whatever the volume of water filtered.

Results of the recovery of thermophilic campylobacters from sediment samples are shown in Table 5.3. Presumptive *Campylobacter* isolates were only recovered following the enrichment of 0.1 ml and 1.0 ml samples. Presumptive thermophilic campylobacters were never recovered from 5.0 ml sediment samples.

5.3.3 Confirmation of presumptive *Campylobacter* isolates

All 24 presumptive isolates from pond water and sediment were found to be Gram negative, gull-wing shaped, both catalase and oxidase positive, and they grew on blood agar incubated at 37 C under microaerobic, but not aerobic conditions. Hence they were all confirmed as *Campylobacter*. DNA extracted from all 24 presumptive isolates was successfully amplified by PCR. The amplified products were between 340 and 380 bp in length (Figs. 5.1 and 5.2). This reinforced the confirmation that these isolates were thermophilic campylobacters, but did not indicate whether they were *C. jejuni* or *C. coli*.

5.3.4 Heterotrophic plate counts, coliform counts, and acridine-orange direct counts of total bacteria

Heterotrophic plate counts, coliform counts and direct microscopic counts of total bacteria were performed on post enrichment cultures derived from water samples from Brantingham, Little Weighton and South Dalton. All *Campylobacter*-negative cultures, from 1000 ml filtration, had higher levels of heterotrophic bacteria and coliforms than did the positive cultures (10 ml, 100 ml) (Table 5.4). The count of total bacteria in *Campylobacter*-negative enrichments was many fold higher than in positive cultures (Table 5.4). The Kruskal-Wallis test showed that there was significant difference in heterotrophic plate counts, coliforms, and total bacteria between enrichments set up using different volumes filtered (Table 5.4).

Table 5.1 Abiotic variables for all five ponds, August-December 2003

	Mean (range) n				
	Brantingham	Little Weighton	South Dalton	Bentley	Sancton
Temperature (°C)	13 (3-22) 3	12 (4-20)3	13 (3-22) 3	7 (4-9) 2	5 (4-7) 2
pH	6.5 (6.5-6.6) 3	6.6 (6.3-7.0) 3	6.6 (6.5-6.7) 3	6.7 (6.5-6.9) 2	6.6 (6.4-6.8) 2
Absorbance (580 nm)	0.7 (0.5-0.9) 3	0.07 (0.05-0.09) 3	0.03 (0.03-0.03) 3	0.011 (0.008-0.014) 2	0.0025 (0.001-0.004) 2
Conductivity ($\mu\text{S cm}^{-1}$)	1517 (1510-1526) 3	1047 (1033-1057) 3	1517 (1510-1520) 3	580 (415-744) 2	1069 (1002-1135) 2
Waterfowl [†]	20 (19-21) 3	20 (20-20) 3	14 (12-19) 3	0 (0-0) 2	0 (0-0) 2

[†] indicates ducks and sometimes geese, n = total number of samples.

Table 5.2 The recovery of thermophilic campylobacters from pond water, August-December 2003

Location of pond	Volume of water filtered		
	10 ml n:p (%)	100 ml n:p (%)	1000 ml n:p (%)
South Dalton [†]	6:5 (83)	6:6 (100)	6:0 (0)
Little Weighton [†]	6:2 (33)	6:2 (33)	6:0 (0)
Brantingham [†]	6:2 (33)	6:2 (33)	6:0 (0)
Bentley [‡]	4:0 (0)	4:0 (0)	4:0 (0)
Sancton [‡]	4:0 (0)	4:0 (0)	4:0 (0)
Total for all ponds	26:9 (35)	26:10 (39)	26:0 (0)

n = total number of samples.

p = number of positive samples (percentage of positive samples in brackets).

[†] Ponds with waterfowl.

[‡] Ponds without waterfowl.

Table 5.3 The recovery of thermophilic campylobacters from pond sediment, September-November 2003

Location of pond	Volume of sediment used for inoculation		
	0.1 ml n:p (%)	1.0 ml n:p (%)	5.0 ml n:p (%)
South Dalton	4:2 (50)	4:2 (50)	4:0 (0)
Little Weighton	4:0 (0)	4:1 (25)	4:0 (0)
Brantingham	4:0 (0)	4:0 (0)	4:0 (0)
Total for all ponds	12:2 (17)	12:3 (25)	12:0 (0)

n = total number of samples.

p = number of positive samples (percentage of positive samples in brackets).

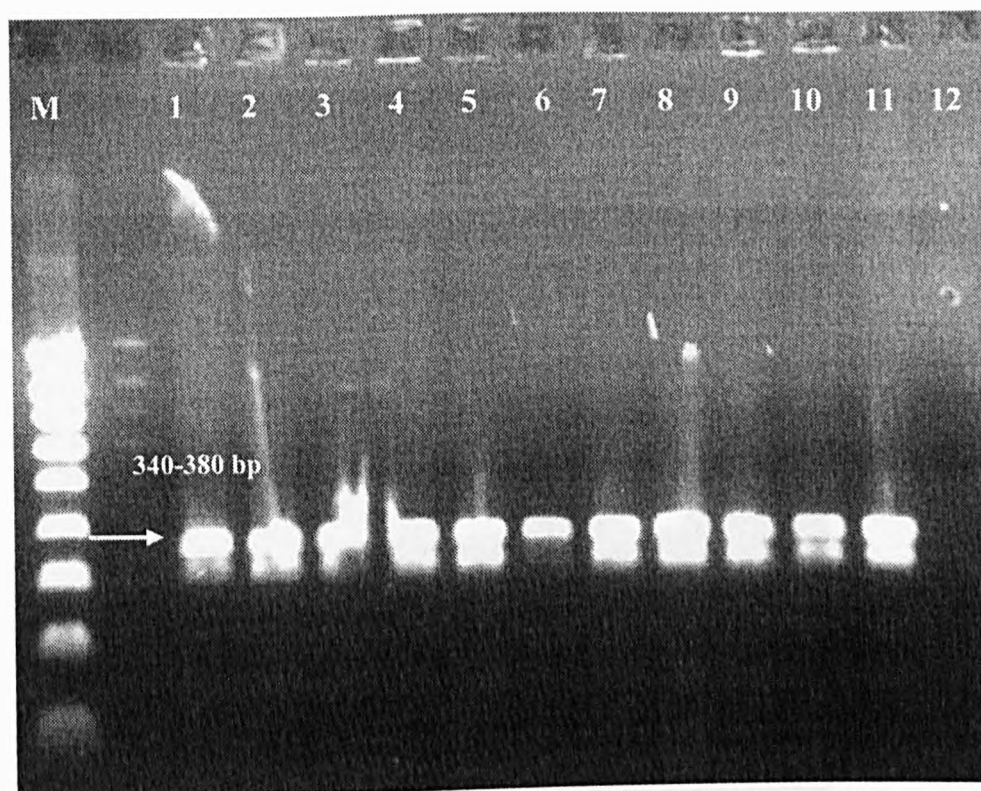


Fig. 5.1 Results of PCR on presumptive *Campylobacter* isolates recovered from pond water. Lane M represents a 100 bp ladder; lanes 1-2 presumptive campylobacters from filtration of 10 ml of South Dalton water; lanes 3-4 from 100 ml of South Dalton water; lanes 5-6 from 10 ml of Little Weighton water; lanes 7-8 from 100 ml of Little Weighton water, lane 9 from 10 ml of Brantingham water, lane 10 from 100 ml of Brantingham water, Lane 11 is a positive control (a clinical *Campylobacter* isolate); lane 12 is a negative control (a DNA-free amplification).

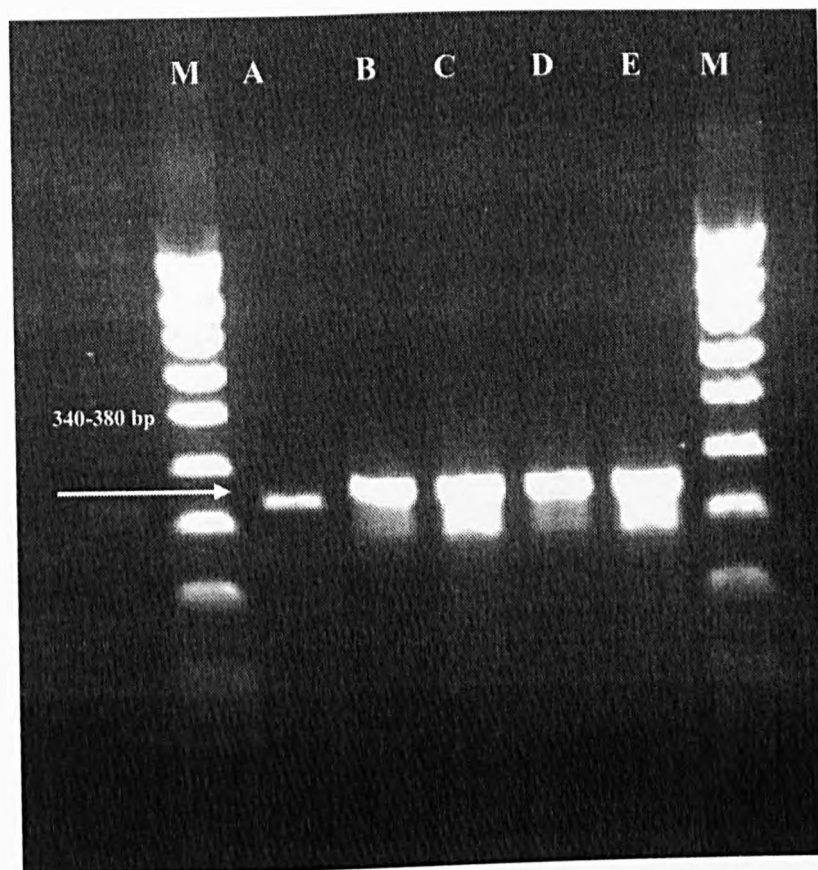


Fig. 5.2 Results of PCR on presumptive *Campylobacter* isolates recovered from pond sediment. Lanes M represents a 100 bp ladder; lane A is a positive control (a clinical *Campylobacter* isolate); lanes B-C presumptive campylobacters from the enrichment of 0.1 ml of South Dalton sediment; lane D from 1.0 ml of South Dalton sediment; lane E from 1.0 ml of Little Weighton sediment.

Table 5.4 Heterotrophic plate counts, coliform counts and acridine-orange direct counts in *Campylobacter* enrichment cultures derived from pond water samples

	Volume of water filtered			<i>P</i>
	10 ml	100 ml	1000 ml	
	Mean (range) n	Mean (range) n	Mean (range) n	
Heterotrophic plate count $\times 10^6$ cfu ml ⁻¹	0.55 (0.013-0.98) 4	1.1 (0.66-1.6) 4	6.4 (1.8-11) 4	< 0.05
Coliforms $\times 10^6$ cfu ml ⁻¹	0.13 (0.12-0.14) 4	0.69 (0.66-0.72) 4	1.6 (1.4-1.8) 4	< 0.05
AODC $\times 10^9$ ml ⁻¹	0.036 (0.032-0.038) 4	0.083 (0.071-0.089) 4	5.8 (5.5-6.1) 4	< 0.05

P is the probability that there is no difference between the number of bacteria in enrichment cultures inoculated with the residue from filtration of different sample volumes (Kruskal-Wallis test).

n = total number of samples.

Culturable and total bacterial counts were performed on *Campylobacter* post enrichment cultures derived from water samples taken from the ponds at Brantingham, Little Weighton and South Dalton.

5.4 DISCUSSION

5.4.1 Detection of thermophilic campylobacters in pond water and sediment

Thermophilic campylobacters are thought to be frequently present in aquatic environments, but their detection can be difficult. This is because the organism may be present in low numbers and/or be sub-lethally injured (i.e. impaired to the extent that they are not readily able to grow on culture media). To overcome these problems, the detection of *Campylobacter* from environmental waters involves initial concentration on a membrane filter followed by incubation of the filters in an enrichment broth (Hunter, 1997; Percival *et al.*, 2004).

In the present study, a combination of a selective enrichment procedure and selective plating was used. This approach has been reported to increase the recovery of campylobacters from environmental waters (Rosef *et al.*, 2001). Preston enrichment broth and mCCDA were used as selective media in the current study, the simultaneous use of these two media was suggested to be useful for the determination of thermophilic campylobacters from environmental samples (e.g. water), where *Campylobacter* numbers are low (Corry *et al.*, 1995). The initial incubation of enrichment cultures at 37 °C followed by selective incubation at 42 °C and then by selective plating seems to enhance significantly the recovery of sub-lethally injured thermophilic campylobacters from environmental waters and foods (Humphrey and Muscat, 1989; Scates *et al.*, 2003).

Since the number of thermophilic campylobacters in environmental waters is believed to be often low, the treatment of a large water volume seems to be an obvious way of increasing recovery (Bolton *et al.*, 1982). Hänninen, *et al.* (2003) found that the filtration of 4000 ml to 10,000 ml of tap water increased the recovery of campylobacters in an investigation of three waterborne outbreaks associated with contaminated drinking

water caused by *Campylobacter jejuni* in Finland. Therefore, the Standard Public Health Laboratory Service protocol recommends the filtration of 1000 ml of water. The results obtained in the present study, however, showed that the filtration of a large volume (1000 ml) of turbid pond water never yielded *Campylobacter* isolates (Table 5.2). These were clearly false-negative results, since the filtration of smaller volumes (10 ml or 100 ml) of water from the same sites frequently gave positive results.

Since the *Campylobacter*-negative enrichment cultures from 1000 ml filtration had high levels of heterotrophic bacteria, coliforms and total bacterial population (Table 5.4), it is likely that high levels of background bacteria, competing for nutrients or releasing inhibitory compounds, prevented the growth of *Campylobacter* during the enrichment procedure. Aquino, *et al.* (1996) evaluated direct plating on selective media and broth enrichment techniques for the recovery of campylobacters from heavily contaminated poultry products. They found that a large proportion of their samples gave *Campylobacter* isolates with direct plating, yet failed to yield positive campylobacters from enrichment cultures. Likewise, they found that *Campylobacter*-negative cultures had high levels of faecal indicators and lactobacilli, which may have hindered the recovery of campylobacters. Thus, the seeding of enrichment culture with the residue from a large volume of turbid water (1000 ml), may lead to overloading by background bacteria and the out-competition of *Campylobacter* to the extent that it is unable to grow to detectable levels. Fricker (1987) reviewed various methods that are used to isolate *Campylobacter* from environmental samples and suggested that overloading by background microflora may prevent the growth of campylobacters during the enrichment stage; this suggestion is supported by the results presented in this chapter. The negative results that were observed for all volumes filtered from the ponds at Bentley and Sancton, which did not harbour waterfowl, did not give further information about the interference of background bacteria on the recovery of thermophilic

campylobacters. However, they suggest that the failure to detect *Campylobacter* from water samples may sometimes reflect their genuine absence. By and large, the results of this chapter further support the conclusions of the previous chapter (Chapter 4) that waterfowl are responsible for poor microbiological quality and the presence of enteric pathogens (i.e. *Salmonella* and/or *Campylobacter*) in village ponds. The results in this chapter clearly show that thermophilic campylobacters were recovered only from ponds with populations of waterfowl.

Similar conclusions may be made with regard to sediment samples. A large volume (5.0 ml) of sediment used for enrichment never gave presumptive campylobacters, whereas smaller volumes did (Table 5.3). The concentration of bacteria in aquatic sediment has been reported to be around a hundred times higher than in overlying water (Cavallo, *et al.* 1999), thus the results in the present study suggest that the use of a large inoculum of microbially-rich sediment to assess the presence of campylobacters may lead to false-negative results, probably because of competition by the background microflora.

It may be suggested that in the processing of large volumes of turbid water (1000 ml), the use of a larger volume of enrichment broth (500 ml or 1000 ml) per sample would prevent the interference of background bacteria and allow *Campylobacter* to grow to detectable levels. However, for a laboratory carrying out routine investigations, such a procedure would be both expensive and impractical, especially when smaller volumes of enrichment broth (150 ml) give positive results provided that appropriate volumes of water are processed, as is shown in this study.

5.4.2 PCR as a rapid confirmatory tool for presumptive campylobacters

The routine confirmation of presumptive *Campylobacter* isolates is based on biochemical tests. These tests are used in almost all laboratories because they are cheap,

rapid and easy to perform. For instance, the United Kingdom Health Protection Agency (HPA) utilises a routine confirmatory route that is based on colony appearance on mCCDA medium, microscopic appearance after Gram staining and the production of oxidase (HPA, 2003). The main disadvantage using of such tests is possible false-positive results. For example, *Campylobacter*-like organisms (i.e. *Helicobacter* and *Arcobacter*) may be falsely confirmed as campylobacters. Consequently, there is interest in the application of molecular approaches, such as PCR, to the identification of campylobacters. Diergaardt, *et al.* (2004) isolated 100 presumptive campylobacters from drinking water, ground water, surface water and raw sewage in South Africa. Out of the 100 isolates, only 22 did not grow under aerobic conditions and were confirmed as *Campylobacter* spp. by biochemical tests. The analysis of the 16S rRNA sequence, however, revealed that only three of the 22 confirmed isolates were *Campylobacter jejuni*, while the remaining 19 isolates were identified as *Arcobacter butzleri* (Diergaardt *et al.*, 2004).

The *Campylobacter* flagellum is believed to be a significant virulence factor (Ketley, 1997; Percival *et al.*, 2004). In this study, a PCR protocol that amplifies the intergenic sequence between the *Campylobacter* flagellin genes, *flaA* and *flaB*, was used as a rapid confirmatory test on presumptive *Campylobacter* isolates. Primers CF03 and CF04 (Wegmüller *et al.*, 1993) were chosen because of their specificity for detecting *C. jejuni* and *C. coli*. The amplification of these primers is thought to give a 340 to 380 bp fragment with *C. jejuni* and *C. coli* (Wegmüller *et al.*, 1993). Although Wegmüller, *et al.* (1993) did suggest that *C. lari* may also give a similar product size with primers CF03 and CF04, the results of Waage, *et al.* (1999), however, indicated that some *C. lari* strains give products of 300 or 450 bp with these primers while other strains of *C. lari* are never amplified. Therefore, only *C. jejuni* and *C. coli* give the 340-380 bp fragment.

The results presented in this chapter showed that the amplification of DNA extracted from all 24 presumptive *Campylobacter*, isolates and also from a clinical isolate, produced PCR fragments of 340-380 bp in length (Figures 5.1; 5.2). Thus, all presumptive isolates that were tested in the present study were confirmed as *Campylobacter* spp. and it can be concluded that they were either *C. jejuni* or *C. coli* on the basis of the work described by Wegmüller, *et al.* (1993) and Waage, *et al.* (1999).

5.6 CONCLUSIONS

- Filtration of a large volume of turbid environmental water or the use of a large volume of sediment may be counterproductive and may not yield presumptive campylobacters.
- This is because of competition by background microflora in enrichment culture.
- For turbid pond water filtration of 10 ml or 100 ml were most appropriate in the ponds studied here.
- Pilot studies to establish appropriate volumes of environmental water or sediment samples should be undertaken before routine determination of *Campylobacter* is begun,

CHAPTER 6

SOURCES AND DISTRIBUTION OF *CAMPYLOBACTER* AND *SALMONELLA* IN VILLAGE PONDS

6.1 INTRODUCTION

The presence of *Campylobacter* and *Salmonella* species in environmental waters constitutes a potential threat to human health, since these enteric bacterial pathogens are involved in many water-borne disease outbreaks worldwide (Hunter, 1997; Percival *et al.*, 2004). *Campylobacters* and *salmonellae* are common inhabitants of the intestinal tract of wild birds and they are frequently detected in sewage effluents and in run-off water from livestock farms (Murray, 2000; Jones, 2001; Refsum *et al.*, 2002a). These sources constitute important avenues of contamination to natural waters and can contribute to the transmission of disease.

6.1.1 Sources of campylobacters and salmonellae to natural waters

Campylobacter and *Salmonella* are not indigenous to aquatic environments. Hence, their presence indicates faecal contamination. These organisms can reach natural waters through point sources (e.g. sewage treatment works) and non-point sources (e.g. run-off or wild fauna) of contamination.

Sewage effluents are a common source of enteric pathogens to natural waters. The discharge of untreated or partially treated effluent from sewage treatment works, abattoirs and meat processing plants into surface water contributes to the overall loading

of campylobacters and salmonellae in natural waters (Bolton *et al.*, 1987; Jones *et al.*, 1990a; Jones *et al.*, 1990c; Geldreich, 1996; Baudart *et al.*, 2000).

Run-off from land is also increasingly being recognised as a major cause of transient deterioration in the microbiological quality of natural waters and is a public health concern. Run-off from pasture and arable land can include faeces from grazing cattle and sheep and slurries and manures and sewage sludge put onto land as fertilizer. Thus run-off was found to have high bacterial loads (Heinonen-Tanski and Uusi-Kämpä, 2001). Such run-off was reported to be a significant source of microbiological pollution in the River Riato, Spain (Fernández-Alvarez *et al.*, 1991), and caused failure to meet water quality standards on shorelines in Southern California (Noble *et al.*, 2003). There is a growing public health concern about the contamination of natural waters by rain-related run-off because it is important in the distribution and dissemination of pathogens in natural waters. For examples, the presence of thermophilic campylobacters at freshwater bathing beaches on the River Lune, UK was attributed, in part, to agricultural run-off (Obiri-Danso and Jones, 1999a). In another study of thermophilic campylobacters in a river system in Lancashire, UK, Bolton, *et al.* (1987) noted a sharp increase in the abundance of *Campylobacter* following rainfall, suggesting that run-off from adjacent agricultural land was a leading source of *Campylobacter*. Likewise, Claudon, *et al.* (1971) isolated 13 *Salmonella* serotypes from Lake Mendota, a lake used for recreation at Madison, Wisconsin. The presence of *Salmonella* in the lake water was due to contamination by run-off. Similarly, Baudart, *et al.* (2000) reported around 6.2×10^{10} 1000 ml⁻¹ *Salmonella* in estuarine waters of the Tech River, France as a result of rain-related run-off.

Wild birds, especially waterfowl, appear to be a major zoonotic reservoir of *Campylobacter* (Luechtefeld *et al.*, 1980; Pacha *et al.*, 1988; Waldenström *et al.*, 2002). Wild birds, including waterfowl, are also recognised as carriers of *Salmonella*, although

the incidence of *Salmonella* carriage appears to be low (Kapperud and Rosef, 1983; Feare *et al.*, 1999; Murray, 2000; Refsum *et al.*, 2002a). Since the presence of waterfowl increases the abundance of faecal indicator bacteria in natural waters, as shown in Chapter 4, it may be suggested that these birds can be an important source of pathogens in surface waters. Thus, mallard ducks (*Anas platyrhynchos*) were, in part, the source of large numbers of thermophilic campylobacters in coastal bathing waters on the River Lune, England (Obiri-Danso and Jones, 1999a).

In general, the presence of campylobacters and salmonellae in natural surface waters can be attributed to a combination of several of the above-mentioned sources. This was observed by Obiri-Danso and Jones (1999a, 1999b), who reported that *Campylobacter* and *Salmonella* in the bathing waters of the River Lune (fresh water) and Morecambe Bay (marine) came from a diversity of sources, primarily, sewage effluent, run-off from agricultural land, and wild birds (ducks and gulls).

6.1.2 Typing of environmental isolates of *Campylobacter* and *Salmonella*

Typing of campylobacters and salmonellae derived from the environment, particularly natural waters, can provide significant information about the source of contamination. In a study of the effects of sewage treatment works effluent on faecal indicators and campylobacters in bathing waters, Obiri-Danso and Jones (1999b) isolated only *Campylobacter lari* and urease-positive thermophilic campylobacters (UPTCS). This indicated that the source of campylobacters was not sewage effluents because *C. lari* and UPTCS are more typical of birds such as gulls. However, Polo, *et al.* (1999) examined 823 isolates of *Salmonella* from sea, river and reservoir waters in North-East Spain. They found 55 different serotypes, the majority of which were the same as those found in clinical specimens, suggesting that human sewage was the most likely source of contamination. In general, typing of pathogenic bacteria from the

environment provides important epidemiological information related to: (i) tracking the route of transmission to humans, (ii) monitoring the geographic and temporal distribution of specific strains and (iii) developing control strategies (Newell *et al.*, 2000).

6.1.2.1 Approaches to the typing of *Campylobacter* and *Salmonella*

Typing methods for both *Campylobacter* and *Salmonella* are based on either phenotype or genotype. The phenotypic methods include phage typing and serotyping. In clinical laboratories, phenotypic methods are preferred because of their ease of use and relatively low cost. Genotypic methods, however, are preferred in research laboratories because of their discriminatory power and high sensitivity.

6.1.2.1.1 Phage typing

Phage typing for *Campylobacter* and *Salmonella* is widely used by reference laboratories (Grajewski *et al.*, 1985; Jayasheela *et al.*, 1987; Jones *et al.*, 2000). Bacteriophages used for typing are isolated from the environment, usually from sewage effluents. The phages are then enumerated and purified using a bacterial lawn on an agar plate (plaque isolation technique). Following isolation, the phages are identified against a wide range of referenced isolates to establish the host range and the plaque morphology. Once the host range and the plaque morphology are being established, the production of large stocks of typing-phages can begin.

6.1.2.1.2 Serotyping

Serotyping schemes are designed to target specific antigens on the surface of bacterial cells. These antigens are the somatic O and/or the flagellar H antigens. The somatic antigens are on the cell wall and are polysaccharides, while the flagellar antigens are proteinaceous. Flagellar antigens have two phases, the H antigens are

referred to as diphasic. Strain-specific antigens are recognised by agglutination with specific agglutinating antibodies (Jones *et al.*, 2000).

Campylobacter serotyping schemes are based on the agglutination of specific antigens with antisera. Two schemes are used for serotyping *Campylobacter* isolates. The Penner scheme (Moran and Penner, 1999) which is based on the presence of soluble, heat-stable (HS), antigens. These antigens are lipopolysaccharides on the cell surface (i.e. somatic O antigens). The other scheme is called after Lior (Lior *et al.*, 1982) and detects diverse heat-labile antigens (these are flagellar H antigens). The Penner scheme was found to fulfil most of the criteria required of a typing method (McKay *et al.*, 2001), hence its worldwide adoption by laboratories undertaking routine typing.

The genus *Salmonella* has perhaps the most elaborate, and certainly the largest, serological typing system among bacteria. *Salmonella* serovars are specifically identified on the basis of their respective O and H antigens using the White-Kauffmann-Le Minor (WKL) scheme (Jones *et al.*, 2000). There are more than 2000 serotypes of *Salmonella* that belong to the aggregate species, *S. enterica*, Table 6.1 shows antigenic profiles of some *Salmonella* species according to the WKL scheme (Timbury *et al.*, 2002).

Because other members of the *Enterobacteraceae* may possess similar antigens as *Salmonella* and, therefore, may give agglutination with *Salmonella* antisera (e.g. *Citrobacter* with Group C antisera), it is recommended that serotyping should be carried out only after presumptive isolates have been confirmed as *Salmonella* using biochemical tests (Jones *et al.*, 2000).

Table 6.1 selected salmonellae within the aggregate species *S. enterica* showing antigenic profiles according to the White-Kauffmann-Li Minor scheme

Serotype (species)	Group	O antigen types	H antigen types	
			Phase 1	Phase 2
<i>S. paratyphi</i> A	A	1, 2, 12	A	-
<i>S. paratyphi</i> B	B	1, 4, 5, 12	B	1, 2
<i>S. agona</i>	B	4, 12	f, g, s	-
<i>S. typhimurium</i>	B	1, 4, 5, 12	I	1, 2
<i>S. paratyphi</i> C	C	6, 7	C	1, 5
<i>S. typhi</i>	D	9, 12	D	-
<i>S. enteritidis</i>	D	1, 9, 12	g, m	-

Adopted with modification from: Timbury, *et al.* (2002).

6.1.2.1.3 Molecular typing methods

Although serotyping methods are widely used, they have the major disadvantage that many untypable strains are encountered. This is especially so for *Campylobacter* isolates. Untypable strains amongst confirmed *Campylobacter* isolates from environmental samples (i.e. water and animal faeces) have been reported (Manser and Dalziel, 1985; Mawer, 1988; Brennhovd *et al.*, 1992). This problem, has led to the development of DNA-based typing methods which have high sensitivity and discriminatory power. Typing by digestion of bacterial DNA using restriction enzymes that cleave the DNA (i.e. pulsed-field gel electrophoresis PFGE) has proved to be useful for both campylobacters (Broman *et al.*, 2004) and salmonellae (Refsum *et al.*, 2002b) from clinical and environmental sources. Other DNA-based methods include: rapid amplification of polymorphic DNA (RAPD); amplified fragment length polymorphism fingerprinting (AFLP); polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP); and, most recently the flagelling typing (*fla* typing) for *Campylobacter*. The value of these DNA-based methods for typing bacterial pathogens,

particularly *Campylobacter* has been addressed by Rivoal *et al.* (1999), Newell *et al.* (2000), and Petersen and On (2000).

Nucleotide sequencing (with or without prior PCR amplification) has also been recently considered as an alternative molecular genomic technique for typing bacterial isolates. Sequencing provides high discrimination power and the results may be interpreted by the aid of appropriate computer programmes. Sequence analysis has been successfully applied to the flagellin locus of *Campylobacter jejuni* (*flaA* gene) (Harrington *et al.*, 1997; Meinersmann *et al.*, 1997).

6.1.3 Aims

Campylobacter and *Salmonella* were isolated from the water and sediments of village ponds using the methods described in previous chapters (Chapters 4 and 5). Recovery of these pathogens was successful only from ponds that harboured waterfowl. Waterfowl were clearly the source of high numbers of faecal indicators in village ponds and it was suggested that they could also be the source of campylobacters and salmonellae. These ponds, however, are also subject to pollution from run-off which is potentially an alternative source of campylobacters and salmonellae. The work described in this chapter tested the hypothesis that waterfowl are a significant source of *Campylobacter* and *Salmonella* in village ponds. It is complementary to the work described in Chapter 4 that supported the hypothesis that general microbiological quality is worse in ponds with waterfowl. The work aimed to isolate campylobacters and salmonellae from the faeces of waterfowl and from the run-off water that enters village ponds. Molecular methods were used to type confirmed isolates of *Campylobacter* and *Salmonella* and to compare them with those previously isolated from the water and sediment (Chapters 4, 5).

6.2 MATERIALS AND METHODS

6.2.1 Sites and sampling

The study sites were three roadside village ponds in East Yorkshire, NE England. These ponds have populations of ducks, and sometimes geese. The ponds are at Brantingham, Little Weighton and South Dalton. All receive run-off from adjacent roads. The ponds are amenity sites and they and their environs were described in Chapter 2.1.

A total of 90 fresh (still moist) faecal droppings of ducks were collected from adjacent to the three ponds during August, October and December 2003. Thirty of these were from each pond. Faecal samples were collected using forceps and were transferred into sterile 30-ml universal bottles. Samples of run-off water were collected during rainfall events, from the ponds' input pipes, into sterile 1000-ml polypropylene bottles. Altogether, 18 pairs of replicate samples of run-off water were collected from the three ponds during December 2003-January 2004. All samples of faeces and run-off water were kept on ice, in darkness, during transportation and microbiological assays were begun later on the same day as sampling. Water and sediment samples (Chapter 5) were collected at the same time as the faecal samples, while run-off samples were collected during rain-fall events.

6.2.2 Abiotic variables

pH, conductivity and turbidity of run-off water samples were measured as described in Chapter 3.2.

6.2.3 Detection of campylobacters and salmonellae in faecal samples

Faecal samples from ducks were screened for campylobacters and salmonellae by means of selective enrichment and selective plating technique. For isolation of

Campylobacter, 0.2 g wet weight of each faecal sample was transferred to a sterile bijou bottle. Preston enrichment broth (about 8.0 ml) was added leaving little air space (less than 0.5 ml), in order to achieve microaerobic conditions, and the procedure was then continued as described in Chapter 3.3.2.1. For isolation of *Salmonella* 0.2 g wet weight of each faecal sample was also used. The samples were transferred to 10 ml buffered peptone water broth and the procedure was continued as described in Chapter 3.3.2.2.

6.2.4 Detection of campylobacters and salmonellae in run-off water

Membrane filtration techniques were employed for the detection of campylobacters and salmonellae in run-off water samples. These were based on the standard methods for the examination of water as used by the Public Health Laboratory Service (PHLS 1998d-e). Run-off water samples, volume 10 ml and 100 ml, were membrane filtered and processed as described in Chapter 3.3.2.1 and 3.3.2.2.

6.2.5 Confirmation of presumptive campylobacters and salmonellae

Presumptive *Campylobacter* colonies were subjected to the following confirmatory tests: Gram stain; cell shape (Chapter 5.4.3); presence of oxidase and catalase; growth on blood agar at 37 °C under microaerobic but not aerobic conditions (Chapter 3.3.2.1); and by PCR (Chapter 3.3.5.1)

Presumptive salmonellae were confirmed by means of biochemical tests (growth on lysine iron agar and urea broth) as described in Chapter 3.3.2.2, and by PCR as described in Chapter 3.3.5.1.

6.2.6 Molecular typing of confirmed campylobacters

DNA sequences were determined using amplified PCR products from *Campylobacter* isolated from faecal samples and run-off water. Amplification was done

using the primers CF03 and CF04 as described in Chapter 3.3.5.1. DNA from campylobacters previously isolated from pond water and sediment (Chapter 5) was also amplified and sequenced. Sequencing was performed using a capillary electrophoresis sequencer, Beckman Coulter CEQ™ 8000 (Appendix 3A). Sequence data were interpreted and aligned using CodonCode Aligner (Codoncode Corporation, Dedham, MA, USA). Capillary electrophoretic sequencing has previously been proved useful for genotyping of bacterial species (Baele *et al.*, 2002).

The sequencing procedure followed four steps, (i) cleaning of the template DNA, (ii) the sequence reactions, (iii) post sequencing clean up with ethanol, and (iv) capillary electrophoresis. The steps were performed as follows:

- (i) *DNA cleaning:* PCR products were cleaned prior to sequencing. For each sample, 1.0 µl of PCR products which contained the template DNA was added to 0.4 µl of exonuclease and shrimp alkaline phosphatase (EXOSAP) (Beckman Coulter Inc., Fullerton, CA), in a sterile microcentrifuge tube (total volume 1.4 µl). The tube was incubated at 37 °C for 15 min, followed by 15 min at 80 °C.
- (ii) *Sequence reaction:* Each of the clean template DNA samples (1.4 µl) was transferred to a sterile microcentrifuge tube which contained 4.0 µl of dye terminator cycle sequencing (DTCS) quick start master mix (Beckman Coulter Inc., Fullerton, CA); 0.5 µl of each primer used (0.25 µmol l⁻¹) (i.e. CF03; CF04 for *Campylobacter*), and 4.1 µl of sterile deionised water (total volume in the tubes 10 µl). The sequencing temperature cycle was as follows: denaturation at 96 °C for 2 min, 30 cycles at 96 °C for 20 sec, 50 °C for 20 sec, and final extension at 60 °C for 4 min. After the sequencing reaction was

complete a volume of 10 μl of each sample was transferred to a separate well on a sequencing microtitre plate containing 20 mg ml^{-1} (1.0 μl) glycogen, 100 mmol l^{-1} EDTA pH 8.0 (2.0 μl) and 3 mol l^{-1} sodium acetate pH 5.2 (2.0 μl).

(iii) *Post-reaction cleaning:* 60 μl of 100 % ethanol was added to each well in the microtiter plate. The plate was covered tightly by foil and vortexed. Next the plate was centrifuged at 2760 RCF for 30 min at 4 °C. Following centrifugation the foil was removed and the plate was inverted over a sink and gently shaken two or three times. Thus the ethanol was discarded leaving the DNA in the bottom of the well. 200 μl of 70 % ethanol was next added to each well in the plate and the above procedure was twice repeated with centrifugation for 5 min. The plate was then inverted and placed on a bed of tissue in a centrifuge and spun gently by hand to ensure that no ethanol remains in the wells. The plate was put in a vacuum dryer for 60 min to remove residual ethanol completely. After the drying, 40 μl of sample loading solution (SLS [Beckman Coulter Inc., Fullerton, CA]) was added to the DNA in each well. The plate was covered tightly with foil and vortexed. The liquid was tapped down to bottom of the wells and overlaid with mineral oil. Another plate was prepared with the same number of wells which contained running buffer (Beckman Coulter Inc., Fullerton, CA).

(iv) *Electrophoretic sequencing:* The plates were then loaded into the Beckman Coulter CEQ™ 8000 and capillary electrophoresis sequencing was begun. Standard sequencing procedures were used

(Anon., 2002): capillary electrophoresis was performed at 50 °C, with constant voltage of 4.2 kV for 85 min; denaturation temperature was 90 °C for 120 s. and injection voltage was 2.0 kV for 15 s.

6.2.7 Recognition of sequence data

Campylobacter species were confirmed on the basis of the nucleotide sequence of the flagellin genes (*flaA* and *flaB*). The sequences that were determined were matched with those available in GenBank (BLAST: <http://www.ncbi.nlm.nih.gov/BLAST>). Accession numbers and complete sequence of all isolates are listed in Appendix 3B.

6.3 RESULTS

6.3.1 Abiotic variables

Results of analysis of run-off water samples are shown in Table 6.2. It is clear that the run-off into Little Weighton and Brantingham ponds was more turbid and had higher conductivity than that which flowed into the pond at South Dalton.

6.3.2 Recovery of campylobacters and salmonellae from duck faeces

In all 90 faecal samples were processed. Presumptive *Campylobacter* was recovered from 11% of the samples (Table 6.3). The recovery rate was highest in samples of faeces collected from South Dalton (27%); compared to (3%) at both Little Weighton and Brantingham. Conversely, presumptive *Salmonella* was never recovered from duck faeces from South Dalton, but was recovered from 7% of the samples from both Little Weighton and Brantingham (Table 6.3). Overall, *Campylobacter* was

recovered from 11 % of faecal samples whereas *Salmonella* was recovered only from 4 % of the samples.

6.3.3 Recovery of campylobacters and salmonellae from run-off water

In all 18 pairs of samples of run-off water (volumes 10 ml and 100 ml) were processed for the recovery of presumptive *Campylobacter* and *Salmonella* isolates. *Campylobacters* were frequently isolated from Little Weighton, less frequently from Brantingham, but not at all from South Dalton (Table 6.4). Presumptive salmonellae, however, were never recovered from any run-off water sample (Table 6.4).

6.3.4 Confirmation of presumptive campylobacters and salmonellae recovered from faecal and run-off water samples.

All 17 presumptive isolates of *Campylobacter* from run-off water and faeces were found to be Gram negative, gull-winged shaped, both catalase and oxidase positive, and they all grew on blood agar incubated at 37 °C under microaerobic, but not aerobic conditions. Hence they were all confirmed as *Campylobacter*. DNA extracted from all presumptive isolates was successfully amplified with primers CF03 and CF04. The amplified products were between 340 and 380 bp in length (Figs. 6.1 and 6.2). This indicated that isolates were either *Campylobacter jejuni* or *C. coli*.

The four presumptive *Salmonella* isolates from duck faeces were found to produce alkaline reaction after growing on lysine iron agar slopes, with H₂S production and were urease negative after growing in urea broth. Hence they were confirmed as *Salmonella*. However, the DNA extracted from these isolates did not amplify with primers S18 and S19 (Fig. 6.3). Hence they appeared to be not *Salmonella*. Furthermore, when these presumptive isolates were sent to PHLS Hull for serotyping they did not give positive reactions with the WKL scheme, hence they were not *Salmonella*.

Table 6.2 Abiotic variables in run-off water samples from three pond sites, December 2003-January 2004

	Mean (range) n		
	Brantingham	Little Weighton	South Dalton
pH	7.6 (7.5-7.7) 3	7.1 (6.9-7.3) 3	7.2 (6.9-7.4) 3
Conductivity ($\mu\text{S cm}^{-1}$)	897 (715-1160) 3	1743 (1728-1811) 3	338 (269-409) 3
Absorbance (at 580 nm)	0.74 (0.70-0.78) 3	0.75 (0.73-0.76) 3	0.46 (0.44-0.48) 3

n = total number of samples

Table 6.3 The recovery of presumptive *Campylobacter* and *Salmonella* from duck faeces August-December 2003

Location of pond	Recovery from faeces	
	<i>Campylobacter</i> n:p (%)	<i>Salmonella</i> n:p (%)
South Dalton	30:8 (27)	30:0 (0)
Little Weighton	30:1 (3)	30:2 (7)
Brantingham	30:1 (3)	30:2 (7)
Total	90:10 (11)	90:4 (4)

n = total number of samples.

p = number of positive samples (percentage of positive samples in brackets).

Table 6.4 The recovery of presumptive *Campylobacter* and *Salmonella* from run-off water, December 2003-January 2004

Location of pond	<i>Campylobacter</i>	
	Volume of water filtered	
	10 ml n:p (%)	100 ml n:p (%)
South Dalton	6:0 (0)	6:0 (0)
Little Weighton	6:3 (50)	6:2 (33)
Brantingham	6:1 (17)	6:1 (17)
Total	18:4 (22)	18:3 (17)

Location of pond	<i>Salmonella</i>	
	Volume of water filtered	
	10 ml n:p (%)	100 ml n:p (%)
South Dalton	6:0 (0)	6:0 (0)
Little Weighton	6:0 (0)	6:0 (0)
Brantingham	6:0 (0)	6:0 (0)
Total	18:0 (0)	18:0 (0)

n = total number of samples.

p = number of positive samples (percentage of positive samples in brackets).



Fig. 6.1 Examples of results of PCR on presumptive *Campylobacter* isolates recovered from duck faeces. Lane M represents a 100 bp ladder; lane 1, a negative control (a DNA-free amplification); lane 2, a positive control (a clinical *Campylobacter* isolate); lanes 3-8, presumptive campylobacters from faecal samples collected from South Dalton; lane 9, from Little Weighton faeces; lane 10 from faeces from Little Weighton; lane 10, from faeces from Brantingham.

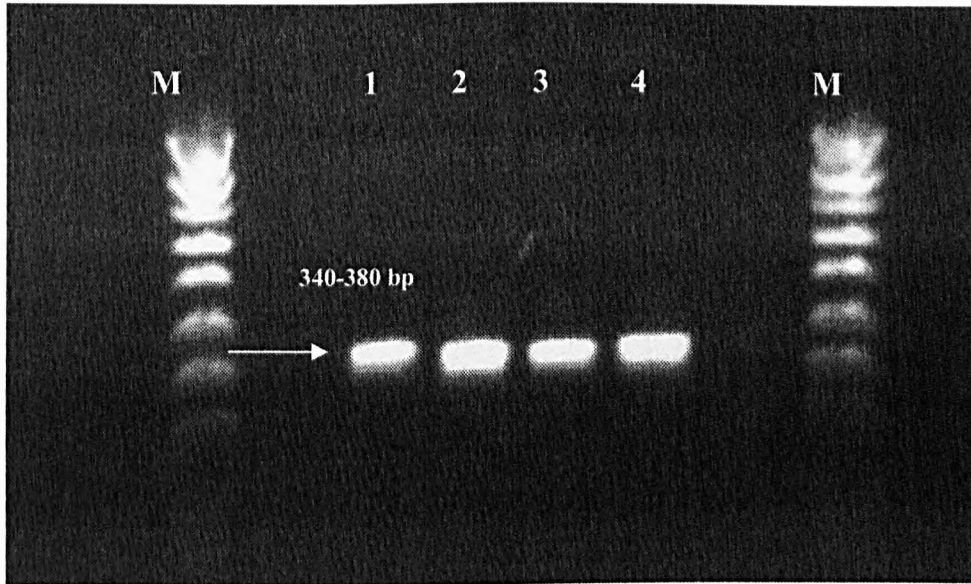


Fig. 6.2 Examples of results of PCR on presumptive *Campylobacter* isolates recovered from run-off water. Lane M represents 100 bp ladder; lanes 1-2, presumptive campylobacters from filtration of 10 ml of run-off water from Little Weighton; lane 3, from filtration of 100 ml of run-off from Little Weighton; lane 4, from filtration of 10 ml of run-off from Brantingham.

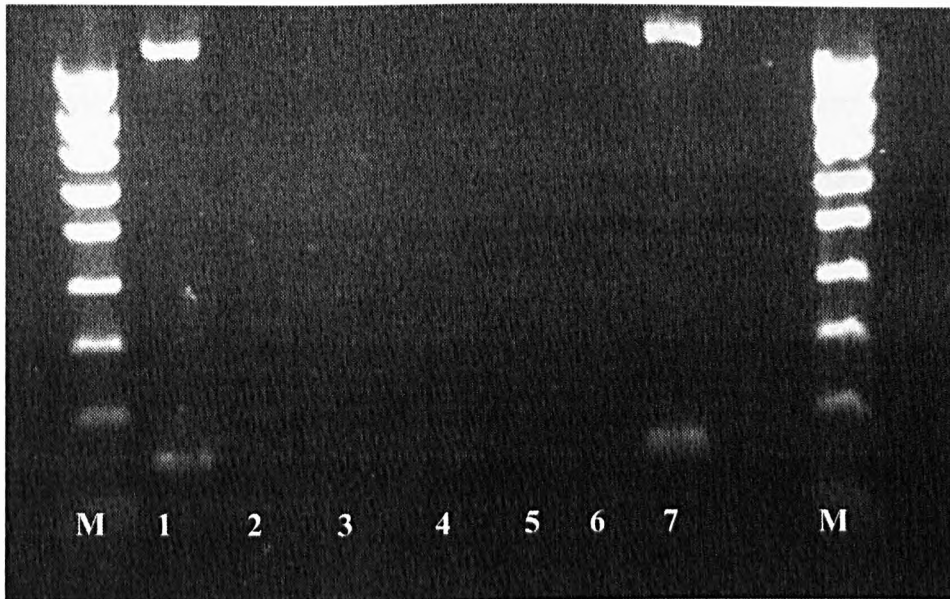


Fig. 6.3 Results of PCR on presumptive *Salmonella* isolates recovered from duck faeces. Lane M represents a 100 bp ladder; lanes 1 and 7 are a positive control (*S. enteritidis* NCTC 12694); lanes 2-3, are presumptive salmonellas from faeces collected from Little Weighton; lanes 4-5, from faeces collected from Brantingham; and lane 6, is a negative control (a DNA-free amplification).

6.3.5 Information from sequencing of *Campylobacter* DNA

DNA sequencing showed that all ten isolates from duck faeces were *Campylobacter jejuni* (Table 6.3). Their sequences all had 93 % similarity with *Campylobacter jejuni* (ATCC43431 [GenBank Accession Number Z29327]). The next nearest (92%) was with *Campylobacter jejuni* (NCTC11168 [GenBank Accession Number AL139078]). In contrast, all seven isolates from run-off water (from Little Weighton and Brantingham, Table 6.4) were shown to be *Campylobacter coli*. Their sequences all had 100 % similarity with *Campylobacter coli* (GenBank Accession Number M64671). The next nearest match (100 %) was with *Campylobacter coli* (GenBank Accession Number M64670).

Amplified DNA from confirmed campylobacters that had been recovered from the water and sediments of the three ponds (see Chapter 5) was also sequenced. All *Campylobacter* isolates from South Dalton water and sediment were *C. jejuni* (Table 6.5). All *Campylobacter* isolates from the water and sediment of the ponds at Little Weighton and Brantingham, however, were found to be *C. coli* (Table 6.5).

It is important to stress here that DNA sequence analysis showed that all *Campylobacter jejuni*, derived from faecal, water and sediment samples from all three pond sites, showed a 100 % identical sequence. Similarly the sequence of all *Campylobacter coli* recovered was also found to be 100 % identical (Appendix 3B).

Table 6.5 Results from sequencing of amplified DNA from *Campylobacter* recovered from pond water and sediment, August-December 2003

Location of pond	Total <i>Campylobacter</i> isolates	<i>C. jejuni</i>	<i>C. coli</i>
In water			
South Dalton	11	11	0
Little Weighton	4	0	4
Brantingham	4	0	4
Total	19	11	8
In sediment			
South Dalton	4	4	0
Little Weighton	1	0	1
Brantingham	1	0	1
Total	6	4	2

6.3.6 Sources of *Campylobacter* species in village ponds

The distribution of *Campylobacter* isolates recovered from the ponds at South Dalton, Little Weighton and Brantingham and their environs was investigated to determine the source of *Campylobacter* contamination. At South Dalton the DNA sequencing of the confirmed campylobacters showed that *Campylobacter jejuni* was the only species to be found in water, sediments and faeces, while no campylobacters were recovered from the run-off water (Table 6.6). Thus duck faeces appear to be the source of *Campylobacter* in the South Dalton Pond.

At Little Weighton and Brantingham ponds, however, only *C. coli* was recovered from the pond water and sediment. This species was also found in the run-off water whereas only *C. jejuni* was found in faeces. Hence at these ponds run-off water was probably the source of campylobacters (Table 6.6).

Table 6.6 The distribution of *Campylobacter* species at village pond sites, East Yorkshire

	Total <i>Campylobacter</i> isolates	<i>C. jejuni</i> [†]	<i>C. coli</i> [†]
South Dalton			
Pond water	11	11	0
Sediment	4	4	0
Faeces	8	8	0
Run-off water	0	0	0
Total	23	23	0
Little Weighton			
Pond water	4	0	4
Sediment	1	0	1
Faeces	1	1	0
Run-off water	5	0	5
Total	11	1	10
Brantingham			
Pond water	4	0	4
Sediment	0	0	0
Faeces	1	1	0
Run-off water	2	0	2
Total	7	1	6

[†] DNA sequence analysis showed that all *C. jejuni* and all *C. coli* shared 100 % identical sequence.

6.4 DISCUSSION

The presence of *Campylobacter* and *Salmonella* in village ponds that are subject to faecal contamination was described in Chapters 4 and 5. Since campylobacters and salmonellae were only recovered from the water and sediment of ponds that harboured large populations of waterfowl, it was concluded that ducks and geese were the major source of these bacteria in the ponds. The primary aim of the present chapter was to investigate the potential sources of *Campylobacter* and *Salmonella* species in village ponds and to determine the influence of duck faeces and run-off water as sources of contamination on the distribution of different species of *Campylobacter* and *Salmonella* in the ponds.

6.4.1 The recovery of campylobacters and salmonellae from duck faeces

Campylobacter and *Salmonella* species are associated with diseases of human and domestic animals. Wild animals, particularly birds, and especially migratory waterfowl, are considered to be a reservoir for *Campylobacter* and *Salmonella* (Percival *et al.*, 2004).

In the present study the incidence of campylobacters in waterfowl was found to be higher than that of salmonellae. Campylobacters were recovered from 11 % of faecal samples while only 4 % of faecal samples harboured salmonellae (Table 6.3) and even these were not confirmed by PCR (Chapter 6.3.4). In general, *Campylobacter* species are known to be common inhabitants of the intestinal tract of a wide range of wild birds including migratory waterfowl. The recovery of campylobacters from 10 out of 90 (11 %) of fresh samples of duck faeces in the present study is in agreement with Luechtefeld, *et al.* (1980) and Pacha, *et al.* (1988) who reported frequent recovery of *Campylobacter* from the faeces of mallard (*Anas platyrhynchos*) and Canada geese

(*Branta canadensis*). The presence of campylobacters in the intestinal tract of ducks and geese may be because the body temperature of these birds is around 42 °C, which is the optimal growth temperature for thermophilic campylobacters (i.e. *C. jejuni*; *C. coli*; *C. lari*). The frequent presence of campylobacters may reflect commensalism rather than infection (Luechtefeld *et al.*, 1980; Park *et al.*, 1991; Walenström *et al.*, 2002). The campylobacters that were isolated from duck faeces at the East Yorkshire ponds were all *Campylobacter jejuni* (Table 6.5). This agrees with Luechtefeld, *et al.* (1980), Pacha, *et al.* (1988), Fallacara, *et al.* (2001), Jones (2001) and Murphy, *et al.* (2005) who reported high prevalence of *Campylobacter jejuni* in the faeces of ducks and geese.

DNA sequence analysis of *Campylobacter jejuni* recovered from duck faeces showed that all isolates had a 100 % similar sequence (Appendix 3B), which indicates overall genetic stability of this species within the East Yorkshire duck population. Genetic stability among environmental *Campylobacter jejuni* isolates has been reported elsewhere. Colles, *et al.* (2003) found that 8 genotypes accounted for 73 % (82 out of 112) of *Campylobacter jejuni* isolated from farm animals and farm environments, suggesting genetic stability that is explained by niche/host adaptation. Likewise, Broman, *et al.* (2004) found that *Campylobacter jejuni* derived from one bird species, or feeding guild, usually exhibited a high genetic similarity, indicating a common source from which the organism was acquired, or an association between particular types of *Campylobacter jejuni* with particular feeding guilds or phylogenetic groups of host birds. The observations by Colles, *et al.* (2003) and Broman, *et al.* (2004) agree and may explain the genetic stability of *Campylobacter jejuni* isolates reported in this chapter.

In contrast, other studies of the population structure of *Campylobacter jejuni* based on multi-locus sequence typing (MLST) have suggested that the organism is highly diverse with a weak clonal population structure (Dingle *et al.*, 2002). Genetic

instability of *Campylobacter* species was observed in *Campylobacter jejuni* isolated from chicken meat and live chicken (Wassenaar *et al.*, 1998; Hänninen *et al.*, 1999).

In general the level of discrimination of the *Campylobacter fla* gene typing is reported to be greater than that of serotyping (Wassenaar and Newell, 2000). However, the level of variation between isolates can be affected by the set of primers used (Newell *et al.*, 2000). Although primers CF03 and CF04 have been used to amplify a conserved region of *flaA* and *flaB* in this study, they did not differentiate between *C. jejuni* and *C. coli* (Chapter 5). Sequencing was, therefore, needed to distinguish between the two species. This sequencing showed little or no variation within species. Had alternative primers been used it is possible that within species differences would have been revealed by the sequencing (Newell *et al.*, 2000).

The apparently low incidence of *Salmonella* in duck faeces reported in this chapter agrees with Fallacara, *et al.* (2001) who found *Salmonella* in only one faecal sample out of 439 from ducks and geese in Ohio, USA. Also, Refsum, *et al.* (2002a) conducted a survey of salmonellas in avian wildlife in Norway between 1969 and 2000 and reported low incidence of salmonellae in ducks and geese compared to other wild birds. Furthermore, *Salmonella* species were not recovered from faeces of Canada geese (*Branta canadensis*) and whistling swans (*Cygnus columbianus columbianus*), nor from the water and sediment collected from four roosting sites with the Chesapeake Bay region, Maryland, USA (Damaré *et al.*, 1979; Hussong *et al.*, 1979). In general the carriage of *Salmonella* in ducks as well as wild birds seems to be low (Mitchell and Ridgwell, 1971; Brittingham *et al.*, 1988; Hubálek *et al.*, 1995; Murray, 2000; Kirk *et al.*, 2002; Hernandez *et al.*, 2004; Fallacara *et al.*, 2004). This low incidence might be attributed to diverse factors. For example, contaminated feeding sites may result in short-term carriage of salmonellae without infection of the host (Murray, 2000). This was clearly shown among gulls in which salmonellae were carried for few days but

infection did not occur (Gridwood *et al.*, 1985). There is strong evidence that *Salmonella* species are unlikely to spread from bird to bird by direct contact (Murray, 2000). This may support the conclusions of Damaré, *et al.* (1979) and Tizard (2004) who suggested that wild birds that live well away from polluted environments may not harbour salmonellae.

The very few isolates (four) of *Salmonella* that were recovered from duck faeces in the current study (Table 6.3) were confirmed by biochemical tests (H₂S production in parallel with the lack of urea hydrolysis). Good practice for the confirmation of salmonellae recommends the parallel use of these two tests because there are other Gram negative bacteria that give a positive reaction with lysine iron agar (H₂S production). These, however, also give a positive reaction with urea broth (urea hydrolysis) (Jones *et al.*, 2000). Although the four isolates recovered in the current study gave the expected biochemical reactions for typical *Salmonella*, they did not give a positive outcome with the WKL serotyping scheme. Furthermore, the DNA extracted from these isolates was not amplified by primers S18 and S19 whereas DNA from a control clinical isolate was amplified (Fig. 6.3). These primers were shown to amplify DNA extracted from 60 isolates representing 40 *Salmonella* species (Kwang *et al.*, 1996). Since the four isolates were not confirmed neither by WKL serotyping nor by PCR, the observed biochemical confirmation as *Salmonella* was probably a false-positive result.

6.4.2 The recovery of campylobacters and salmonellae from run-off water

With regard to *Campylobacter* species, their presence in run-off water (Table 6.4) demonstrated that run-off is a potential source of campylobacters in these ponds. These results are in agreement with Stelzer, *et al.* (1991) and Jones (2001) who concluded that the contamination of surface waters with campylobacters is dependent,

in part, on rain-related run-off. Molecular sequence analysis of confirmed *Campylobacter* species isolated from run-off water showed that *Campylobacter coli* was the only species present (Table 6.6). It was most frequently recovered from run-off water at Little Weighton, less frequently from run-off at Brantingham. The *Campylobacter coli* isolates from run-off water were also genetically stable (Table 6.6). This genetic stability may reflect a common source of contamination, or niche adaptation, i.e. a particular species may be localised in a particular area (Colles *et al.*, 2003; Broman *et al.*, 2004).

The apparent absence of *Salmonella* species from run-off water (Table 6.4) may indicate an overall low incidence of the organism in the area surrounding the ponds. Similar sparcity of salmonellas has been reported elsewhere. Over a two-year study, only one *Salmonella* isolate was recovered from a bathing site in Lancashire that received continuous faecal pollution from sewage-works effluent, run-off water from surrounding agricultural land, and had permanent flocks of waterfowl (Obiri-Danso and Jones, 1999b). Similarly, in Italy, Pianetti, *et al.* (1998) reported a low prevalence of *Salmonella* species in environmental samples, mainly waters.

6.4.3 The sources and distribution of *Campylobacter jejuni* and *Campylobacter coli* in village ponds

The DNA sequencing of confirmed *Campylobacter* isolates recovered from pond water and sediment, and of those from faeces and run-off, suggested that different ponds might have different sources of *Campylobacter*.

In the pond at South Dalton, *Campylobacter jejuni* was the only species recovered and was found in pond water, sediment and duck faeces (Table 6.6). Also, all run-off water samples that were collected from the pond input pipes at South Dalton were *Campylobacter*-negative. It appears, therefore, that ducks are the source of

Campylobacter at South Dalton. This suggestion is supported by the high similarity amongst DNA sequences of isolates from water, sediment and faeces. This suggestion agrees with Jones (2001) who reported that mallard (*Anas platyrhincos*) were the source of up to 430 campylobacters per 100 ml of water, in a pond in NW England, and that 90 % of campylobacters isolated from faeces were *C. jejuni*.

At Little Weighton pond, *Campylobacter jejuni* was recovered only rarely from duck faeces and was absent from the water, sediment and run-off water (Table 6.6). *Campylobacter coli*, however, was found in run-off water and strains with identical DNA sequence were recovered from water and sediment samples (Table 6.6). This suggests that the source of contamination by *Campylobacter* in the pond at Little Weighton is run-off water rather than ducks. The prevalence of *Campylobacter coli* in run-off water may indicate the source of contamination. At Little Weighton, run-off water flows over the road, verges and footpaths before entering the pond; the faeces of dogs and horses on the roads and paths surrounding the pond are possible sources of *C. coli*.

At Brantingham pond, few campylobacters were isolated (Table 6.6), hence few conclusions can be made. The one isolate from faeces was *C. jejuni* whereas *C. coli* was found in run-off water (2 isolates) and in the pond water (4 isolates). Thus it is perhaps most likely that run-off was, as at Little Weighton, the source of pond's contamination by *Campylobacter*.

6.5 CONCLUSIONS

- Campylobacters may be isolated from duck faeces and run-off water as well as from the water and sediment of village ponds.
- Thus the faeces of waterfowl and rain-related run-off are both potential sources of campylobacters to the ponds.

- Different *Campylobacter* species in the ponds may have different origin: *C. jejuni* from ducks, *C. coli* from run-off.
- Salmonellae, in contrast, were not reliably isolated from duck faeces or run-off, nor were they found in pond water, and only occasionally in sediment.

CHAPTER 7

PCR ASSAY FOR DIRECT DETECTION OF THERMOPHILIC CAMPYLOBACTERS IN POND WATER

7.1 INTRODUCTION

The contamination of natural waters and of drinking water, by infectious agents has become a major worldwide public health concern. In order to prevent the spread of water-borne illnesses the detection of infectious agents in water is essential. The routine microbiological detection of water-borne pathogens particularly bacterial agents, by culture techniques faces several limitations. In general, the methods are laborious and time consuming. Also, since the majority of pathogenic bacteria are exogenous to aquatic environments, they are likely to be present in relatively low numbers. Such low numbers can generate errors in both sampling and detection (Fleisher, 1990), i.e. the sample volume used and the choice of media have to be appropriate. Thus problems are frequently encountered, especially with the enumeration of thermophilic campylobacters in environmental waters. This is because no adequate universal method for detecting campylobacters in all types of samples has been developed. Therefore, the choice of methods and culture media may depend on the expected abundance of campylobacters and on the background microflora that are present in the sample. Another limitation that affects the routine detection of water-borne bacteria, particularly enteric infectious agents, is the likelihood that they will lose culturability in hostile environments (e.g. water). When bacteria enter the viable but non-culturable stage

(VBNC), they retain basal metabolic activities, yet they become unable to grow or multiply in microbiological culture media (Xu *et al.*, 1982). Many enteric bacteria have been reported as exhibiting non-culturable forms in environmental waters, including *Campylobacter* species (Rollins and Colwell, 1986; Thomas *et al.*, 2002). The ability of water-borne bacterial pathogens to enter the VBNC stage might result in either underestimation of their number or the complete failure to detect them in environmental waters. Given all the obstacles encountered in the detection of pathogenic bacteria, particularly *Campylobacter*, by standard culture methods, the development of alternative, sensitive, methods is highly desirable.

Since the beginning of the 1990s, there has been growing interest in the development of molecular genomic methods and in their application to the detection and identification of infectious agents, particularly bacterial pathogens, from both clinical and environmental samples. Molecular methods such as PCR have been suggested as being rapid, specific, and highly sensitive tools for the detection of bacterial infectious agents in many types of samples (e.g. environmental and clinical). In general, PCR amplifies a small amount of target DNA or RNA in a sample using a thermostable polymerase enzyme from *Thermus aquaticus* (*Taq* enzyme), to obtain a million-fold increase in the numbers of DNA copies (Pepper *et al.*, 1995). This amplification is achieved by using short (15-30 nucleotides), synthetic, specific, single-stranded lengths of DNA known as oligonucleotide primers. Primers can be designed for the detection of specific species or a phylogenetic group (Burlage, 1998). They can be based upon parts of the sequences of specific genes, such as verotoxin-genes; flagellin genes; virulence plasmids and 16S rRNA sequences (Olsen, 2000).

With various degrees of success and using diverse methods, the detection of pathogenic bacteria by PCR has been reported for coliforms and *E. coli*, including pathogenic strains (Fricker and Fricker, 1994; Li and Drake, 2001), *Salmonella* (Kwang

et al., 1996; Rychlik *et al.*, 1999), *Vibrio* (Kock *et al.*, 1993), and campylobacters (Birkenhead *et al.*, 1993).

7.1.1 Detection of thermophilic campylobacters by PCR

PCR has been applied to the detection of *Campylobacter* species. The range of targeted genes for PCR amplification used for the detection of *Campylobacter* species includes, 16S rRNA (Vanniasinkam *et al.*, 1999), 23S rRNA (Fermér and Engvall, 1999), the flagellin genes (*flaA* and *flaB*) (Oyofu *et al.*, 1992; Wegmüller *et al.*, 1993; Rasmussen *et al.*, 1996) and the hippuricase gene (*hipO*) (Denis *et al.*, 1999; Englen and Kelley, 2000). This wide range of available primers, allows not only the detection of the most frequent species that cause enteritis, i.e. *C. jejuni* and *C. coli*, but also the newly emerged pathogenic *Campylobacter* species, i.e. *C. lari* and *C. upsaliensis* (Fremér and Engvall, 1999).

Various PCR protocols, based on the use of various sets of primers, have been developed and have been reported as detecting campylobacters in water (Kirk and Rowe, 1994; Waage *et al.*, 1999), wastewater (Alxandrino *et al.*, 2004), milk and soft cheese (Wegmüller *et al.*, 1993; Jackson *et al.*, 1996), poultry products (Winters *et al.*, 1998), poultry carcass rinses (Englen and Kelley, 2000), and fruit and vegetables (Winters *et al.*, 1998). It is important to stress that the above protocols were applied to the recovery of *Campylobacter* from samples that had been liberally seeded with cultured *Campylobacter*. PCR has also been applied to the detection of *Campylobacter* in un-seeded human faeces (Vanniasinkam *et al.*, 1999) and faecal samples from a wide range of wild and domestic animals (Rasmussen *et al.*, 1996; Steinhäuserova *et al.*, 2000; Inglis and Kalischuk, 2003). Detection of naturally-occurring *Campylobacter* in drinking and swimming pool water by direct PCR has been reported (Jackson *et al.*, 1996; Moore *et al.*, 2001). The development of a PCR protocol that can be used to

detect naturally-occurring *Campylobacter* in environmental samples, such as turbid pond water is a new goal.

7.1.2 Aims

Some of the problems associated with the detection of campylobacters in pond water and sediment using conventional culture methods emerged in the work described in Chapter 4. The development of culture methods to overcome these problems was described in Chapter 5. That work confirmed the presence of thermophilic campylobacters in the water and sediment of ponds that harboured a large number of waterfowl. In the work described in Chapters 4 and 5 the detection of thermophilic campylobacters from pond water using culture methods proved to be laborious, time consuming and liable to the generation of false negative results. The aim of the work described in this chapter was to explore the use of a rapid and sensitive PCR assay for direct detection of thermophilic campylobacters in village pond water.

7.2 MATERIALS AND METHODS

7.2.1 Site and sampling

The study site was a roadside village pond at South Dalton in East Yorkshire, NE England. The pond harbours a permanent population of ducks, receives run-off from adjacent roads, and is an amenity site. A detailed description of the pond and its environs is given in Chapter 2.1. Surface water samples were collected in the mornings during in March 2003. The samples were collected in sterile polypropylene bottles as described in Chapter 3.1.1.

7.2.2. Direct PCR assay for detection of campylobacters in pond water

A PCR assay was applied directly to eight 100 ml pond water samples with the aim of detecting thermophilic campylobacters. Microbial DNA was extracted by four different approaches. These were: (1) Bead-beating, (2) the use of Proteinase K, (3) boiling, and (4) the use of Chelex[®] 100. Each approach was applied to two of the 100 ml pond water samples. In parallel with the molecular approaches, another two 100 ml water samples from the same pond were used to detect *Campylobacter* by conventional culture methods as described in Chapter 3.3.2.1.

7.2.2.1 Preparation of cell pellets

A volume of 100 ml of pond water was centrifuged at 10,000 RCF for 10 min. The supernatant was discarded and the pellets were washed and re-centrifuged twice in 500 µl phosphate-buffered saline (8 g NaCl, 0.2 g KCl, 1.44 g KH₂PO₂ in 800 ml sterile pure water [pH 7.4], Sambrook *et al.*, 1989).

7.2.2.2a DNA extraction by the bead-beating method

After preparation and washing of the pellets, DNA was extracted by the bead-beating method (More *et al.*, 1994; Kuske *et al.*, 1998). The procedure was as follows: 0.5 ml of 0.1 mol l⁻¹ sodium phosphate buffer (pH 8.0), 1.0 g sterile 0.1 mm diameter glass beads, and 0.25 ml of 100 mmol l⁻¹ NaCl: 500 mmol l⁻¹ Tris-HCl (pH 8.0): 10 % sodium dodecyl sulphate (SDS) were added to the pellets in a 2-ml sterile bead-beater tube (Ogram, 1998). The samples were homogenised for 2 min at 2,500 rpm in a mini-bead beater disruptor (Biospec, Oklahoma, USA). The mixture was then transferred to a sterile microcentrifuge tube and centrifuged for 2 min at 11,600 RCF.

After centrifugation, the aqueous phase was transferred to a sterile microcentrifuge tube and protein impurities were extracted into an equal volume of a

mixture of phenol:chloroform:isoamylalcohol (25:24:1) (v/v/v). The extraction protocol was repeated until no protein precipitate was observed at the aqueous-organic interface (Wawer and Muyzer, 1995). Following the phenol extraction, a one step extraction of protein impurities with chloroform was applied.

The aqueous phase that was left after chloroform extraction, and which contained the DNA was mixed with 900 μl of 10 mol l^{-1} ammonium acetate (final concentration 2.5 mol l^{-1}) and 2 volumes of ice-cold ethanol, and was maintained at -20°C overnight. This step was to precipitate the extracted DNA from the aqueous phase and was followed by centrifugation at 11,600 RCF for 20 min. After centrifugation, the DNA pellet was rinsed with 70 % ethanol and dried overnight in air at room temperature. The following day, the DNA was dissolved in 100 μl TE buffer (10 mmol l^{-1} Tris:HCl: 1 mmol l^{-1} EDTA [pH 8.0], Sambrook *et al.*, 1989).

To reduce humic materials and other organic contaminants, the extracted DNA was cleaned using a Wizard DNA Clean Up Resin Kit (Promega, Madison, USA). DNA purification was performed as follows: a volume of 100 μl of the DNA preparation was added to 1.0 ml of the resin (pre-heated at 37°C and cooled to 30°C), in a sterile microcentrifuge tube. The tube was inverted gently to mix the contents well. The mixture was transferred to a 3-ml sterile disposable syringe attached at the tip of a Mini-column (Promega, Madison, USA). The slurry was then pushed gently through the Mini-column by the aid of the syringe plunger. At the end of this stage, the DNA was trapped in the Mini-column. The Mini-column was washed with 2 ml of 80 % isopropanol and then centrifuged at 3,360 RCF for 2 min, to ensure that the resin was dried. A volume of 50 μl of pre-warmed (70°C) TE buffer was then added to the Mini-column and left for 1 min then centrifuged at 3,360 RCF for 20 s. The resulting solution contained sufficiently pure DNA to use as a template for PCR.

7.2.2.2b DNA extraction by Proteinase K

Proteinase K is a non-specific serine protease from the fungus *Tritirachium album*, which is used to purify target materials from contaminating proteins and for the isolation of DNA (Anon., 1997). The washed pellets that had been prepared by centrifugation of 100 ml of pond water were resuspended in 100 µl of Proteinase K buffer (0.002 g Proteinase K [Sigma, St. Louis, USA], 0.075 g KCl, 0.012 g MgCl₂, 0.048 g Tris, 0.1 ml Tween[®] 80 in 10 ml sterile pure water). The suspension was incubated at 55 °C for 60 min, followed by incubation at 99.9 °C for 10 min to denature the Proteinase K. The mixture, now containing lysed microbial cells, was centrifuged at 1260 RCF for 5 min. The supernatant was used as template DNA for PCR.

7.2.2.2c DNA extraction by boiling

The washed pellets prepared by centrifugation of 100 ml of pond water were resuspended in 100 µl sterile pure water. The mixture was boiled for 5 min, and then centrifuged at 1260 RCF for 5 min. The supernatant was then used as template DNA for PCR.

7.2.2.2d DNA extraction by Chelex[®] 100

Chelex[®] 100 is a chelating resin that has a high affinity for polyvalent metal ions (Malorny and Helmuth, 2003). The washed pellets prepared from centrifugation of 100 ml of pond water were resuspended in 100 µl of Chelex[®] 100 solution (Bio-Rad, Hercules, CA, USA). The mixture was then incubated at 56 °C for 20 min, followed by incubation at 99 °C for 10 min. The mixture was then centrifuged at 1260 RCF for 5 min. The supernatant was used as template DNA for PCR.

7.2.3 PCR assay

The PCR assay was carried out with the aim of amplifying the flagellin genes (*flaA* and *flaB*) of *Campylobacter jejuni* and *C. coli* from DNA extracted using the above four protocols. PCR was carried out as described in Chapter 3.3.5.1

7.2.4 Detection limits of PCR

In order to determine the least number of *Campylobacter* cells that can be detected by the PCR method employed, DNA was extracted and amplified from sterile pure water and from fresh pond water that had both been spiked with known concentrations of *Campylobacter* cells. The *Campylobacter* isolates used for spiking water samples were isolated from the pond at South Dalton by membrane filtration and selective enrichment as described in Chapter 3.3.2.1, and confirmed as either *C. jejuni* or *C. coli* by colony-PCR as described in Chapter 3.3.5.1. A thick suspension of *Campylobacter* was prepared and serially diluted. A volume of 1.0 ml from each dilution was added to 100 ml samples of sterile pure water and fresh pond water. *Campylobacter* concentrations in each spiked sample were calculated from the result of acridine-orange direct counts made on the stock *Campylobacter* suspension as described in Chapter 5.3.6. Spiked samples (100 ml) were centrifuged at 10,000 RCF and pellets were washed as described previously. Microbial DNA was extracted from each spiked sample by the Proteinase K, boiling, and Chelex[®] 100 methods as described above.

7.2.5 Detection of thermophilic campylobacters in un-spiked pond water by using PCR after selective enrichment culture

Pond water samples of different volumes (10 ml, 100 ml, 1000 ml) were filtered and the filters were incubated overnight in Preston enrichment broth as described in Chapter 3.3.2.1. After the enrichment period, 500 µl of each enrichment culture were

transferred to a sterile microcentrifuge tube, and spun at 10,000 RCF for 10 min. The supernatant was discarded and the pellets were washed twice in 500 µl of phosphate-buffered saline. DNA was extracted from the pellets by the Proteinase K method. Template DNA was amplified by PCR as described in Chapter 3.3.5.1. At the same time, a loopful of each enrichment was plated on mCCD agar to confirm the presence of thermophilic campylobacters in pond water by conventional culture techniques (see Chapter 3.3.2.1) and by PCR applied to colonies on the mCCD agar.

7.3 RESULTS

7.3.1 Direct PCR assay for detection of campylobacters in pond water

Four different methods of DNA extraction were performed on pellets prepared from eight 100 ml pond water samples that had suffered natural contamination by thermophilic campylobacters. Despite the successful extraction of DNA from pond water, no PCR amplification with primers CF03 and CF04 was obtained. Thus, all eight-100 ml pond water samples were recorded as *Campylobacter*-negative by the direct PCR approach (Fig. 7.1). The detection of *Campylobacter* by the conventional enrichment method in two 100 ml samples of pond water, however, yielded positive results (Table 7.1).

7.3.2 Detection limits of PCR

The investigation of detection limits of PCR showed that when the Proteinase K extraction method was used as few as 50 *Campylobacter* cells per 1.0 ml were detected in spiked sterile pure water by direct PCR (Fig. 7.2). The lower limit of PCR detection in spiked pond water following Proteinase K extraction was around 400 *Campylobacter* cells per 1.0 ml (Fig. 7.2).

The DNA extracted by the Proteinase K method from spiked sterile pure water and from spiked fresh pond water was frequently amplified with primers CF03 and CF04 (Fig. 7.2). In contrast, the treatment of pellets by boiling as a method of DNA extraction gave fewer positive PCR amplifications (Fig. 7.3). The DNA extracted by Chelex[®] 100 gave even poorer PCR amplification. Thus DNA from spiked fresh pond water was never amplified, while DNA extracted by Chelex[®] 100 from sterile pure water amplified only when the highest concentration of spike ($5 \times 10^5 \text{ ml}^{-1}$) was used (Fig 7.4). A summary of the results of direct PCR detection on pure water and fresh pond water that had been spiked with *Campylobacter* cells is given in Table 7.2.

7.3.3 Detection of thermophilic campylobacters in un-spiked pond water by PCR after selective enrichment

PCR amplification of DNA from pellets harvested from Preston enrichment broth culture was always obtained when the enrichments were inoculated with the residue on filters that were from the filtration of 100 ml of pond water. In contrast, PCR amplification was only sometimes obtained when 10 ml samples of pond water were filtered and was never obtained when 1000 ml samples were filtered (Figs. 7.5; 7.6; Table 7.3). When PCR amplification was obtained using DNA from the enrichment broth, the DNA from colonies on mCCD agar also gave positive PCR amplification (Fig. 7.6; Table 7.3).

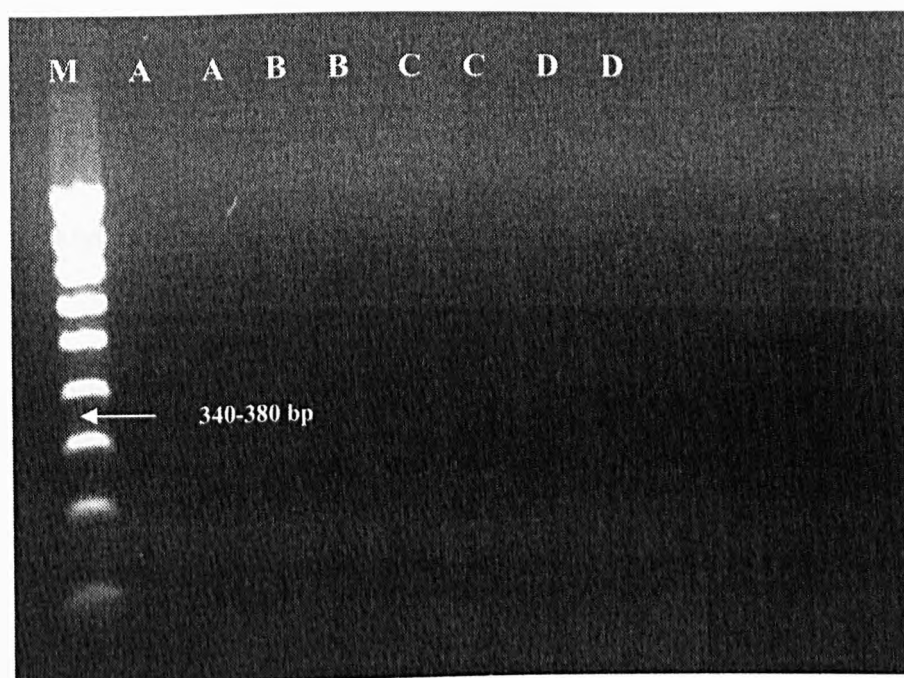


Fig. 7.1 The results of PCR assay for *Campylobacter* conducted on DNA extracted directly from 100 ml samples of pond water, using four different methods of DNA extraction. Lane M represents a 100 bp ladder; lanes A are from amplification of DNA extracted by the bead-beating method; lanes B by the Proteinase K method; lanes C by the boiling method and lanes D by the Chelex[®] 100 method. No amplification was obtained.

Samples were from South Dalton pond in February 2004.

Table 7.1 The detection of naturally-occurring *Campylobacter* in 100 ml pond water samples by direct PCR and by enrichment culture

Method used	Total number of samples [†]	Number of positive samples
Bead-beating and direct PCR	2	0
Proteinase K and direct PCR	2	0
Boiling and direct PCR	2	0
Chelex [®] 100 and direct PCR	2	0
Enrichment culture and selective plating	2	2

[†] Samples were from South Dalton Pond in February 2004.

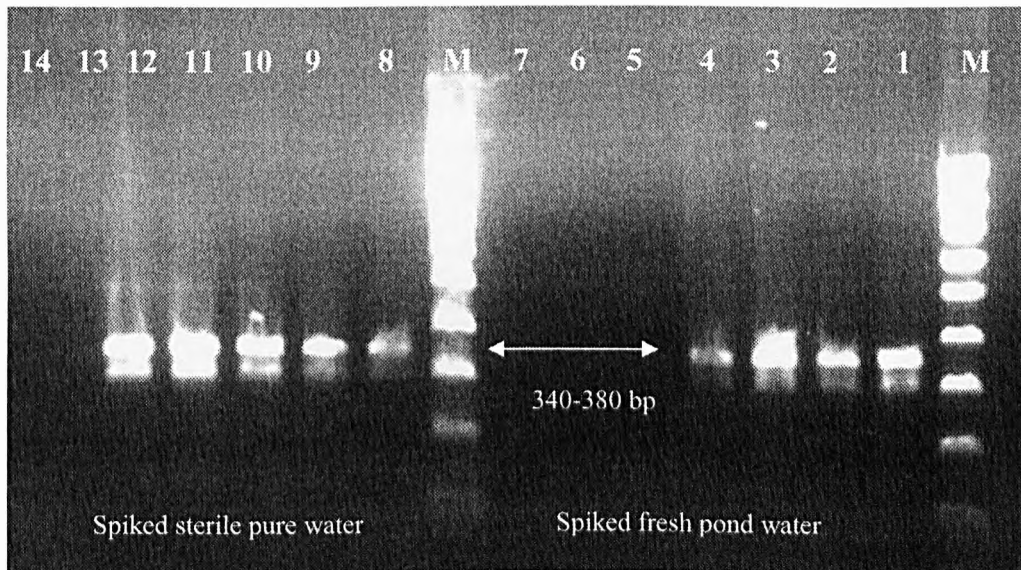


Fig. 7.2 Investigation of the PCR detection limit in sterile pure water and fresh pond water, from South Dalton in March 2004, spiked with known concentration of *Campylobacter*. DNA was extracted by the Proteinase K method. Lane M represents a 100 bp ladder; lanes 1-7 were for amplification of DNA extracted from 100 ml of spiked pond water and lanes 8-14 from 100 ml of spiked sterile pure water. Lanes 1 and 8 had 5×10^7 *Campylobacter* cells 100 ml^{-1} ; lanes 2 and 9, 3×10^6 cells 100 ml^{-1} ; lanes 3 and 10, 3×10^5 cells 100 ml^{-1} ; lanes 4 and 11, 4×10^4 cells 100 ml^{-1} ; lanes 5 and 12, 5×10^3 cells 100 ml^{-1} ; lanes 6 and 13, 8×10^2 cells 100 ml^{-1} and lanes 7 and 14, 9×10^1 cells 100 ml^{-1} .

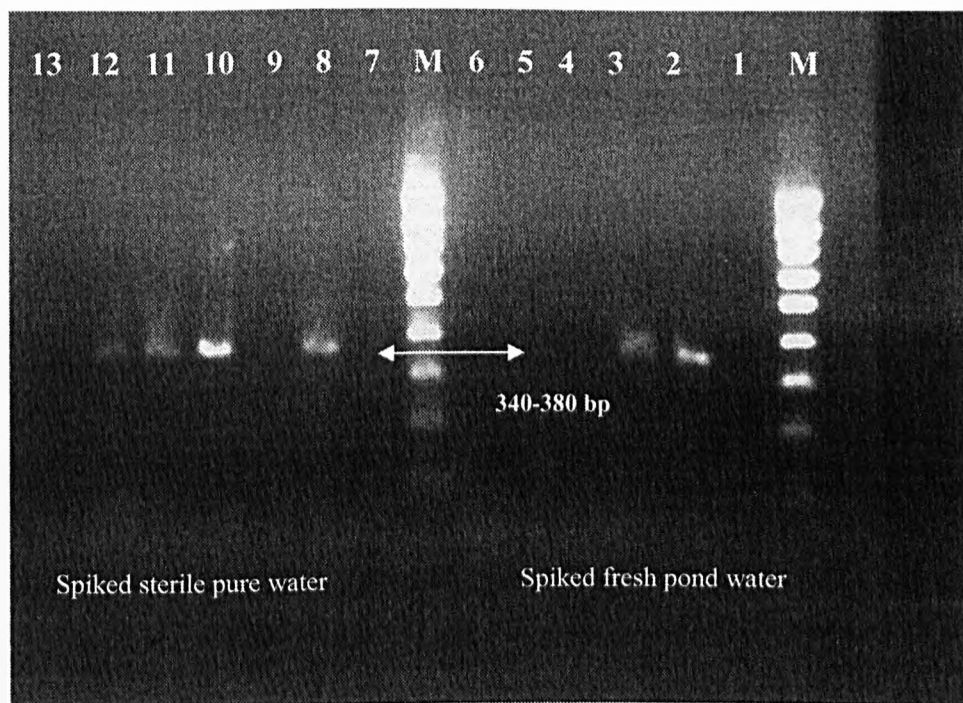


Fig. 7.3 Investigation of the PCR detection limit in sterile pure water and pond fresh water, from South Dalton in March 2004, spiked with known concentrations of *Campylobacter*. DNA extracted by boiling. Lane M represents a 100 bp ladder; lanes 1-6 were from amplification of DNA extracted from 100 ml of spiked pond water; lanes 7-13 were DNA extracted from 100 ml of spiked sterile pure water. Lanes 1 and 7 had 5×10^7 *Campylobacter* cells 100 ml^{-1} ; lanes 2 and 8, 3×10^6 cells 100 ml^{-1} ; lanes 3 and 9, 3×10^5 cells 100 ml^{-1} ; lanes 4 and 10, 4×10^4 cells 100 ml^{-1} ; lanes 4 and 10, 4×10^4 cells 100 ml^{-1} ; lanes 5 and 11, 5×10^3 cells 100 ml^{-1} ; lanes 6 and 12, 8×10^2 cells 100 ml^{-1} ; lane 13, 9×10^1 cells 100 ml^{-1} .

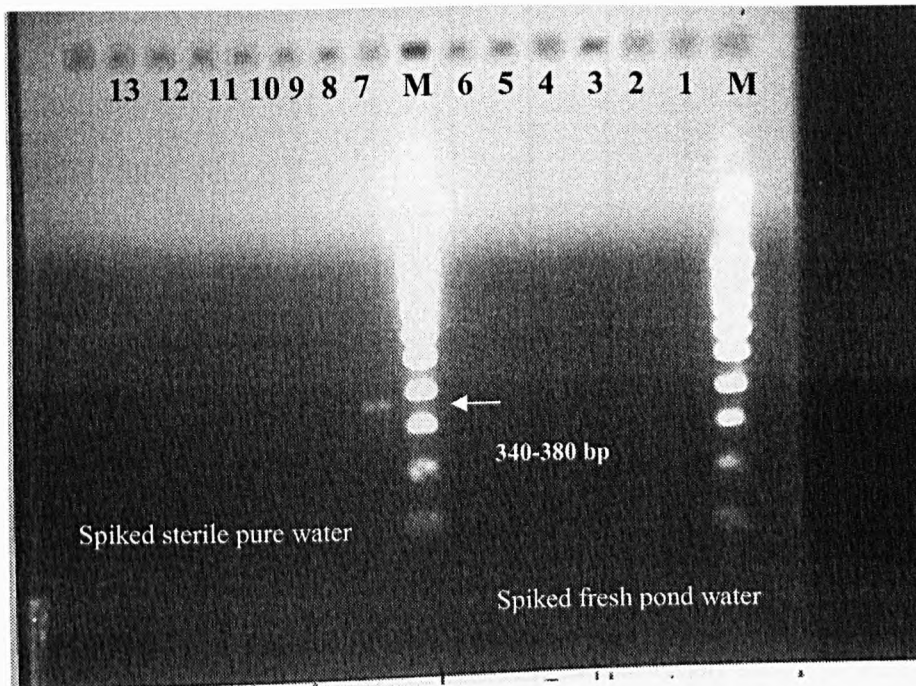


Fig. 7.4 Investigation of the PCR detection limit in sterile pure water and fresh pond water, from South Dalton in March 2004 spiked with known concentrations of *Campylobacter*. DNA extracted by Chelex[®] 100. Lane M represents a 100 bp ladder; lanes 1-6 were from amplification of DNA extracted from 100 ml of spiked pond water; lanes 7-13 were DNA extracted from 100 ml of spiked sterile pure water. Lanes 1 and 7 had 5×10^7 *Campylobacter* cells 100 ml^{-1} ; lanes 2 and 8, 3×10^6 cells 100 ml^{-1} ; lanes 3 and 9, 3×10^5 cells 100 ml^{-1} ; lanes 4 and 10, 4×10^4 cells 100 ml^{-1} ; lanes 4 and 10, 4×10^4 cells 100 ml^{-1} ; lanes 5 and 11, 5×10^3 cells 100 ml^{-1} ; lanes 6 and 12, 8×10^2 cells 100 ml^{-1} ; lane 13, 9×10^1 cells 100 ml^{-1} .

Table 7.2 Summary of the result of direct PCR applied to sterile pure water and fresh pond water spiked with *Campylobacter*

Concentration of <i>Campylobacter</i> cells added (ml ⁻¹)	Proteinase K extraction		Boiling extraction		Chelex [®] 100 extraction	
	In sterile pure water	In fresh pond water	In sterile pure water	In fresh pond water	In sterile pure water	In fresh pond water
5×10^5	+	+	-	-	+	-
3×10^4	+	+	+	+	-	-
3×10^3	+	+	-	+	-	-
4×10^2	+	+	+	-	-	-
50	+	-	+	-	-	-
8	-	-	-	-	-	-
0.9	-	-	-	-	-	-

+ Indicates detection of *Campylobacter* by direct PCR; - indicates not detected.

The data in this table summarise the results shown in Figs. 7.2, 7.3 and 7.4.

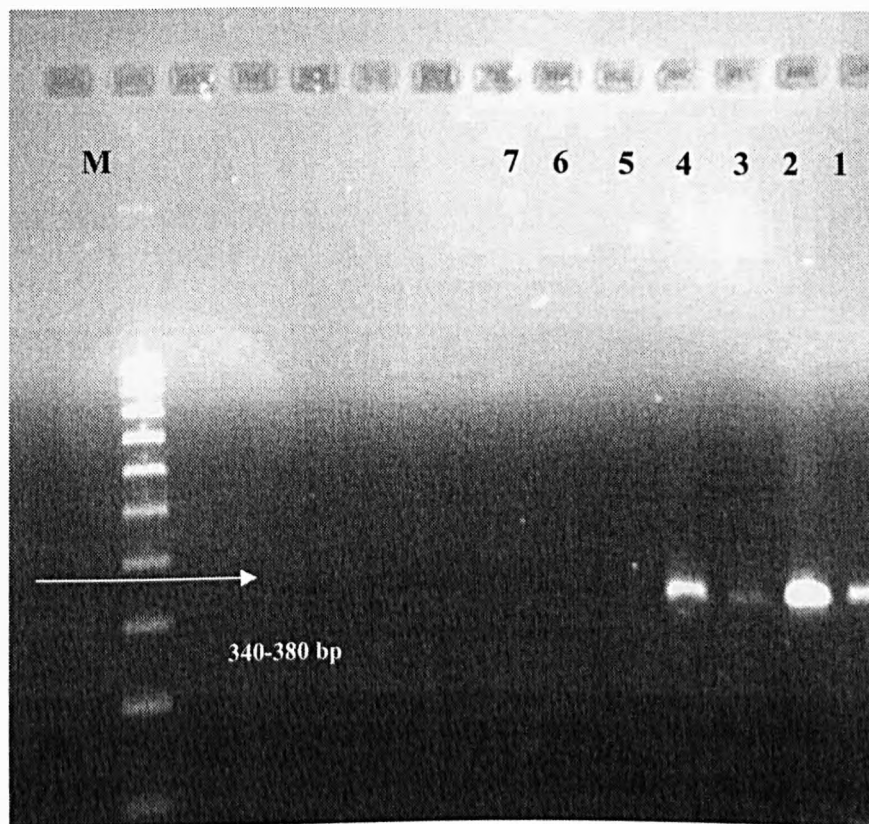


Fig. 7.5 PCR performed on DNA extracted from pellets after selective enrichment of filtered pond water in Preston broth. DNA extracted with Proteinase K. Lane M is a 100 bp ladder; lanes 1 and 3, pellets harvested from the enrichment of 10 ml filtration of pond water; lanes 2 and 4 from the enrichment of 100 ml filtration of pond water; lanes 5-6 from the enrichments of 1000 ml filtration of pond water. Samples were from the pond at South Dalton in May 2004.

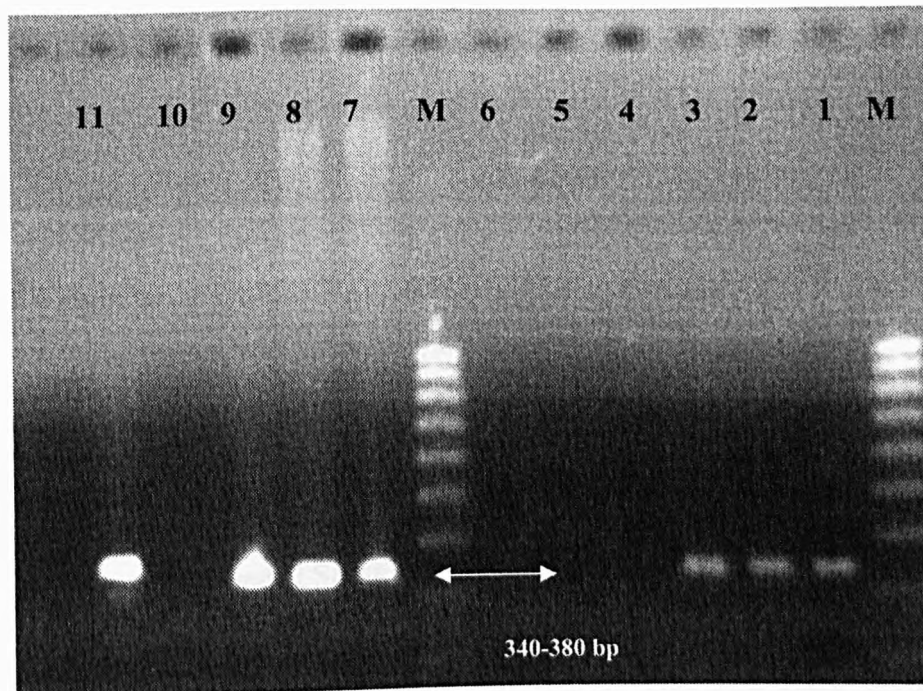


Fig. 7.6 PCR performed on DNA extracted from pellets after selective enrichment of filtered pond water in Preston broth and from colonies growing on selective agar treated by Proteinase K. Lanes M are 100 bp ladder; lanes 1-6, pellets from enrichment broth; lanes 7-9, colonies growing on selective agar after enrichment in Preston broth. Lanes 1-2 from the enrichments following the filtration of 100 ml pond water; lanes 3-4 from the enrichments following the filtration of 10 ml of pond water; lanes 5-6 from the enrichments following the filtration of 1000 ml of pond water; lanes 7-8 from the colonies growing on selective agar after the enrichment of 100 ml of filtered pond water; lane 9 from the colonies on selective agar after the enrichment of 10 ml of filtered pond water; lane 10 DNA-free amplification; lane 11 a positive control (a clinical *Campylobacter* isolate).

Samples were from the pond at South Dalton in May 2004.

Table 7.3 Detection of thermophilic campylobacters in un-spiked pond water by PCR after selective enrichment

	Volumes filtered [†]		
	10 ml n:p	100 ml n:p	1000 ml n:p
PCR with Proteinase K extraction from pellets after selective enrichment	4:2	4:4	4:0
Confirmation by selective plating after enrichment [‡]	4:2	4:4	4:0

[†] Un-spiked pond water was filtered and filters were incubated overnight in Preston enrichment broth.

[‡] This represents positive confirmation of colonies on mCCD agar by both conventional biochemical/morphological methods and by PCR.

n = total number of samples.

p = total number of positive samples.

Samples were from the pond at South Dalton in May 2004.

7.4 DISCUSSION

7.4.1 Direct PCR assay for detection of campylobacters in pond water

In this chapter the direct application of PCR to fresh turbid pond water, to detect thermophilic campylobacters, was investigated. DNA that was extracted from eight 100 ml pond water samples, by four different methods, failed to give PCR amplification with primers CF03 and CF04 (Fig. 7.1). This negative result may have been due to the presence of humic substances that have an inhibitory effect on the DNA polymerase enzyme, and/or colloidal material that has a high affinity for DNA (Way *et al.*, 1993; Wilson, 1997). Alternatively, the negative result may be because low numbers of thermophilic campylobacters were present or because they were absent in the samples. Since one of the four protocols used for DNA extraction included the use of the wizard DNA clean up kit, the inhibition should have been partially removed. Other work on DNA from aquatic environments (upland streams) has shown that the wizard kit is effective at removing contaminant humic materials from DNA samples prior to PCR (Mahmoud, Goulder and Carvalho, in press). It follows that the negative results with PCR were probably due to the absence or low numbers of *Campylobacter*. However, since filtration, enrichment and selective plating of 100 ml samples from the same pond yielded confirmed *Campylobacter* isolates (Table 7.1) this strongly suggests that *Campylobacter* was present but in such low numbers that it was not readily detectable by PCR.

7.4.2 Detection limits of PCR

To determine the detection limit of direct PCR assay, both sterile pure water and fresh pond water samples were spiked with known concentrations of *Campylobacter*

cells. This procedure was also designed to allow comparison of the efficacy of three methods of DNA extraction (i.e. the use of Proteinase K, boiling, and Chelex[®] 100). At best, as few as 50 *Campylobacter* cells ml⁻¹ were detected by PCR in sterile pure water (Fig. 7.2 and Table 7.2). This result is close to that reported by Kirk and Rowe, (1994), who achieved a PCR detection limit of 10-20 *Campylobacter* cells ml⁻¹ in spiked pure water. Lower PCR detection limits of pathogenic bacteria in water have been reported, for example, as few as 12 cells ml⁻¹ of *Legionella* were detected by PCR in un-spiked cooling tower water (Koide *et al.*, 1993). When PCR was applied to spiked turbid pond water, however, the lowest concentration of *Campylobacter* cells detectable by direct PCR was higher, at around 400 cells ml⁻¹ (Fig. 7.2 and Table 7.2). This result supports the suggestion that *Campylobacter* cells in low numbers in turbid environmental waters are not easily detectable by direct PCR.

To investigate the efficacy of different DNA extraction methods, the Proteinase K, boiling, and Chelex[®] 100 protocols for DNA extraction were compared. The three methods were applied to pellets harvested from 100 ml samples of spiked sterile pure water and spiked fresh pond water. Extraction with Proteinase K was much the most successful method (Table 7.2). With the boiling method, DNA was amplified from fewer samples (Table 7.2). This result supported the suggestion of Mohran, *et al.* (1998) that some *Campylobacter* strains are resistant to lysis by boiling. When the DNA was extracted by Chelex[®] 100, PCR almost always gave a negative result (Table 7.2). Unlike in my study, the successful use of Chelex[®] 100 for the extraction of prokaryotic and eukaryotic DNA has been reported (Werner and Mergenhagen, 1998; Brasher *et al.*, 2002; Shah *et al.*, 2002). However, Chelex[®] 100 is actually a chelating resin that has affinity for polyvalent metal ions that is usually applied to remove PCR inhibitors and

to protect extracted DNA from degradation (Malorny and Helmuth, 2003). It appears not to be appropriate for extraction of DNA from campylobacters.

7.4.3 Detection of thermophilic campylobacters in un-spiked pond water by PCR after selective enrichment

The many problems associated with the detection of thermophilic campylobacters in naturally contaminated environmental waters by culture methods have encouraged the development of molecular techniques to detect these pathogens without the need for conventional culturing methods. PCR has successfully detected thermophilic campylobacters in naturally-contaminated drinking water and swimming-pool water without the need for enrichment culture (Jackson *et al.*, 1996; Moore *et al.*, 2001). More recently, Yang, *et al.* (2003) claimed to have detected *Campylobacter jejuni* in naturally contaminated environmental water by direct PCR without the need for culturing the samples. The description of methods in that paper, however, clearly suggests that there was enrichment culture of the water samples prior to PCR. Thus, although direct PCR detection of *Campylobacter* in clean water may be feasible, the direct application of PCR to turbid pond water for detection of thermophilic campylobacters poses problems and may not give positive results. Unlike in contaminated drinking water or swimming-pool water, thermophilic campylobacters in turbid pond water are present in relatively low numbers against an abundant background microflora and high concentrations of potential PCR inhibitors.

In the work described in this chapter, when 10 ml and 100 ml samples of turbid pond water were filtered, and the filters were incubated overnight in a selective enrichment culture, the DNA subsequently extracted almost always amplified with primers CF03 and CF04 (Table 7.3), giving a product size of 340-380 bp (Figs. 7.5; 7.6). Thus, the results showed that selective enrichment culture followed by PCR is a

reliable method for the relatively rapid detection of thermophilic campylobacters in naturally contaminated turbid environmental waters. This conclusion agrees with many authors who have used PCR after an enrichment step to detect *Campylobacter* in spiked estuarine and river waters (Hernandez *et al.*, 1995; Waage *et al.*, 1999), naturally contaminated and spiked sewage (Koenraad *et al.*, 1995; Waage *et al.*, 1999), naturally contaminated and spiked food samples (Giesendorf *et al.*, 1992; Denis *et al.*, 2001; Sails *et al.*, 2003), spiked chicken rinse water (Ng *et al.*, 1997; Josefsen *et al.*, 2004) and from naturally-contaminated human and poultry faecal samples (Rasmussen *et al.*, 1996; Denis *et al.*, 1999; Vanniasinkam *et al.*, 1999). In general, enrichment incubation after sample collection has been shown to provide higher level of PCR detection by increasing the number of target cells. This approach was reported to be successful not only for the detection of campylobacters, but also for the detection of other bacteria such as *Salmonella* from spiked faeces and meat (Rychlik *et al.*, 1999) and *Vibrio cholerae* from spiked drinking, surface and ground water and treated sewage (Theron *et al.*, 2000).

Thus, it has been suggested that the combination of selective enrichment culture followed by a PCR assay is a more rapid method, with higher detection ability, than conventional enrichment culture followed by confirmation using biochemical and morphological tests (Koenraad *et al.*, 1995). My results reported in this chapter support this suggestion.

Another potential disadvantage of direct PCR assay on environmental samples without an enrichment step is the possibility that naked DNA fragments or DNA from dead cells might be detected (Mandrell and Wachtel, 1999). The presence of dead *Campylobacter* cells in environmental waters may suggest that the water has been contaminated, but no longer poses any public health threat (Theron and Cloete, 2004). Since the detection of viable *Campylobacter* cells in environmental waters is the point

of concern to public health authorities, the application of an enrichment step prior to PCR assay will allow only the detection of viable cells.

In an earlier chapter (Chapter 5), the conventional detection of thermophilic campylobacters was successful following the filtration of 10 ml and 100 ml of pond water. The filtration of 1000 ml of pond water, however, never gave *Campylobacter*-positive cultures. It was suggested that competition from the background microflora in large-volume sample prevented the growth of *Campylobacter* cells to detectable levels. The results of enrichment culture followed by PCR that is reported in the present chapter further supports the findings of Chapter 5. PCR amplification was achieved only from the enrichment culture derived from the filtration of 10 ml and 100 ml of turbid pond water (Figs. 7.5; 7.5). In contrast, the filtration and enrichment of 1000 ml of pond water never gave any PCR amplification (Figs. 7.5; 7.6). When enrichment cultures were plated on mCCDA selective agar, confirmed *Campylobacter* colonies were only obtained from the enrichments that also gave positive PCR amplification, i.e. from the filtration of 10 ml and 100 ml of pond water (Fig. 7.6 and Table 7.3). This clearly shows that the filtration of a large volume of turbid pond water is counterproductive and does not lead to the growth of *Campylobacter* in enrichment culture.

7.5 CONCLUSIONS

- The detection of thermophilic campylobacters in naturally-contaminated, turbid, pond water was not achieved by direct PCR assay although positive cultures were obtained by conventional methods.
- This was most likely due to low numbers of *Campylobacter* in the samples.
- The presence of humic material and other PCR inhibitors may also have contributed. Selective enrichment, however, facilitated the detection of

Campylobacter by PCR, especially when Proteinase K was used to extract DNA from cells.

- The combination of selective enrichment and PCR for detecting thermophilic campylobacters in turbid pond water is recommended.
- The method only gave positive results when an appropriate volume (10 ml or 100 ml, but not 1000 ml) of pond water was used for enrichment. This was probably because of competition by background bacteria during enrichment culture.

CHAPTER 8

GENERAL DISCUSSION

In previous chapters, the influence of waterfowl on the bacteriological quality of amenity ponds, and the distribution of bacterial pathogens in these ponds was discussed. The present chapter aims to review the main findings and to identify directions for future work.

The results reported in Chapters 4, 5, and 6 supported the hypothesis that dense populations of waterfowl are responsible for poor microbiological quality of amenity ponds (Chapter 4) and act as a source of enteric bacterial pathogens through the contamination of these ponds (Chapter 6). Although amenity ponds in East Yorkshire are not used for bathing, they failed the EU guidelines and also mandatory requirements for bathing water (Chapter 4), hence skin contact or accidental ingestion of these pond waters should be considered as potentially hazardous. Furthermore, *Salmonella* and *Campylobacter* were recovered only from the water and/or sediments of ponds with waterfowl (Chapters 4 and 5). Initial failure to detect pathogenic bacteria in amenity ponds that are subject to faecal contamination from waterfowl was attributed to shortcomings in standard culture techniques (Chapter 5). These shortcomings can be overcome by using small sample volumes to reduce competition from background bacteria in enrichment culture. PCR improved on conventional confirmatory tests and gave accurate results in a much shorter time (Chapters 5 and 6). The work described in Chapter 7 showed that direct detection by PCR of *Campylobacter* in turbid pond water may not be feasible. The use of a selective enrichment step prior to PCR, however, facilitated the detection of campylobacters.

8.1 Waterfowl, faecal indicators, and pathogenic bacteria in amenity ponds: concluding remarks and future work

8.1.1 The influence of waterfowl on amenity ponds

The impact of wild birds on the microbiological quality of surface water has received considerable attention from a public health standpoint. The role of gulls in the deterioration of bacteriological quality in drinking water reservoirs and on bathing beaches is well documented (Gould and Fletcher, 1978; Lévesque *et al.*, 1993; Alderisio and Deluca, 1999; Lévesque *et al.*, 2000; Wither *et al.*, 2005). Poor microbiological quality of recreational waters brought about by waterfowl (i.e. ducks and geese) has been studied to a far lesser extent (Standridge *et al.*, 1979; Hussong *et al.*, 1979). Studies that address the deterioration in microbiological quality of non-bathing recreational water (i.e. amenity ponds), and the possible health risks caused by waterfowl, are rare.

The results reported in my study clearly showed that high abundance of *E. coli* and faecal streptococci was only found in the water and sediment of ponds that harboured waterfowl (Tables 4.6, 4.7 and 4.9). The abundance of *E. coli* in pond waters was strongly associated with waterfowl numbers per unit pond area (Table 4.10). These results agree with Hussong, *et al.* (1979) and Standridge, *et al.* (1979), who concluded that large numbers of waterfowl can cause an increase in faecal coliforms in water and sediments. Such increase is probably inevitable since a gram dry weight of mallard faeces can contain up to 4.9×10^{11} faecal coliforms and 6.3×10^8 faecal streptococci (Obiri-Danso and Jones, 1999a) and 3.3×10^7 *E. coli* (Taylor, 2003). Pathogenic bacteria (*Campylobacter* and *Salmonella*) were only recovered from the water and/or

sediment of ponds with waterfowl (Tables 4.13, 5.2 and 5.3). This is not surprising since ducks and geese have been reported to harbour *Campylobacter* and *Salmonella* (Luechtefeld *et al.*, 1980; Pacha *et al.*, 1988; Feare, *et al.*, 1999; Fallacara *et al.*, 2001; Kassa *et al.*, 2001; Murphy *et al.*, 2005). The high abundance of faecal indicator bacteria found in pond waters (Tables 4.6 and 4.9) led to ponds with waterfowl failing the EU guidelines and also mandatory requirements for safe bathing water. It is important to emphasise, however, that amenity ponds studied here are not bathing sites. The poor microbiological quality of these ponds, however, suggests that skin contact or accidental exposure to pond water could pose a health threat. Children should probably be discouraged from playing in the margins of these ponds.

The presence of large numbers of waterfowl in the vicinity of amenity ponds reduces aesthetic value. There is an element of subjectivity in assaying aesthetic values; some observers prefer rich and diverse vegetation, while others might prefer observing waterfowl. The birds eat and damage the aquatic vegetation (Table 2.1) and the water is always turbid as a result of their disturbing bottom sediment (Table 4.5). Defecation from waterfowl litters the areas around the ponds and poses a health hazard to visitors due to the presence of pathogenic bacteria (Table 6.3). It has been estimated that the faecal dropping of one goose per day may be equal to around two pounds of waste (about 1 kg). Such gross amounts of faecal material will inhibit children's play and discourage lying or sitting on grassy margins (Dieter *et al.*, 2001). Other problems associated with the presence of ducks and geese in the vicinity of amenity ponds include possible attacks on elderly people or children especially when there are ducklings or goslings to protect. The birds also tend to stray onto adjacent roads, potentially causing traffic accidents (Dieter *et al.*, 2001).

Waterfowl have been considered to be an important reservoir of campylobacters in nature (Percival *et al.*, 2004). This was further supported by the results reported in my study (Chapter 6).

Little is known about waterfowl and pathogenic strains of *E. coli* (i.e. *E. coli* O157) which have recently emerged as a cause of bloody diarrhoea associated with vomiting in humans (Percival *et al.*, 2004). *E. coli* O157 has been reported in cases related to drinking water and recreational contact with water (Hunter, 1997). Livestock (e.g. cattle and sheep) are regarded as the most important reservoirs for *E. coli* O157 (Duffy, 2003; Ogden *et al.*, 2005) and little is known about any association between waterfowl with pathogenic *E. coli*. Palmgren, *et al.* (1997) failed to detect pathogenic *E. coli* in 151 faecal samples from gulls, starlings, blackbirds, song thrushes and other wild birds but they did not exclude the possibility of a bird reservoir of *E. coli* O157. The speculation of Palmgren and her colleagues about a possible reservoir of pathogenic *E. coli* within wild birds was proved by Wallace, *et al.* (1997) who reported for the first time the isolation of *E. coli* O157 from gull faeces. Recently, Kullas, *et al.* (2002) were the first to report the presence of pathogenic strains of *E. coli* in the faeces of Canada geese. It would be interesting to study the prevalence of *E. coli* O157 in the waterfowl populations and in the water of the ponds that were the focus of the present study (i.e. South Dalton, Little Weighton and Brantingham).

In Saudi Arabia, gastrointestinal tract infections in humans due to bacterial agents are increasing (MOH, 2001; Al-Mazrou, 2004). Although reports concerning the distribution of *Salmonella* serotypes within farm animals and poultry in Saudi Arabia are available (Barbour and Nabbut, 1982; Nabbut *et al.*, 1982; Al-Nakhli *et al.*, 1999), studies concerning the distribution of enteric bacterial pathogens (e.g. *Campylobacter*) in the environment, particularly in wild birds, are lacking. This dearth of information is important because, for example, the City of Makkah (Western Saudi Arabia) has more

pigeon flocks than comparable cities elsewhere. These flocks of pigeons roost on the roofs of houses, near drinking water reservoirs, and sometimes near dining places and food outlets. Campylobacters and salmonellae have been recovered from the faeces of urban pigeons (*Columba livia* var.) in Barcelona, Spain, and were regarded as a potential source of human infection (Casanovas *et al.*, 1995). It would be of value to study the distribution of various serotypes of campylobacters, salmonellae and *E. coli* O157 within wild bird populations and in surface waters in the City of Makkah and other regions of Saudi Arabia, and to compare these types with those of clinical origin.

In addition to bacterial pathogens, waterfowl are reported to carry a wide range of pathogens including viruses (e.g. avian influenza virus, West Nile virus), parasites (e.g. *Cryptosporidium*, *Toxoplasma*) and fungi (e.g. *Candida albicans*, *Aspergillus fumigatus*) (Hubálek, 2004). In 2004 an outbreak of avian influenza (influenza A virus subtype H₅N₁) that specific is to birds began in south-east Asia. In mid 2005 the same strain of the virus was isolated from migratory waterfowl in Eastern Europe. The British Veterinary Association warned that the virus is very likely to reach the UK by migrating birds (Jones, 2005).

8.1.2 Conventional and molecular-genomic methods for the detection and confirmation of enteric bacteria in environmental samples

The detection of a few, possibly injured, cells of enteric bacteria (e.g. *Campylobacter*, *Salmonella*) amongst the large and diverse natural bacterial flora of environmental water samples poses many difficulties. The results obtained from the present work (Chapters 5, 6 and 7) address some of the problems encountered.

8.1.2.1 Detection and confirmation of campylobacters in environmental samples

The general procedure for the detection of campylobacters in environmental waters involves the concentration of large volumes of water on membrane filters and the incubation of these filters in a selective enrichment broth at 37 °C followed by incubation at 42 °C (Percival *et al.*, 2004). Such procedures have been employed as a standard method for the determination of these organisms in all types of water (PHLS, 1998d). In order to increase the sensitivity of the filtration procedure (i.e. increase the number of *Campylobacter* cells concentrated on the filters), the filtration of larger volumes of water samples seems appropriate. Thus, Hänninen, *et al.* (2003) showed that the filtration of up to 10 litres of tap water increased the number of recovered campylobacters and they suggested that sample volumes of 1000 ml are too small to detect campylobacters in tap waters. However, the filtration of large volumes (1000 ml) of turbid pond water can lead to false-negative results (Table 5.2). This is due to the growth of high levels of background heterotrophs and coliforms during the enrichment stage (Table 5.4) which prevent the growth of campylobacters to detectable levels, probably as a result of competition for nutrients. Similar problems were encountered with the processing of a large volume (5.0 ml) of microbially-rich sediment to assess the presence of campylobacters (Table 5.3). Interference by background bacteria in *Campylobacter* enrichment cultures, derived from the filtration of large water samples, was suggested as a problem by Fricker (1987) in his review of diverse methods that may be applied to detect campylobacters and salmonellas in environmental samples. Studies that have addressed this problem are few. Aquino, *et al.* (1996) found that all seemingly *Campylobacter*-negative enrichment cultures derived from contaminated poultry samples had high levels of faecal indicators and lactobacilli, while plates streaked from the same samples yielded campylobacters. The results reported in the present work

(Tables 5.2, 5.3 and 5.4) support the suggestion of Fricker (1987) and reinforce the findings of Aquino, *et al.* (1996) with evidence from a different sampling milieu.

The recovery of *Campylobacter* from duck faeces (Table 6.3), and from run-off water (Table 6.4) at pond sites, confirm the significant roles of both waterfowl and rain-related run-off in the dissemination and distribution of *Campylobacter* in the environment, particularly in surface waters. Roles that have been identified by Stelzer, *et al.* (1991) and Jones (2001).

Presumptive campylobacters that were recovered from pond water (19 isolates), sediment (5 isolates), duck faeces (10 isolates) and run-off water (7 isolates) (Table 6.6) were all confirmed as thermophilic campylobacters by conventional morphological and biochemical tests and also by PCR (Figs. 5.1, 5.2, 6.1 and 6.2). Conventional confirmatory tests were limited to the identification of presumptive isolates to genus level. In contrast, PCR reinforced the confirmation of the presumptive isolates as thermophilic campylobacters and provided further information on the species recovered (i.e. *Campylobacter jejuni* or *C. coli*). DNA from other thermophilic campylobacters (e.g. *C. lari* and *C. upsaliensis*) do not normally amplify with the primers used.

The work described in Chapter 5 on the detection of thermophilic campylobacters in turbid pond water showed that the application of conventional enrichment methods is liable to generate false-negative results. Thus, the aim was to develop a molecular-based approach that provides more reliable results. Direct PCR detection of naturally-occurring *Campylobacter* in drinking water and swimming-pool water has been achieved without prior enrichment culture (Jackson *et al.*, 1996; Moore *et al.*, 2001). With turbid pond-water samples, however, the detection of naturally-occurring campylobacters by direct PCR assay seems not be feasible (Fig. 7.1 and Table 7.1). This is probably due to inadequate number of the target organism along with the presence of humic material and other PCR inhibitors. This suggestion was supported by

the application of direct PCR to pellets prepared by centrifugation of sterile pure water samples and fresh pond water samples, both spiked with known concentrations of *Campylobacter* cells. In spiked sterile pure water the lowest detection limit with direct PCR was 50 *Campylobacter* cells ml⁻¹, which is close to that reported by Kirk and Rowe (1994) (10-20 cells ml⁻¹). In contrast, in spiked fresh pond water the lowest detection limit was much higher at 400 *Campylobacter* cells ml⁻¹ (Fig. 7.2 and Table 7.2).

When fresh pond water samples (10 ml and 100 ml) were filtered and the filters were incubated overnight in selective enrichment culture, the DNA extracted from pellets prepared from the cultures was successfully amplified with primers CF03 and CF04 (Figs. 7.5, 7.6 and Table 7.3). Thus, selective enrichment culture followed by PCR is a reliable and relatively rapid method for the detection of naturally-occurring campylobacters in turbid environmental waters. This conclusion is in agreement with other studies, where PCR was used after an enrichment step for the detection of *Campylobacter*, in spiked estuarine and river waters (Hernandez *et al.*, 1995; Waage *et al.*, 1999). The detection of campylobacters in naturally-contaminated environmental waters by PCR after selective enrichment has many advantages; for example, enrichment culture will allow target cells to increase in numbers and will also dilute PCR inhibitors, thus providing increase of sensitivity of PCR detection. Also the application of PCR assay after a selective enrichment step will allow only the detection of viable cells that are capable of causing infection. Thus PCR assay after selective enrichment is proposed as a standard method for the detection of campylobacters in environmental samples. This proposal conforms to that of Josefsen, *et al.* (2004).

The detection and confirmation of *Campylobacter* in water samples by conventional methods takes up to 88 h (PHLS, 1998d). The purpose of the development of PCR assay for the detection of campylobacters, and other pathogens, in

environmental samples is to obtain reliable and accurate results in a relatively short time. The detection of campylobacters by selective enrichment followed by PCR was shown to give confirmed results within around 44 h, i.e. half the time required by conventional methodology (Chapter 7). This is a valuable improvement in technique, it shortens the time needed for results and eliminates the need for conventional confirmatory tests. Other studies have shown that the amplification of DNA from *Campylobacter* pellets obtained from selective enrichment culture can be achieved after reducing the incubation period. Hernandez, *et al.* (1995) achieved a positive PCR amplification of *Campylobacter* DNA after 24 h incubation, of the filtered residue of spiked estuarine water samples, in enrichment culture. With spiked river water, amplification of DNA from pellets obtained by centrifugation, was achieved after 14 h of incubation (Waage *et al.*, 1999). Similarly, in spiked sewage, *Campylobacter* DNA was amplified after 18 h of incubation in selective enrichment (Koenraad *et al.*, 1995), and with spiked chicken-rinse samples (Josefsen *et al.*, 2004) *Campylobacter* DNA was amplified after 20 h of incubation in enrichment culture.

All the above examples of the successful detection of *Campylobacter* by PCR, after a short enrichment culture step, used spiked environmental samples. It would be useful to develop a PCR protocol for the detection of *Campylobacter* in naturally-contaminated surface waters (e.g. ponds). This might involve perhaps 4-20 h of selective enrichment incubation prior to PCR. Such protocol would require less time and materials than for conventional detection and confirmation of campylobacters and would increase the sensitivity and reliability of the results.

8.1.2.2 Detection and confirmation of salmonellae in environmental samples

Salmonella was not recovered from pond water samples (Table 4.13). This result confirms the difficulties that arise in culturing *Salmonella* from natural aquatic environments, both freshwater and marine (Alcaide *et al.*, 1984; Hussong *et al.*, 1984;

Morinigo *et al.*, 1989; Obiri-Danso and Jones, 1999a, 1999b). *Salmonella* was recovered only from sediment, from a pond that harboured waterfowl, on three occasions during summer (Table 4.13). Greater recovery of salmonellae from bottom sediments than from overlying water has been reported. Hendricks (1971) found that, of 195 samples of sediment and river water, 90 % of the *Salmonella* recovered were from sediment. This was probably a result of concentration of the bacteria through sedimentation (Hendricks, 1971; Van Donzel and Geldreich, 1971). The recovery of *Salmonella* from the sediment of the pond at South Dalton only during the summer (June-August) is superficially explained by the seasonal pattern of distribution shown by this organism, it being most readily found in environmental samples during the summer and autumn (Pianetti *et al.*, 1998).

The low incidence of recovery of *Salmonella* from duck faeces (Table 6.3) agrees with other studies from different parts of the world. They have shown either very low numbers of *Salmonella* in the faeces of ducks and geese, or their absence not only from faeces but also from the water and sediment of roosting sites (Damaré *et al.*, 1979; Hussong *et al.*, 1979; Hubálek *et al.*, 1995; Fallacara *et al.*, 2001, 2004; Refsum *et al.*, 2002a). The low incidence of *Salmonella* in ducks and geese reported in Chapter 6 supports the suggestion that the overall carriage of salmonellae by wild birds is low (Brittingham *et al.*, 1988; Palmgren *et al.*, 1997; Murray, 2000; Kirk *et al.*, 2002; Hernandez *et al.*, 2003; Dovč *et al.*, 2004). Such low carriage can be attributed to a number of factors. For example, wild birds can acquire *Salmonella* through contaminated feeding sites when the carriage of the organism may last only for a few days and there is no pathogenic infection of the host bird (Gridwood *et al.*, 1985; Murray, 2000). Thus it is suggested that wild birds that live well away from polluted environments may not harbour *Salmonella* (Damaré *et al.*, 1979; Tizard, 2004).

It is also important to stress that all the 11 presumptive *Salmonella* isolates that were recovered from sediment (7 isolates) and from duck faeces (4 isolates) (Tables 4.13 and 6.3) were confirmed as *Salmonella* only by biochemical tests (H_2S production in parallel with the lack of urea hydrolysis). The four *Salmonella* isolates recovered from duck faeces did not give a positive reaction with the White-Kauffmann-Li Minor (WKL) serotyping scheme, nor was their DNA amplified with primers S18 and S19 (Fig. 6.3). Thus the observed positive biochemical confirmation of the presumptive isolates as *Salmonella* may have been a false-positive result. Also, the seven *Salmonella* isolates that were recovered from sediment samples from the pond at South Dalton (Table 4.13) were confirmed only by biochemical tests, and were not tested by the WKL scheme or by PCR. Thus it is not known whether these really were *Salmonella*. The positive biochemical confirmation of these isolates may also have been a false-positive result. Thus, results in the literature that are based on conventional confirmation of presumptive salmonellae may need reevaluation.

Problems with false-positive results associated with conventional detection of *Salmonella* from environmental waters have been reported elsewhere. Xylose lysine desoxycholate agar (XLD) has been suggested as the best plating medium for the recovery of salmonellae from polluted water after selective enrichment (Fricker, 1984; Morinigo *et al.*, 1989) and has been used by public health authorities in their standard protocols (HMSO, 1984; PHLS, 1998e; APHA, 1998). These seem to be inadequate in differentiating *Salmonella* from some other bacteria (e.g. *Proteus*) and a high rate of false-positive results has been associated with the use of XLD (Rambach, 1990; Waltman, 2000). In recent years, a completely new agar medium has been developed that contains a chromogenic substrate (Cooke *et al.*, 1999). This medium (CHROMagar *Salmonella*) showed high efficacy in differentiation between reference isolates of *Salmonella* and other genera such as *Proteus*, *Pseudomonas* and *Citrobacter*, and is

thought to be sufficiently selective to allow confirmation of the presence of *Salmonella* without the subsequent use of biochemical confirmatory tests. When the chromogenic medium was compared with conventional plating media for the screening of naturally-occurring salmonellae in human stool samples (Gaillot *et al.*, 1999) it was shown to be superior and had a high selectivity. Thus it was suggested as suitable for use as a primary plating medium for the recovery of salmonellae from faeces. It would be of interest to study the prevalence of *Salmonella* in amenity ponds and their environs utilising the new chromogenic medium and to evaluate its selectivity in the recovery of *Salmonella* from naturally contaminated surface waters and duck faeces. This would need to be done in conjunction with confirmation by PCR assay.

In the present work PCR was used on presumed *Salmonella* colonies that had been confirmed by biochemical methods – they turned out not to be *Salmonella*. PCR might, however, be used in further searching for *Salmonella* in ponds with waterfowl. Both direct PCR, and PCR after an initial, short, enrichment culture, might be explored. A successful protocol would be faster than conventional enrichment culture and selective plating.

8.1.3 Typing of faecally-derived bacteria and tracking the source of contamination in environmental waters

Molecular sequence analysis of amplified DNA from confirmed *Campylobacter* isolates recovered from pond water and sediments, duck faeces, and run-off water (Table 6.6) indicated that the distribution of campylobacters in ponds is dependent on both waterfowl and rain-related run-off. This conclusion is in agreement with Stelzer, *et al.* (1991) and Jones (2001).

The sequence analysis of *Campylobacter flaA* and *flaB* genes showed interesting results in the context of *Campylobacter* ecology. *Campylobacter jejuni* was found in

duck faeces at all pond sites from which faeces were collected (Table 6.6). However, it was found in pond water and sediment only at South Dalton (Table 6.6). In contrast, *Campylobacter coli* was found in the run-off water and in the water and sediment at Little Weighton and Brantingham (Table 6.6). These results suggest that different *Campylobacter* species in pond water and sediment had different sources; i.e. the source of *C. jejuni* in South Dalton pond was waterfowl, whereas the source of *C. coli* at Little Weighton and Brantingham was run-off water. This suggestion is supported by the 100 % identical sequences of all *C. jejuni* isolates from both waterfowl and ponds, and likewise by the identical sequences of all *C. coli* isolates from both ponds and run-off water (Table 6.6). This genetic stability at the East Yorkshire sites may reflect niche/host adaptation, i.e. particular *Campylobacter* strains may be associated with particular phylogenetic groups or geographic populations of host birds (Colles *et al.*, 2003; Broman *et al.*, 2004).

In general, the results of molecular DNA sequencing of *Campylobacter* isolated from the East Yorkshire amenity ponds and their environs (Chapter 6) demonstrated the ecological relevance of typing of campylobacters below the genus level. It is important to stress that routine typing (e.g. biotyping, serotyping) of campylobacters derived from clinical and environmental sources, is not undertaken in public health laboratories in the UK. The practice is that the identification procedure is terminated once presumptive isolates are confirmed as *Campylobacter* spp. by morphological and biochemical tests (Frost, 2001; HPA, 2003; Reilly and Browning, 2004).

The use of conventional tests for the identification of *Campylobacter* spp. from environmental sources has two major limitations. First, the generation of false-positive results. For example, *Campylobacter*-like species (i.e. *Helicobacter* and *Arcobacter*) may be falsely identified as campylobacters. Diergaardt, *et al.* (2004) isolated 100 presumptive campylobacters from drinking water, ground water, and raw sewage in

South Africa. Of these 22 did not grow under aerobic conditions and were thus confirmed as *Campylobacter* spp. The analysis of a 16S rRNA sequence, however, showed that only three of the 22 confirmed isolates were actually *Campylobacter jejuni*, while the remaining 19 isolates were shown to be *Arcobacter butzleri*. Second, lack of knowledge about the degree of similarity between *Campylobacter* strains that are found in clinical specimens and those found in the faeces of waterfowl and in environmental waters. Thus, it is not straightforward for public health authorities in England to determine whether or not the strains of *Campylobacter jejuni* and *C. coli* that can be recovered from amenity sites (e.g. village ponds) play a role in the increase in reported cases of *Campylobacter* enteritis in the human community?

Mawer (1988) studied *Campylobacter* species in the ponds and drains of Hull, as well as strains from local clinical sources, using the Penner serotyping scheme. The results of that study showed that 53 % of the environmental strains were confirmed as *C. jejuni* by morphological and biochemical tests, but were not typable by the Penner scheme (i.e. they were regarded as atypical *C. jejuni*). Thus it was concluded that ponds and drains in the city were not the source of campylobacteriosis in the people living near these water bodies (Mawer, 1988). Given the high discriminatory power of molecular typing methods, it would be worth using them to study the prevalence of *Campylobacter* types derived from diarrhoea patients and from the environment in Hull and surrounding areas to further investigate the conclusions of Mawer (1988).

Based on significant statistical association, it was concluded that waterfowl are the leading cause of high numbers of faecal indicators in some amenity ponds (Table 4.10). In order to reinforce this conclusion a study which tracked the source of faecal indicators by a molecular-genomic approach or by a rapid computerised biochemical fingerprinting approach (Möllby *et al.*, 1993; Blanch *et al.*, 2004; Ahmed *et al.*, 2005) would be of interest. The typing of *E. coli* and faecal streptococci isolated from the

faeces of waterfowl, and those from the water and sediments would reveal more information about the prevalence of different/similar types of faecal indicators in both birds and the pond environments.

Taken together, the results of the present study show that waterfowl are not necessarily benign occupants of amenity ponds. As well as destroying aquatic vegetation and fouling margins, they are a source of faecal indicators and pathogenic bacteria. It might be appropriate for local authorities (e.g. civil parish councils) with responsibilities for amenity ponds to review their policy towards large populations of waterfowl. For example, numbers of waterfowl could be culled, this would need agreement from local people. Also, a programme of educating local people about advantages and disadvantages of waterfowl, and signage near ponds to indicate potential hazards might be appropriate.

MAIN CONCLUSIONS

- Significant correlations were found between abundance of *E. coli* in pond water and number of waterfowl.
- Faecal indicators in the water of East Yorkshire ponds with ducks and geese always exceeded not only EU guidelines, but the absolute requirement for bathing waters (EU, 1994) of < 2000 *E. coli* per 100 ml and < 400 faecal streptococci per 100 ml.
- Skin contact and accidental ingestion of water from ponds with waterfowl is probably best avoided.
- When determining campylobacters the filtration of a large volume of turbid environmental water or the use of a large volume of sediment may be counterproductive and may not yield presumptive campylobacters; this is because of competition by background microflora in enrichment cultures.

- Pilot studies to establish appropriate volumes of environmental water or sediment samples should be undertaken before routine determination of *Campylobacter* is begun,
- *Campylobacter*s may be isolated from duck faeces and run-off water as well as from the water and sediment of village ponds.
- The faeces of waterfowl and rain-related run-off are both potential sources of *Campylobacter*s to the ponds.
- Different *Campylobacter* species may have different origin: *C. jejuni* from ducks, *C. coli* from run-off.
- *Salmonellae*, in contrast, were not reliably isolated from duck faeces or run-off, nor were they found in pond water, and only occasionally in sediment.
- The detection of thermophilic *Campylobacter*s in naturally-contaminated, turbid, pond water was not achieved by direct PCR assay although positive cultures were obtained by conventional methods.
- This was most likely due to low numbers of *Campylobacter* in the samples.
- The presence of humic material and other PCR inhibitors may also have contributed. Selective enrichment, however, facilitated the detection of *Campylobacter* by PCR, especially when Proteinase K was used to extract DNA from cells.
- The combination of selective enrichment and PCR for detecting thermophilic *Campylobacter*s in turbid pond water is recommended.
- It shortens the time need for detection and eliminates the conventional methods needed for confirmation of presumptive isolates.
- The method only gave positive results when an appropriate volume (10 ml or 100 ml, but not 1000 ml) of pond water was used for enrichment. This was

probably because of competition by background bacteria during enrichment culture.

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APPENDIX 1A

ABIOTIC VARIABLES OF EAST YORKSHIRE VILLAGE PONDS JUNE 2001 – JANUARY 2002

Table A1.1 Abiotic variables of the ponds at South Dalton and Holme-on-Spalding-Moor, June 2001- January 2002

	Location of ponds	
	South Dalton	Holme-on-Spalding-Moor
Water temperature °C		
19-06-01	14	14
11-07-01	18	16
07-08-01	19	19
18-09-01	13	13
31-10-01	10	10
23-11-01	4.0	4.0
23-01-02	7.0	6.2
pH		
19-06-01	7.4	7.5
11-07-01	8.2	7.2
07-08-01	7.52	7.58
18-09-01	7.24	7.23
31-10-01	7.02	7.04
23-11-01	6.33	6.86
23-01-02	6.3	6.2
Absorbance (580 nm)		
19-06-01	0.14	0.12
11-07-01	0.391	0.0385
07-08-01	0.31	0.23
18-09-01	0.165	0.0197
31-10-01	0.079	0.028
23-11-01	0.053	0.026
23-01-02	0.13	0.023
Conductivity $\mu\text{S cm}^{-1}$		
19-06-01	440	630
11-07-01	480	807
07-08-01	475	853
18-09-01	539	917
31-10-01	458	756
23-11-01	479	826
23-01-02	490	915
Sediment dry weight (g)		
19-06-01	2.3	4.1
11-07-01	3.339	4.094
07-08-01	2.239	0.63

18-09-01	2.06	2.4
31-10-01	4.42	0.29
23-11-01	2.59	0.49
23-01-02	4.41	0.29
Waterfowl		
19-06-01	Ducks (13)	Moorhens (5)
11-07-01	Ducks (31)/geese (6)/moorhens (2)	Moorhens (2)
07-08-01	Ducks (28)/moorhens (4)	0
18-09-01	Ducks (31)/moorhens (2)	0
31-10-01	Ducks (30)/moorhens (4)	Coots (2)
23-11-01	Ducks (41)/moorhens (4)	Coots (2)
23-01-02	Ducks (33)/moorhens (2)	0

Table A1.2 Abiotic variables of the ponds at Brantingham, Garton-on-the-wolds and Little weighton, August, October and November 2001

	Location of ponds		
	Brantingham	Garton-on-the-wolds	Little Weighton
Temperature °C			
29-08-01	20	18	18
16-10-01	13	13	13
13-11-01	8	8	7
pH			
29-08-01	7.95	7.65	6.88
16-10-01	6.87	6.75	6.83
13-11-01	6.5	6.42	6.7
Absorbance (580 nm)			
29-08-01	0.58	0.31	0.58
16-10-01	0.034	0.07	0.078
13-11-01	0.098	0.067	0.048
Conductivity $\mu\text{S cm}^{-1}$			
29-08-01	410	822	460
16-10-01	601	648	400
13-11-01	913	866	922
Waterfowl			
29-08-01	Ducks (75)	Ducks (45)	Ducks (23)/goose (1)
16-10-01	Ducks (13)	Ducks (35)	Ducks (24)/goose (1)
13-11-01	Ducks (14)/coot (1)	Ducks (37)	Ducks (21)/goose (1)

Table A1.3 Abiotic variables of the ponds at Bentley and Sancton, August, October and November 2001

	Location of ponds	
	Bentley	Sancton
Temperature °C		
29-08-01	15	15
16-10-01	14	13
13-11-01	7	11
pH		
29-08-01	6.85	6.75
16-10-01	6.83	6.6
13-11-01	6.51	6.4
Absorbance (580 nm)		
29-08-01	0.008	0.002
16-10-01	0.014	0.004
13-11-01	0.03	0.001
Conductivity $\mu\text{S cm}^{-1}$		
29-08-01	440	1138
16-10-01	415	1002
13-11-01	744	1113
Waterfowl		
29-08-01	Coots (4)	0
16-10-01	Coots (3)	0
13-11-01	Coots (1)	0

APPENDIX 1B

COUNTS OF FAECAL INDICATORS, HETEROTROPHIC BACTERIA AND TOTAL BACTERIA IN WATER AND SEDIMENTS OF THE EAST YORKSHIRE VILLAGE PONDS, JUNE 2001-JANUARY 2002

Table A1.4 Faecal indicators, heterotrophic plate counts and total bacterial counts in the water and sediment of the ponds at South Dalton and Holme-on-Spalding-Moor, June 2001-January 2002

Water	Volume filtered	Mean count (CV%) number of samples	
		South Dalton ¹	Holme-on-Spalding-Moor [†]
<i>E. coli</i>			
19-06-01	10 ml [†] / 10 ml [‡]	300 (0%) 3	0.66 (18.2%) 3
11-07-01	0.1 ml / 100 ml	81 (31.5%) 2	70 (6.1%) 2
07-08-01	0.1 ml / 10 ml	45.3 (8.9%) 3	49.5 (1.43%) 3
18-09-01	0.1 ml / 1.0 ml	188 (69.2%) 2	83 (13.6%) 2
31-10-01	0.1 ml / 1.0 ml	62.5 (49%) 2	113 (16.3%) 2
23-11-01	0.1 ml / 1.0 ml	21 (13.5%) 2	40 (35.4%) 2
23-01-02	0.1 ml / 1.0 ml	86.5 (12.3%) 2	115.5 (0.61%) 2
Faecal streptococci			
19-06-01	10 ml [†] / 10 ml [‡]	160.6 (8.03%) 3	0 (0%) 3
11-07-01	10 ml / 100 ml	ND	134 (7.4%) 2
07-08-01	1.0 ml / 10 ml	170.6 (15.9%) 3	7 (14.3%) 3
18-09-01	0.1 ml / 1.0 ml	492 (31%) 2	187.5 (29%) 2
31-10-01	0.1 ml / 1.0 ml	51 (19.4%) 2	16.3 (4.30%) 2
23-11-01	0.1 ml / 1.0 ml	124.5 (2.8%) 2	110 (38.6%) 2
23-01-02	0.1 ml / 1.0 ml	18.5 (14.4%) 2	42 (77.4%) 2
<i>Cl. perfringens</i>			
19-06-01	10 ml [†] / 10 ml [‡]	114.6 (19.5%) 3	9 (62.2%) 3
11-07-01	10 ml / 10 ml	300 (0%) 2	44 (16.13%) 2
07-08-01	10 ml / 10 ml	300 (0%) 3	77 (17.5%) 3
18-09-01	0.1 ml / 1.0 ml	977.5 (25%) 2	690 (47.1%) 2
31-10-01	0.1 ml / 0.1 ml	800 (18%) 2	469 (20.2%) 2
23-11-01	0.1 ml / 0.1 ml	1005 (9.4%) 2	603 (15.7%) 2
Heterotrophic plate count			
19-06-01		330.8 (51%) 10	820 (1.6%) 10
11-07-01		158.8 (42.1%) 10	837 (1.8%) 10
07-08-01		250.4 (3%) 10	2446 (3%) 10
18-09-01		318.6 (29.4%) 10	1620 (6.4%) 10
31-10-01		19.11 (43%) 10	438 (1.9%) 10
Total bacterial (DAPI) count × 10 ⁷ ml ⁻¹			
19-06-01		3.4	1.1
11-07-01		6.4	2.5
07-08-01		7.4	3.0
18-09-01		3.4	1.2
31-10-01		4.7	1.1

Sediment	Mean count (CV%) (n) number of samples	
	South Dalton	Holme-on-Spalding-Moor
<i>E. coli</i>		
19-06-01	14 (37.8%) 3	0.66 (17.6%) 3
11-07-01	25.5 (25%) 2	0 (0%) 2
07-08-01	238 (4.5%) 3	221.6 (61.2%) 3
18-09-01	69.5 (21.3%) 2	9.5 (67.4%) 2
31-10-01	236 (36%) 2	73.5 (2.9%) 2
23-11-01	469 (20.5%) 2	92.5 (16.12%) 2
23-01-02	108 (24.1%) 2	66.5 (54.3%) 2
Faecal streptococci		
19-06-01	4.3 (14%) 3	0 (0%) 3
11-07-01	3 (0%) 2	0 (0%) 2
07-08-01	300 (0%) 3	300 (0%) 3
18-09-01	152.5 (48%) 2	100 (56.6%) 2
31-10-01	16.5 (47.3%) 2	6 (0%) 2
23-11-01	1139 (8.3%) 2	469 (20.2%) 2
23-01-02	99 (51%) 2	6.5 (32.6%) 2
<i>Cl. perfringens</i>		
19-06-01	89.7 (20.1%) 3	24 (19.2%) 3
11-07-01	6.5 (54%) 2	85.5 (9.12%) 2
07-08-01	300 (0%) 3	300 (0%) 3
18-09-01	575 (57%) 2	460 (35.4%) 2
31-10-01	460 (15.4%) 2	260 (32.7%) 2
23-11-01	1139 (8.3%) 2	871 (10.9%) 2
Heterotrophic plate count		
19-06-01	1691 (2.6%) 10	171.3 (1.2%) 10
11-07-01	2149 (3.22%) 10	2015 (2.8%) 10
07-08-01	2328 (3.11%) 10	2754 (2.7%) 10
18-09-01	1612 (2.42%) 10	1706 (3.5%) 10
31-10-01	1032 (3.6%) 10	1306 (3.2%) 10

Table A1.5 Faecal indicator bacteria in water from ponds with waterfowl, August-November 2001

	Mean (CV%) n		
	Little Weighton	Garton-on-the-wolds	Brantingham
<i>E. coli</i>			
29-08-01	ND	ND	300 (0%) 2
16-10-01	118 (21.6%) 2	116.5 (14%) 2	88 (12.9%) 2
13-11-01	378 (2.3%) 2	302 (2.8%) 2	213 (72%) 2
Faecal streptococci			
29-08-01	195 (15.9%) 2	193.5 (15.7%) 2	300 (0%) 2
16-10-01	74 (15.3%) 2	42.5 (3.5%) 2	187.5 (12.33%) 2
13-11-01	13.5 (25.9%) 2	16 (100%) 2	13.5 (5.3%) 2
<i>Cl. perfringens</i>			
29-08-01	15.5 (22.7%) 2	300 (0%) 2	300 (0%) 2
16-10-01	938 (20.2%) 2	670 (23.6%) 2	804 (20.2%) 2
13-11-01	1139 (8.3%) 2	1005 (9.4%) 2	804 (23.6%) 2

Table A1.6 Faecal indicator bacteria in water from ponds without waterfowl, August-November 2001

	Mean (CV%) n	
	Bentley	Sancton
<i>E. coli</i>		
29-08-01	4.5 (16%) 2	2.5 (28.4%) 2
16-10-01	250 (19.3%) 2	13.5 (15.7%) 2
13-11-01	153.5 (12.5%) 2	3.5 (61%) 2
Faecal streptococci		
29-08-01	1 (14%) 2	1 (14%) 2
16-10-01	365 (16%) 2	14.5 (14.6%) 2
13-11-01	27.5 (7.71%) 2	4 (73%) 2
<i>Cl. perfringens</i>		
29-08-01	9 (16%) 2	1.5 (14%) 2
16-10-01	1276 (8%) 2	9.5 (7.5%) 2
13-11-01	1005 (9.5%) 2	9 (48%) 2

APPENDIX 2A

ABIOTIC VARIABLES OF VILLAGE PONDS AUGUST- DECEMBER 2003

Table A2.1 Abiotic variables of village ponds with waterfowl August-December 2003

	Location of pond		
	South Dalton	Little Weighton	Brantingham
Water temperature °C			
12-Aug-03	22	20	22
02-Oct-03	14	13	13
15-Dec-03	5	4	3
pH			
12-Aug-03	6.7	6.3	6.6
02-Oct-03	6.6	6.5	6.5
15-Dec-03	6.5	7.0	6.6
Conductivity ($\mu\text{S cm}^{-1}$)			
12-Aug-03	1520	1047	1526
02-Oct-03	1515	1033	1510
15-Dec-03	1510	1057	1520
Absorbance ($A_{580 \text{ nm}}$)			
12-Aug-03	0.03	0.07	0.5
02-Oct-03	0.03	0.05	0.9
15-Dec-03	0.03	0.09	0.7
Ducks			
12-Aug-03	12	19	21
02-Oct-03	12	14	20
15-Dec-03	19	19	19
Geese			
12-Aug-03	0	1	0
02-Oct-03	0	1	0
15-Dec-03	0	1	0

Table A2.2 Abiotic variables of village ponds without waterfowl October-November 2003

	Location of pond	
	Bentley	Sancton
Water temperature °C		
20-Oct-2003	7	9
27-Nov-2003	4	4
pH		
20-Oct-2003	6.5	6.4
27-Nov-2003	6.9	6.8
Conductivity ($\mu\text{S cm}^{-1}$)		
20-Oct-2003	415	1002
27-Nov-2003	744	1135
Absorbance ($A_{580 \text{ nm}}$)		
20-Oct-2003	0.008	0.004
27-Nov-2003	0.014	0.001
Ducks		
20-Oct-2003	0	0
27-Nov-2003	0	0
Geese		
20-Oct-2003	0	0
27-Nov-2003	0	0

APPENDIX 2B

RECOVERY OF THERMOPHILIC CAMPYLOBACTERS FROM POND WATER AND SEDIMENTS

**Table A2.3 The recovery of thermophilic campylobacters from pond water
August-December 2003**

Location of pond	Volume of water filtered		
	10 ml n:p	100 ml n:p	1000 ml n:p
South Dalton			
12-Aug-03	2:2	2:2	2:0
02-Oct-03	2:1	2:2	2:0
15-Dec-03	2:2	2:2	2:0
Little Weighton			
12-Aug-03	2:2	2:2	2:0
02-Oct-03	2:0	2:0	2:0
15-Dec-03	2:1	2:0	2:0
Brantingham			
12-Aug-03	2:2	2:0	2:0
02-Oct-03	2:0	2:0	2:0
15-Dec-03	2:0	2:0	2:0
Bentley			
20-Oct-03	2:0	2:0	2:0
20-Nov-03	2:0	2:0	2:0
Sancton			
20-Oct-03	2:0	2:0	2:0
20-Nov-03	2:0	2:0	2:0

n = total number of samples.

p = number of positive samples.

Table A2.4 The recovery of thermophilic campylobacters from pond sediment September-November 2003

Location of pond	Volume of sediment used for inoculation		
	0.1 ml	1.0 ml	5.0 ml
	n:p	n:p	n:p
South Dalton			
12-Sep-03	2:0	2:2	2:0
07-Nov-03	2:2	2:0	2:0
Little Weighton			
12-Sep-03	2:0	2:0	2:0
07-Nov-03	2:0	2:1	2:0
Brantingham			
12-Sep-03	2:0	2:0	2:0
07-Nov-03	2:0	2:0	2:0

n = total number of samples.

p = number of positive samples.

APPENDIX 3A

CAPILLARY ELECTROPHORESIS

A1. PRINCIPLES

The principle of capillary electrophoresis is similar to gel electrophoresis, whereby charged molecules in a solution (e.g. proteins, nucleic acids) migrate in response to an electrical field. In capillary electrophoresis, when an electric field is applied to an electrolyte solution within a capillary, negatively charged DNA fragments will migrate towards the anode (+). As DNA fragments migrate, they will be separated by a size exclusion sieving effect. These DNA fragments are dye-labelled and are detected by fluorescence, and in turn can be rendered into a sequence. Polyacrylamide gels are commonly used as the electrolyte solution to provide the sieving medium for the separation (Anon., 2002). The concept of capillary electrophoresis is the principle behind the separation mechanism for the Beckman Coulter CEQ 8000 Genetic Analysis System.

REFERENCE

Anon. (2002) *Beckman Coulter CEQ™ Genetic Analysis System Operating Manual*. Beckman Coulter Inc., Fullerton, CA.

APPENDIX 3B

CAMPYLOBACTER JEJUNI/CAMPYLOBACTER COLI flaA, flaB GENE SEQUENCES

All sequence and accession numbers were obtained from GenBank. It should be noted that all *C. jejuni* and *C. coli* isolated from different sources had high within-species genetic similarity. The following sequences and accession numbers applied to all *C. jejuni* and *C. coli* isolated from all sources mentioned in this thesis.

Campylobacter jejuni (ATCC43431)

Accession number Z29327

C. jejuni TGH9011 (ATCC43431) *flaA* and *flaB* genes for flagellin A and flagellin B
Length = 3879 bp

Identities = 180/192 (93%)

Query: is the submitted sequence

Subject: is the nearest match available in the GenBank

This sequence represents all 25 *C. jejuni* isolates from faeces, pond water and sediment.

Query: 15 aagacctgaactaagtctacttaagaacttatccagc-ctctagcattaacaactgaatt 73

|||||

Sbjct: 2061 aagacctgaactaagtctgcttaagaacttatccagctctctagcattaacaactgaatt 2002

Query: 74 tgcattgctggttaat-caccgaag-tagtg-ttatcctaaaaccca--ttaatccttt 128

|||||

Sbjct: 2001 tgcattgctggttaatgcaccgatgttggtgttatcctaaaaccattttaatccttt 1942

Query: 129 caaaatattgcatcgaaaagattaaagaagaagtg-tccaagtttagtttaattaaaa 187

|||||

Sbjct: 1941 caaaatattgcatcgaaaagattaaagaagaagtgtccaagtttagtttaattaaaa 1882

Query: 188 attatagagttt 199

|||

Sbjct: 1881 atttagagttt 1870

Campylobacter coli

Accession number M64671

C. coli flagellin (*flaA* and *flaB*) genes, complete cds. Length = 4200 bp

Identities = 166/166 (100%)

Query: is the submitted sequence

Subject: is the nearest available match in the GenBank

This sequence represents all 16 *C. coli* isolates from run off, pond water and sediment.

Query: 1 aagacctgagctaagtctacttaaagactgtcaagctccctagcattaacaactgaatt 60

|||||

Sbjct: 2308 aagacctgagctaagtctacttaaagactgtcaagctccctagcattaacaactgaatt 2249

Query: 61 tgcattgctgcaatgcaccgatgttggtgtttattctaaaaccattttaaatccttt 120

|||||

Sbjct: 2248 tgcattgctgcaatgcaccgatgttggtgtttattctaaaaccattttaaatccttt 2189

Query: 121 caaaatattgcatcgaaaagattaaagaagaagtgtccaagttt 166

|||||

Sbjct: 2188 caaaatattgcatcgaaaagattaaagaagaagtgtccaagttt 2143

APPENDIX 3C

ABIOTIC VARIABLES OF RUN-OFF WATER DECEMBER 2003 – JANUARY 2004

Table A3.1 Abiotic variables of run-off water collected from input pipes December 2003-January 2004

	Location of pond		
	South Dalton	Little Weighton	Brantingham
pH			
11-Dec-03	6.9	6.9	7.5
08-Jan-04	7.3	7.4	7.7
31-Jan-04	7.2	7.2	7.7
Conductivity ($\mu\text{S cm}^{-1}$)			
11-Dec-03	409	1728	1160
08-Jan-04	269	1811	715
31-Jan-04	335	1690	815
Absorbance (580 nm)			
11-Dec-03	0.48	0.75	0.73
08-Jan-04	0.46	0.73	0.70
31-Jan-04	0.44	0.76	0.78

APPENDIX 3D

THE RECOVERY OF CAMPYLOBACTERS AND SALMONELLAS FROM DUCKS FAECES

**Table A3.2 The recovery of campylobacters and salmonellas from ducks faeces
August-December 2003**

Location of pond	Faecal samples used for inoculation	
	n:p	n:p
	<i>Campylobacter</i>	<i>Salmonella</i>
South Dalton		
12-Aug-03	10:3	10:0
02-Oct-03	10:1	10:0
15-Dec-03	10:4	10:0
Little Weighton		
12-Aug-03	10:0	10:2
02-Oct-03	10:0	10:0
15-Dec-03	10:1	10:0
Brantingham		
12-Aug-03	10:0	10:2
02-Oct-03	10:0	10:0
15-Dec-03	10:1	10:0

n = total number of samples.

p = number of positive samples.

APPENDIX 3E

THE RECOVERY OF CAMPYLOBACTERS AND SALMONELLAS FROM RUN-OFF WATER

**Table A3.3 The recovery of campylobacters from run-off water December 2003-
January 2004**

Location of pond	Volume of water filtered	
	10 ml n:p	100 ml n:p
South Dalton		
11-Dec-03	2:0	2:0
08-Jan-04	2:0	2:0
31-Jan-04	2:0	2:0
Little Weighton		
11-Dec-03	2:0	2:0
08-Jan-04	2:1	2:0
31-Jan-4	2:2	2:2
Brantingahm		
11-Dec-03	2:0	2:0
08-Jan-04	2:0	2:1
31-Jan-04	2:1	2:0

n = total number of samples.

p = number of positive samples.

**Table A3.4 The recovery of salmonellas from run-off water December 2003-
January 2004**

Location of pond	Volumes of water filtered	
	10 ml n:p	100 ml n:p
South Dalton		
11-Dec-03	2:0	2:0
08-Jan-04	2:0	2:0
31-Jan-04	2:0	2:0
Little Weighton		
11-Dec-03	2:0	2:0
08-Jan-04	2:0	2:0
31-Jan-04	2:0	2:0
Brantingham		
11-Dec-03	2:0	2:0
08-Jan-04	2:0	2:0
31-Jan-04	2:0	2:0

n = total number of samples.

p = number of positive samples.

APPENDIX 4

HETEROTROPHIC PLATE COUNTS, COLIFORM COUNTS AND ACRIDINE-ORANGE DIRECT MICROSCOPIC COUNTS IN *CAMPYLOBACTER* ENRICHMENT CULTURES

Table A4.1 Heterotrophic plate counts, coliform counts and acridine-orange direct microscopic counts (ODC) in *Campylobacter* enrichment culture derived from the filtration of different volumes of pond water

	Mean (CV%) n		
	10 ml	100 ml	1000 ml
Heterotrophic plate counts	136.8 (25.3 %) 10	668.2 (7.3 %) 10	1906 (9 %) 10
0.1 ml ⁻¹ and 0.0 ml ⁻¹	134.8 (13.4 %) 10	688 (6.6 %) 10	1832.1 (5.2 %) 10
	98.9 (11.3 %) 10	142.4 (24.5 %) 10	1154.4 (12.5 %) 10
	98.9 (8.5 %) 10	169.6 (16.5 %) 10	1107.4 (6.13%) 10
Coliform counts 0.1 ml ⁻¹	129.2(8.5 %) 10	726 (11.5 %) 10	1414.8 (12.8 %) 10
	142.5 (9.8 %) 10	667.5 (10.8 %) 10	1520.2 (6.11 %) 10
	133.8 (20 %) 10	688.8 (10.4 %) 10	1787 (5.6 %) 10
	137.5 (17.6 %) 10	675.6 (6.4 %) 10	1741.9 (5.8 %) 10
AODC [†] counts × 10 ⁹	0.032	0.07	5.5
	0.035	0.083	5.8
	0.037	0.089	6.1
	0.038	0.087	5.9

CV = coefficient of variation (standard deviation / mean × 100).

n = total number of replicate plates.

† AODC represents the actual count obtained from each preparation.



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Recovery of thermophilic campylobacters from pond water and sediment and the problem of interference by background bacteria in enrichment culture

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Abstract

The aim of this study was to address problems in the determination of thermophilic campylobacters in turbid pond water and sediment. Thirty sets of three samples of pond water (volumes 10, 100, 1000 ml) or sediment (0.1, 1.0, 5.0 ml) were examined for the presence of thermophilic campylobacters. The different volumes of pond water were processed by membrane filtration followed by selective enrichment. The samples of sediment were subjected directly to selective enrichment. Presumptive isolates were confirmed by Gram stain, cell morphology, presence of oxidase and catalase, growth under microaerobic but not aerobic conditions, and PCR. Confirmed *Campylobacter* species were recovered only from 10 and 100 ml samples of water and from 0.1 and 1.0 ml samples of sediments. The 1000 ml samples of water and 5.0 ml samples of sediment never gave positive isolates. PCR indicated that the confirmed isolates were all either *Campylobacter jejuni* or *C. coli*. Enrichment cultures from 1000 ml filtrations contained the highest number of background bacteria. It is suggested that the processing of large volumes of turbid environmental water samples or of sediment is counterproductive and may not yield positive *Campylobacter* cultures. This is probably due to antagonistic effects of large numbers of background bacteria out-competing campylobacters during the enrichment stage.

Pilot studies to establish appropriate volumes of pond water or sediment samples should be undertaken before routine determination of campylobacters is begun.

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Keywords: Campylobacter; Enrichment culture; Pond sediment; Pond water

1. Introduction

Campylobacter is possibly the leading worldwide cause of acute bacterial gastroenteritis (Thomas et al., 1999; Frost, 2001). In England and Wales in

2001 there were around 60,000 reported cases of gastroenteritis caused by *Campylobacter* (Humphrey, 2002). The true incidence of food-borne and water-borne campylobacteriosis may be under-reported since a study on infectious intestinal diseases in the UK showed that there might be around 500,000 cases of *Campylobacter* infection (Humphrey, 2002). *Campylobacter* species that are implicated in water-borne and food-borne human infections are *Campylobacter jejuni* and *C. coli* (Percival et al., 2004).

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Undercooked meat, raw milk and contaminated water are potential sources of *Campylobacter* infection; handling of pets (e.g. dogs and cats) may also be a source of infection (Bolton et al., 1987; Baker et al., 1999; Frost, 2001).

The consumption of untreated or contaminated drinking water is the most common vehicle in large *Campylobacter*-associated outbreaks around the world (Leclerc et al., 2004). In UK, *Campylobacter* has been the main cause of private water supply outbreaks where water was not adequately disinfected (Furtado et al., 1998). *Campylobacters* are widespread in the environment. Sewage effluents, livestock farming and wild birds are established sources of *Campylobacter* to environmental water as well as drinking water (Jones, 2001).

The United Kingdom Public Health Laboratory Service (PHLS) standard protocol for the detection of presumptive campylobacters in all types of water (i.e. drinking and recreational) recommends the membrane filtration of 1000 ml samples followed by overnight incubation in selective enrichment broth (Anon, 1998). The confirmation of presumptive isolates is conventionally based on a range of morphological and biochemical tests. Recently, PCR has been extensively applied to the detection and identification of a wide range of pathogenic bacteria including *Campylobacter* species (Waage et al., 1999). PCR primers that amplify a conserved region of the *flaA* and *flaB* genes have been successfully applied to the detection of *Campylobacter jejuni* and *C. coli* (Oyofe et al., 1992; Wegmüller et al., 1993).

Amenity ponds in parks and villages that harbour large populations of waterfowl are usually of poor microbiological quality and could pose a threat to public health as a source for enteric pathogens. Abulreesh et al. (2004) found that water samples from amenity ponds with waterfowl had high abundance of background bacteria; for example, in a pond at South Dalton, which is one of the ponds examined in the present study, heterotrophic plate counts ranged from 1.6 to $3.3 \times 10^5 \text{ ml}^{-1}$ while direct counts of bacteria made by epifluorescence microscopy ranged from 3.4 to $7.4 \times 10^7 \text{ ml}^{-1}$. Also they found that faecal indicator bacteria and *Salmonella* were related to waterfowl populations in amenity ponds but they failed to detect *Campylobacter*. They suspected that the apparent absence of *Campylobacter* was related to the shortcomings of the PHLS standard method for *Campylobacter* determination. The aim of the present work was to develop the UK PHLS (Anon, 1998) protocol for the detection of presumptive thermophilic campylobacters to allow its application to turbid pond water and bottom sediments, and to evaluate PCR for the rapid confirmation of campylobacters from these habitats.

2. Materials and methods

2.1. Sites and sampling

The study sites were three roadside village ponds in East Yorkshire, NE England. All three ponds have resident semi-tame ducks, and sometimes geese, and receive run-off from adjacent roads. All three ponds are amenity sites. The ponds are at Brantingham (National Grid Reference SE 941 296, pond area 240 m²), Little Weighton (SE 988 338, 640 m²), and South Dalton (SE 969 454, 2410 m²). The waters of these ponds are turbid and aquatic vegetation is sparse or absent.

Water and sediment samples were collected in the morning during August–December 2003. Surface water was collected into sterile polypropylene bottles; superficial sediment was scooped from shallow water areas, with depth of less than 10 cm, and transferred to sterile 30 ml universal bottles. All samples were kept on ice and in darkness during transportation; microbiological assays were begun on the same day as sampling.

2.2. Detection and confirmation of thermophilic campylobacters

Membrane filtration techniques were employed for the detection of thermophilic campylobacters in water samples, following the standard methods for the examination of water as used by the Public Health Laboratory Service (Anon, 1998). Water samples (10, 100, 1000 ml) were filtered through 0.45 µm, diameter 47 mm, cellulose nitrate and mixed ester membrane filters (Labsales, Cambridge, UK). Membranes were transferred to 150 ml of Preston enrichment broth (Oxoid, Basingstoke, UK), containing 5% lysed horse blood (Oxoid) and growth supplement consisting of ferrous sulphate (0.125 g l⁻¹), sodium metabisulphite (0.125 g l⁻¹) and sodium pyruvate (0.125 g l⁻¹) (FBP) (Oxoid), and incubated for 22 h at 37 °C, followed by 22 h at 42 °C. Microaerobic conditions were achieved by incubation in screw-top bottles with minimal air-space (less than 1.0 ml). Broth cultures were then subcultured onto blood-free *Campylobacter*-selective modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) (Oxoid) and incubated in a microaerobic atmosphere (N₂ 85%, O₂ 5%, CO₂ 10%) using Oxoid CampyGen, at 37 °C for 48 h.

Aliquots of 1.0 ml of ten times diluted sediment, and 1.0 ml, and 5.0 ml of undiluted sediment samples were transferred to sterile 30 ml universal bottles. Preston enrichment broth was added and the procedure was then continued as described above.

Presumptive colonies were subjected to the following confirmatory tests: Gram stain with 0.85% carbol fuchsin as counter stain; cell morphology; presence of

oxidase and catalase; growth on blood agar at 37 °C under microaerobic but not aerobic conditions and finally PCR.

2.3. Determination of heterotrophic plate counts and coliform counts

Spread plate counts of culturable heterotrophic bacteria and coliforms in *Campylobacter* enrichment cultures were made on Nutrient Agar (Oxoid) and MacConkey Agar (Oxoid) respectively. Ten replicate plates were inoculated with 0.1 ml sub-samples of serially diluted enrichment broth and were incubated at 25 °C for 48 h for the heterotrophic plate counts and at 37 °C for 24 h for the coliform counts.

2.4. Direct counts of total bacteria

Counts of total bacteria in *Campylobacter* enrichment cultures were performed using epifluorescence microscopy after acridine-orange staining (Hobbie et al., 1977). Stained bacteria were concentrated on black 0.2 µm polycarbonate filters (Labsales) and at least 600 cells per preparation were counted at ×1250 magnification.

2.5. PCR assay

PCR was performed on the presumptive colonies to confirm that they were thermophilic campylobacters. DNA was extracted by suspending a loop of presumptive *Campylobacter* colony in 100 µl sterile, pure water and boiling for 5 min. The suspension was then centrifuged for 5 min at 1260 g and 10 µl of the supernatant were used as target DNA. Primers CF03 and CF04 (Invitrogen, Paisley, UK) from the *Campylobacter jejuni flaA* and *flaB* gene sequence were used (Wegmüller et al., 1993). Their sequences are: CF03, 5'-GCT CAA AGT GGT TCT TAT GCN ATG G-3' (forward); CF04, 5'-GCT GCG GAG TTC ATT CTA AGA CC-3' (reverse).

The PCR reaction mixture (50 µl total volume) contained: 0.25 µmol l⁻¹ of each primer; 75 mmol l⁻¹ Tris-HCl; 20 mmol l⁻¹ (NH₄)₂SO₄; 2.0 mmol l⁻¹ MgCl₂; 0.01% (v/v) Tween[®] 20; 0.2 mmol l⁻¹ each of dATP, dCTP, dGTP, and dTTP; 1.25 units of Thermoprime Plus DNA Polymerase (ABgene, Surrey, UK). DNA amplification used the following temperature cycle: denaturation at 94 °C for 4 min, 30 cycles at 95 °C for 1 min, 53 °C for 1 min, 72 °C for 1 min, and final extension at 72 °C for 5 min. A total of 10 µl of PCR products was analysed by 1.5% (w/v) agarose gel (BioLine, London, UK) electrophoresis and made visible by ethidium bromide (0.5 µg ml⁻¹) staining and UV transillumination.

2.6. Statistical analysis

To test the null hypothesis that enrichments from different volumes filtered did not have different concentrations of background bacteria the Kruskal-Wallis non-parametric test was used.

3. Results

Thirty sets of three samples of pond water (volumes 10, 100, 1000 ml) or sediment (0.1, 1.0, 5.0 ml) were examined for the presence of thermophilic campylobacters. For pond water samples, presumptive thermophilic campylobacters were recovered only from 10 and 100 ml samples. Presumptive *Campylobacter* isolates were never recovered following the filtration of 1000 ml samples (Table 1). For sediment samples, presumptive *Campylobacter* isolates were only recovered following the enrichment of 0.1 and 1.0 ml samples. Presumptive thermophilic campylobacters were never recovered from 5.0 ml samples (Table 1).

Heterotrophic plate counts, coliform counts and acridine-orange direct microscopic counts (AODC) of total bacteria were performed on enrichment cultures derived from the filtration of water samples. The counts were made at the end of enrichment-culture incubations (44 h). All *Campylobacter*-negative cultures (from 1000 ml filtration) had higher levels of heterotrophic bacteria and coliforms than positive cultures (10, 100 ml)

Table 1

The recovery of thermophilic campylobacters from pond water and sediment

Location of pond	Volume of water filtered		
	10 ml n:p (%)	100 ml n:p (%)	1000 ml n:p (%)
South Dalton	6:5 (83)	6:6 (100)	6:0 (0)
Little Weighton	6:2 (33)	6:2 (33)	6:0 (0)
Brantingham	6:2 (33)	6:2 (33)	6:0 (0)
Total for all ponds	18:9 (50)	18:10 (56)	18:0 (0)
	Volume of sediment used for inoculation		
	0.1 ml n:p (%)	1.0 ml n:p (%)	5.0 ml n:p (%)
South Dalton	4:2 (50)	4:2 (50)	4:0 (0)
Little Weighton	4:0 (0)	4:1 (25)	4:0 (0)
Brantingham	4:0 (0)	4:0 (0)	4:0 (0)
Total for all ponds	12:2 (17)	12:3 (25)	12:0 (0)

n = total number of samples; *p* = number of positive samples (percentage of positive samples in brackets). All presumptive isolates were confirmed as *Campylobacter*.

Table 2

Heterotrophic plate counts, coliform counts and acridine-orange direct counts (AODC) in *Campylobacter* enrichment cultures prepared using pond water samples

	Volume of water filtered			P
	10 ml Mean (range) n	100 ml Mean (range) n	1000 ml Mean (range) n	
Heterotrophic plate count $\times 10^6$ cfu ml ⁻¹	0.55 (0.013–0.98) 4	1.1 (0.66–1.6) 4	6.4 (1.8–11) 4	<0.05
Coliforms $\times 10^6$ cfu ml ⁻¹	0.13 (0.12–0.14) 4	0.69 (0.66–0.72) 4	1.6 (1.4–1.8) 4	<0.05
AODC $\times 10^9$ ml ⁻¹	0.036 (0.032–0.038) 4	0.083 (0.071–0.089) 4	5.8 (5.5–6.1) 4	<0.05

P is the probability that there is no difference between the number of bacteria in enrichment culture inoculated with the residue from filtration of different sample volumes (Kruskal–Wallis test), n = number of samples.

(Table 2). The count of total bacteria in *Campylobacter*-negative enrichments was many fold higher than in positive cultures (Table 2). The Kruskal–Wallis test showed that there was significant difference in heterotrophic plate counts, coliforms, and total bacteria between enrichments set up using different volumes filtered (Table 2).

All 24 presumptive isolates from pond water and sediment were found to be Gram negative, gull-wing shaped, both oxidase and catalase positive, and they grew on blood agar incubated at 37°C under micro-aerobic, but not aerobic conditions. Thus according to conventional criteria they all were confirmed as *Campylobacter* species. DNA extracted from all 24 confirmed isolates was successfully amplified by PCR. The amplified products were between 340 and 380 bp in length. This reinforced the confirmation that these isolates were thermophilic campylobacters.

4. Discussion

Thermophilic campylobacters are thought to be ubiquitous in aquatic environments, but their detection can be difficult. This is because the organism may be present in low numbers and/or sub-lethally injured. To overcome these problems, the detection of *Campylobacter* from environmental waters involves initial concentration on a membrane filter followed by incubation of the filters in an enrichment broth (Hunter, 1997; Percival et al., 2004).

In the present study, a combination of a selective enrichment procedure and selective plating was used. This approach has been reported to increase the recovery of campylobacters from environmental waters (Rosef et al., 2001). Preston enrichment broth and mCCDA selective agar were used as selective media in the current study; the successive use of these two media was suggested to be useful for the determination of thermophilic campylobacters from environmental sam-

ples (e.g. water), where *Campylobacter* numbers are low (Corry et al., 1995). The initial incubation of enrichment cultures at 37°C followed by selective incubation at 42°C and selective plating seems to significantly enhance the recovery of sub-lethally injured thermophilic campylobacters from environmental waters (Humphrey and Muscat, 1989).

Since the number of thermophilic campylobacters in environmental water is believed often to be low, the examination of a large water volume seems to be an obvious way of increasing recovery (Bolton et al., 1982; Hänninen et al., 2003). Therefore, the standard PHLS protocol recommends the filtration of 1000 ml of water. The results obtained in the present study, however, showed that the filtration of a large volume (1000 ml) of turbid pond water never yielded *Campylobacter* isolates (Table 1). These were clearly false-negative results, since the filtration of smaller volumes (10 or 100 ml) of water from the same sites frequently gave positive results (Table 1). Since the *Campylobacter*-negative cultures from 1000 ml filtration had high levels of heterotrophic bacteria, coliforms and total bacterial populations (Table 2), it is likely that high levels of background bacteria competing for nutrients prevented the growth of *Campylobacter* during the enrichment procedure. Similarly, Aquino et al. (1996) found that the majority of *Campylobacter*-negative cultures from heavily contaminated poultry products, had high levels of faecal indicators and lactobacilli, which may have hindered the recovery of campylobacters. Thus, the seeding of enrichment culture with the residue from a large volume of turbid water (1000 ml), may lead to overloading by background bacteria and the out-competition of *Campylobacter* to the extent that it is unable to grow to detectable levels. Fricker (1987) reviewed various methods that are used to isolate *Campylobacter* from environmental samples and suggested that overloading by background microflora may prevent the growth of campylobacters during the enrichment stage; this suggestion is supported by our results.

Similar observations were obtained from our examination of sediment samples. A large volume (5.0 ml) of sediments did not give any presumptive campylobacters (Table 1). The concentration of bacteria in aquatic sediment has been reported to be around a hundred times higher than in overlying water (Cavallo et al., 1999), thus we suggest that the use of a large inoculum of microbially rich sediment to assess the presence of campylobacters may lead to false-negative results, probably because of competition by the background microflora.

It may be suggested that in the processing of large volumes of turbid water (1000 ml), the use of a larger volume of enrichment broth (500 or 1000 ml) per sample would prevent the interference of background bacteria and allow *Campylobacter* to grow to detectable levels. However, for a laboratory carrying out routine investigations, such a procedure would be both expensive and impractical, especially when smaller volumes of enrichment broth (150 ml) give positive results provided that appropriate volumes of water are processed, as is shown in this study.

The *Campylobacter* flagellum is believed to be a significant virulence factor (Percival et al., 2004). In this study, we used a PCR protocol that amplifies the intergenic sequence between the *Campylobacter* flagellin genes, *flaA* and *flaB*, as a rapid confirmatory test on *Campylobacter* presumptive isolates. We choose the primers CF03 and CF04 (Wegmüller et al., 1993) because of their specificity for detecting *Campylobacter jejuni* and *C. coli*. Our results showed that the amplification of DNA extracted from all 24 presumptive *Campylobacter* isolates, and also from a clinical isolate, produced PCR fragments of 340–380 bp in length. Thus, all presumptive isolates were confirmed as *Campylobacter* spp. and we conclude that they were either *C. jejuni* or *C. coli* on the basis of the work described by Wegmüller et al. (1993) and Waage et al. (1999), who showed that the amplification with CF03 and CF04 gives 340–380 bp fragments with *C. jejuni* and *C. coli*.

5. Conclusions

Standard methods for the detection of *Campylobacter* spp. recommend the processing of water samples of large volume. Our results suggest, however, that the filtration of large volumes of turbid environmental water, or of sediments, may be counterproductive and may not yield presumptive campylobacters. Pilot studies to establish appropriate volumes of environmental water or sediment samples should be undertaken before routine determination of *Campylobacter* is begun.

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Waterfowl and the bacteriological quality of amenity ponds

H. H. Abulreesh, T. A. Paget and R. Goulder

ABSTRACT

This study investigated the impact of waterfowl on the bacteriological quality of village ponds in East Yorkshire, north-east England. Water and sediment samples were collected from ponds with and without resident ducks and geese; faecal indicator and potentially pathogenic bacteria were assayed by membrane filtration and by selective enrichment. *Escherichia coli*, faecal streptococci and, to a degree, *Clostridium perfringens* were more abundant in ponds with waterfowl; *Salmonella* was isolated in June–August from the sediment of a pond with waterfowl. The results suggested that the bacteriological quality of village ponds might be adversely affected by waterfowl. All water samples from ponds with waterfowl had faecal indicators at higher concentrations than EU requirements for bathing waters. Although these ponds are not bathing waters we suggest skin contact and accidental ingestion of water should be avoided.

Key words | *Escherichia coli*, faecal indicators, faecal streptococci, ponds, *Salmonella*, waterfowl

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INTRODUCTION

Waterfowl are gross excretors of faecal coliforms and faecal streptococci (Ashbolt *et al.* 2001) and their association with the contamination of water bodies that are used for recreation has been described. Thus, a bathing beach on a Wisconsin, USA, lake was closed because of a high concentration of faecal coliforms that was attributed to mallard ducks (Standridge *et al.* 1979). Likewise, in the UK, mallard ducks, and other birds were found to be, in part, responsible for high densities of faecal coliforms at Morecambe Bay, which failed to comply with EU guidelines for bathing water (Jones 2002).

Waterfowl also harbour bacteria in their intestinal tract that are potential human pathogens. *Salmonella* and *Campylobacter* are major causative agents of bacterial gastroenteritis in both the UK and USA (Rusin *et al.* 2000; Timbury *et al.* 2002). Both organisms have been found in the intestinal tract of ducks and geese (Pacha *et al.* 1988; Ridsdale *et al.* 1998; Feare *et al.* 1999; Aydin *et al.* 2001; Dieter *et al.* 2001; Refsum *et al.* 2002). The carriage of these pathogens by ducks and geese suggests that waterfowl may act as a reservoir for their transmission through the

contamination of water. Contaminated water is a potential source of *Salmonella* and *Campylobacter* and may be a vehicle for their transmission to domestic animals and humans (Bolton *et al.* 1987; Melloul & Hassani 1999; Thomas *et al.* 1999).

High numbers of waterfowl are often found on village ponds in England. These ponds and their environs are typically recreational and amenity sites. Visitors and residents frequently encourage waterfowl by feeding them, and direct contact with faecal material or contaminated water is likely to occur. The present study aimed to investigate the impact of waterfowl on the bacteriological quality of some amenity village ponds in East Yorkshire, north-east England.

MATERIALS AND METHODS

Sites and sampling

The study sites were seven roadside village ponds. Four of these had resident semi-tame ducks and sometimes geese:

South Dalton (National Grid Reference SE 969 454, pond area 2,410 m²), Brantingham (SE 941 296, 240 m²), Garton-on-the-wolds (SE 983 594, 1,800 m²) and Little Weighton (SE 988 338, 640 m²). Waterfowl (ducks and geese) numbers were variable but some birds were always present; numbers ranged from 13 to 75 birds; the water was turbid and aquatic vegetation was sparse or absent. Ducks and geese were never observed at the other three ponds. These were at Holme-on-Spalding-Moor (SE 827 390, 1,530 m²), Bentley (TA 019 359, 220 m²) and Sancton (SE 901 392, 85 m²). Their water was clear, and submerged and emergent aquatic plants were abundant.

Water and sediment samples were collected from South Dalton and Holme-on-Spalding-Moor in the late morning at about monthly intervals from June 2001 to January 2002. Water samples were taken from the other five ponds on one day in August, one day in October and one day in November 2001. Surface water was collected from pond margins into sterile polypropylene bottles. Two replicate sediment cores with overlying water were taken in 44 mm internal diameter, sterile glass tubes (Carr & Goulter 1990). The top 1 cm of sediment was siphoned off. That from one core was used for *Salmonella* and *Campylobacter* enrichment culture. That from the other core was diluted ten times with sterile pond water and used for the other sediment assays. This diluted sediment was homogenized by treatment in a stomacher (Colworth 400; A.J. Seward Ltd, London) for 5 min. All samples were packed in ice and kept in darkness during transport; bacteriological assays were begun on the same day as sampling.

Bacteriological assays

Protocols for faecal indicators and potential human pathogens were based on those used by the Public Health Laboratory Service (PHLS 1998a–e) and, for *Clostridium*, on HMSO (1983). Media were from Oxoid Ltd, Basingstoke, UK, unless otherwise indicated. *Escherichia coli*, faecal streptococci and *Clostridium perfringens*, in pond water and appropriately diluted sediment slurries, were assayed by colony counts on 0.45 µm membrane filters. At least two replicate filters were counted per

sample. The presence of *Salmonella* and *Campylobacter* was tested for by selective enrichment. The enrichment cultures were seeded with membrane filters bearing the residue from filtration of 1 litre of pond water, or with 5 ml of sediment.

Membranes for *E. coli* counts were incubated on absorbent pads with lauryl sulphate broth; plates were initially incubated at 30°C for 4 hours, followed by 14 hours at 44°C. Colonies of faecal streptococci were counted after incubation of membranes on Slanetz and Bartley agar at 37°C for 4 hours, then at 44°C for 40 hours. Assays for *C. perfringens* used pond water and slurry that had been heat-treated at 75°C for 10 min prior to filtration to ensure that only *C. perfringens* spores remained in the samples. Membranes were incubated anaerobically (AnaeroGen, Oxoid) on reinforced clostridial agar for 24 hours at 37°C.

Representative colonies, five or six from each membrane, were subjected to confirmatory tests. *E. coli* was confirmed by culture in Fluorocult broth (Merck, Darmstadt, Germany); incubation was at 37°C for 24 hours, to verify production of both β-galactosidase and β-glucuronidase. Confirmation of faecal streptococci was by incubation on bile aesculin agar plates at 44°C for 16 hours. *C. perfringens* was confirmed by anaerobic incubation in Crossley milk medium for 24 hours at 37°C.

Membranes and sediment subsamples for *Salmonella* assay were subjected to pre-enrichment incubation in buffered peptone water for 18 hours at 37°C. Selective enrichment was then done separately in both Rappaport-Vassiliadis soya peptone broth, with incubation at 41.5°C for 20 hours, and in selenite cystine broth, with incubation at 37°C for 20 hours. These cultures were plated on both xylose lysine desoxycholate agar (XLD) and brilliant green agar (BGA) and the plates were incubated at 37°C for 22 hours. Suspected *Salmonella* colonies (red with black centres on XLD, and red with a bright red halo on BGA) were subcultured on to cystine lactose electrolyte deficient (CLED) agar and were incubated at 37°C for 18 to 22 hours. Flat, blue colonies on CLED agar were confirmed by incubation on lysine iron agar slopes and in urea broth; incubation at 37°C for 14–18 hours.

Enrichment for *Campylobacter* utilized Preston *Campylobacter* enrichment broth; cultures were initially

Table 1 | Bacteria in water and sediment from ponds at South Dalton and Holme-on-Spalding-Moor, June 2001–January 2002

	Median (range) <i>n</i>		<i>P</i>
	South Dalton	Holme-on-Spalding-Moor	
In water			
<i>E. coli</i> ($\times 10^3$ cfu 100 ml ⁻¹)	81 (3–180) 7	4 (0.01–11) 7	< 0.01
Faecal streptococci ($\times 10^3$ cfu 100 ml ⁻¹)	34 (1.6–490) 6	11 (0–24) 7	< 0.1
<i>C. perfringens</i> ($\times 10^3$ cfu 100 ml ⁻¹)	6.4 (0.11–100) 6	3.9 (0.01–60) 6	NS
Heterotrophic plate count ($\times 10^5$ cfu ml ⁻¹)	2.5 (1.6–3.3) 5	0.83 (0.32–2.4) 5	< 0.05
Total bacteria ($\times 10^7$ ml ⁻¹)	4.7 (3.4–7.4) 6	1.2 (1.1–3.0) 6	< 0.05
In sediment			
<i>E. coli</i> ($\times 10^3$ cfu ml ⁻¹)	24 (0.14–105) 7	4.6 (0–9.25) 7	< 0.01
Faecal streptococci ($\times 10^3$ cfu ml ⁻¹)	9.9 (0.3–113) 7	0.65 (0–50) 7	< 0.1
<i>C. perfringens</i> ($\times 10^3$ cfu ml ⁻¹)	38 (0.65–113) 6	17.3 (0.6–87) 6	< 0.05
Heterotrophic plate count ($\times 10^6$ cfu ml ⁻¹)	1.6 (1.1–2.3) 5	1.7 (1.3–2.8) 5	NS

There were up to about 40 ducks and geese at South Dalton; none was present at Holme-on-Spalding-Moor.

P values are from the χ^2 test which tested agreement between observations and the null hypothesis that when ponds are compared pairwise the numerical abundance of an indicator bacterium should equally often be lower as greater in the pond with waterfowl; *n*=number of samples; NS=*P*>0.1.

incubated at 37°C for 22 hours, followed by 22 hours at 42°C. Microaerobic conditions were achieved by incubation in screw-top bottles with minimal air-space (PHLS 1998e). These broth cultures were then subcultured onto *Campylobacter* selective agar (mCCDA) and incubated in a microaerobic atmosphere (CampyGen, Oxoid) at 37°C for 48 hours. Colonies were then subjected to a confirmation route of a positive oxidase test plus growth on blood agar in a microaerobic but not an aerobic atmosphere.

Spread plate counts of culturable heterotrophic bacteria were made on casein peptone starch agar (Jones 1970). Ten replicate plates were inoculated with 0.1 ml subsamples of diluted water or slurry and were incubated at 20°C for 14 days. Direct counts of total bacteria in water were performed using epifluorescence microscopy (Daley 1979). Bacteria were stained with DAPI (Yu *et al.* 1995) and then concentrated on black 0.2 μ m polycarbonate

membrane filters and at least 600 cells per preparation were counted at $\times 1,250$ magnification.

Mean values of specific bacterial variables in ponds with and ponds without waterfowl were often skewed by extreme values, hence median values are given.

RESULTS AND DISCUSSION

The faecal indicators, *E. coli* and streptococci, were more abundant in water from South Dalton, which had waterfowl (up to about 40 ducks and geese), than in water from Holme-on-Spalding-Moor (Table 1). Plate counts of heterotrophic bacteria and direct counts of total bacteria in pond water were also greater at South Dalton (Table 1). In sediment, *E. coli*, faecal streptococci and *C. perfringens*

Table 2 | Bacteria in water from five additional East Yorkshire ponds, August–November 2001

	Median (range) <i>n</i>		
	Ponds with waterfowl*	Ponds without waterfowl†	<i>P</i>
<i>E. coli</i> ($\times 10^3$ cfu 100 ml ⁻¹)	21 (8.8–300) 7	0.009 (0.003–1.5) 6	< 0.01
Faecal streptococci ($\times 10^3$ cfu 100 ml ⁻¹)	1.6 (1.3–300) 9	0.009 (0.001–0.36) 6	< 0.01
<i>C. perfringens</i> ($\times 10^3$ cfu 100 ml ⁻¹)	80 (0.16–930) 9	0.01 (0.009–16.6) 6	< 0.01

P values are from the Mann-Whitney U-test; *n*=number of samples.

*Ponds at Brantingham, Garton-on-the-wolds and Little Weighton; up to 75 ducks and geese were present.

†Ponds at Sancton and Bentley.

were more abundant at South Dalton (Table 1). Faecal indicators in the water from five additional ponds were also more abundant in those ponds with waterfowl (Table 2). All presumptive colonies of *E. coli* and faecal streptococci that were tested confirmed as positive, but only about 50% of presumptive *C. perfringens* were positive, hence our results for *C. perfringens* represent presumptive isolates.

Values of the Spearman's correlation coefficient showed that there were significant relationships between faecal indicators in pond water and waterfowl number and waterfowl number per unit pond area (Table 3). The closest relationship was between *E. coli* and number of waterfowl.

Table 3 | Relationships between faecal indicators and waterfowl number and waterfowl per unit pond area, June 2001–January 2002

	Relationship with number of waterfowl		Relationship with waterfowl per unit pond area	
	<i>r_s</i>	<i>P</i>	<i>r_s</i>	<i>P</i>
<i>E. coli</i> *	0.84	< 0.01	0.75	< 0.01
Faecal streptococci†	0.63	< 0.01	0.49	< 0.01
<i>C. perfringens</i> *	0.42	< 0.05	0.50	< 0.01

Values are Spearman's correlation coefficients; a two-tailed test was used

**n*=27 samples from 7 ponds, †*n*=29 samples from 7 ponds

Salmonella and *Campylobacter* assays were performed on water from all seven ponds and on sediment from South Dalton and Holme-on-Spalding-Moor. *Salmonella* was recovered only from South Dalton sediment during summer (June, July, August); *Campylobacter* was recovered from none of the ponds.

High counts of *E. coli* and faecal streptococci in pond water (Tables 1 and 2) were probably caused by waterfowl; duck faeces contain about 3.3×10^7 faecal coliforms and 5.4×10^7 streptococci per gram wet weight (Ashbolt *et al.* 2001). The lower and very variable background numbers of faecal indicators found in ponds without ducks and geese perhaps originated from small family groups of coot (*Fulica atra*) and moorhen (*Gallinula chloropus*) and from rainfall-related runoff from adjacent roads and soil. Such runoff, especially from land carrying livestock, may be an appreciable source of faecal indicators to natural waters (Alvarez *et al.* 1991; Jones 2002). Of the ponds that were surveyed in the present study, however, those with more faecal indicators were not obviously more liable to contamination by runoff, hence we suggest that the dense populations of waterfowl were the leading cause of high counts of faecal indicators.

There was sometimes a closer relationship between faecal indicators in pond water and number of waterfowl, than there was with waterfowl per unit pond area (Table 3). This was probably due to non-uniform horizontal distribution of both waterfowl and bacteria. Waterfowl gather to be fed, and will defecate at pond margins adjacent to roadsides; i.e. where the samples were taken. The

lack of a significant difference between *C. perfringens* in the water at South Dalton and Holme-on-Spalding-Moor (Table 1) and its relatively weak correlation with waterfowl abundance (Table 3) suggest that waterfowl were not necessarily the principal source of presumptive *C. perfringens*. Contaminated soil may also be a significant source. The high values of heterotrophic plate counts and total counts in the water at South Dalton (Table 1) probably reflect general nutrient enrichment by waterfowl.

High counts of faecal indicators in the sediment at South Dalton were most likely due to sunken faecal material. Concentrations in sediments were much higher than in overlying water (Table 1) thus the sediments contain a reservoir of bacteria that is potentially recyclable into the water column, perhaps by foraging waterfowl or by extreme weather.

The recovery of *Salmonella* from South Dalton sediment in June–August agrees with Hendricks (1971) who observed greater recovery of *Salmonella* from river sediments than from water, and with Pianetti *et al.* (1998) who most readily found *Salmonella* in rivers in summer and autumn. Its presence in South Dalton sediment might be attributed to its concentration through sedimentation of faecal material. The low frequency of *Salmonella* isolation may be because healthy waterfowl do not necessarily harbour enteric pathogens (Damare *et al.* 1979; Hussong *et al.* 1979); although *Salmonella* has been isolated from a wide range of apparently healthy birds (Kapperud & Rosef 1983). The present study utilised a present/absent assay for the enumeration of *Salmonella* hence absolute number was not determined. Infective doses for salmonellosis in humans are as little as between 10^1 and 10^3 bacterial cells (Blaser & Newman 1982); hence the presence of this potential pathogen might indicate a possible health threat.

The apparent absence of *Campylobacter* from the East Yorkshire ponds that had waterfowl was unexpected; post-enrichment colonies on mCCDA were culturable on blood agar under both microaerobic and aerobic conditions and hence were not confirmed as *Campylobacter*. In contrast, clinical isolates of *Campylobacter*, supplied by Hull PHLS, did confirm as *Campylobacter*. The non-isolation of *Campylobacter* in summer, when morning water temperature was up to 19°C, is compatible with seasonal trends for its recovery from the environment;

Obiri-Danso & Jones (1999) found this organism to be sparse in river water in summer. The apparent absence of *Campylobacter* in winter, however, is puzzling. The standard PHLS protocol used in the present study required the filtration of 1,000 ml of water sample. The seeding of enrichment cultures with the residue from such a large volume of water, or with 5 ml of microbially rich sediment, may have led to out-competition of *Campylobacter* to the extent that it was unable to grow to detectable levels (Fricker 1987).

Campylobacter has been found in the intestinal tract of many species of waterfowl (Pacha *et al.* 1988; Ridsdale *et al.* 1998; Aydin *et al.* 2001), although Hill & Grimes (1984) failed to isolate it in caecal samples from 50 waterfowl from a Mississippi lake and suggested that *Campylobacter* may exhibit sporadic distribution in response to feeding habits, geographical distribution and mixing of waterfowl with other birds and animals. Feare *et al.* (1999) also did not manage to isolate *Campylobacter* from 600 faecal droppings of ducks and geese that were collected from 12 sites located in London, south-east England, Yorkshire and northern England over a period of 2 years. This variation between studies is perhaps explained by differences in the methods used to assess *Campylobacter*.

CONCLUSIONS

Waterfowl are often regarded as a positive feature of amenity ponds in English villages; adults and children enjoy feeding them. However, faecal indicators in the water of East Yorkshire ponds with ducks and geese (Tables 1 and 2) always exceeded the EU requirements for bathing waters (EU 1994) of <2000 *E. coli* per 100 ml and <400 faecal streptococci per 100 ml. These ponds are not used for bathing, nevertheless with these high concentrations of faecal indicators there is a potential hazard from pathogens – and *Salmonella* was found in some samples. Skin contact and accidental ingestion of water is probably best avoided. Ponds without waterfowl were better than EU requirements for bathing waters or only intermittently infringed them.

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Waterfowl and human-pathogenic bacteria at amenity sites: a review

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Waterfowl on ponds in civic parks and at other amenity sites are often seen as a positive feature. Large flocks may however become a cause for concern. They may foul lawns and gardens, destroy vegetation, can cause physical damage at roosting sites and may become a public nuisance. Furthermore, waterfowl may be a reservoir for potentially infectious viral, bacterial and parasitic agents. Bacterial pathogens, such as *Campylobacter*, *Salmonella*, *Escherichia coli*, *Helicobacter* and others, have been isolated from the faeces of apparently healthy waterfowl. These pathogens are also frequently found in the water and/or sediments of sites with abundant waterfowl. Migratory waterfowl potentially play a role in the spread of pathogenic bacteria across geographic locations. Specific links to human bacterial infectious diseases have been difficult to prove, perhaps because of a lack of routine typing of bacteria from both clinical and environmental sources. Nevertheless, the contamination of surface waters and their environs by waterfowl faeces is a potential source of bacterial infections to the human community. Thus, waterfowl are not necessarily benign; it might be appropriate for statutory authorities with responsibility for amenity sites to review their policy towards large populations of waterfowl.

Keywords: *Campylobacter*, ducks, *Escherichia coli*, geese, pathogenic bacteria, public health, *Salmonella*, waterfowl

Introduction

Introduced populations of waterfowl at amenity sites, particularly ducks and geese, have notably increased since the 1960s (Gibbons *et al.*, 1994). High numbers of waterfowl are often found on ponds in civic parks and in villages in England. These ponds and their environs are typically recreational and amenity sites. The presence of waterfowl at such amenity sites is often seen as a positive feature; visitors and residents frequently encourage waterfowl by feeding them. However, large flocks of waterfowl can have negative impact; they may foul lawns and gardens, may destroy aquatic and water-side vegetation, may cause physical damage to roosting sites and become a public nuisance (Conover, 1985). Furthermore, waterfowl may be a reservoir for potentially infectious viral, bacterial and parasitic agents within a context where direct human contact with faecal material or contaminated water is highly likely to occur. This review addresses the carriage of zoonosis-associated bacteria by waterfowl, the role of waterfowl in the distribution of these pathogens in the environment, and possible routes of transmission to humans. This review is not intended to address diseases in waterfowl caused by bacterial infectious agents.

Campylobacter

Campylobacter is possibly the leading worldwide cause of bacterial gastroenteritis (Frost, 2001). *C. jejuni*

and *C. coli* are the species most often implicated in water-borne and food-borne infections (Percival *et al.*, 2004). Avian carriers of *Campylobacter* include chicken (Yogasundram *et al.*, 1989), gulls (Kapperud, 1983; Quessy and Messier, 1992; Kaneko *et al.*, 1999), pigeons (Mégraud, 1987; Kapperud, 1983; Casanovas *et al.*, 1995) and crows (Kapperud, 1983; Ito *et al.*, 1988). In waterfowl *C. jejuni*, and to a lesser extent *C. coli* have frequently been isolated from faeces and/or cloacal swabs derived from apparently healthy ducks and geese worldwide (Luechtefeld *et al.*, 1980; Pacha *et al.*, 1988; Yogasundram *et al.*, 1989; Aydin *et al.*, 2001; Fallacara *et al.*, 2001, 2004; Kassa *et al.*, 2001; Murphy *et al.*, 2005). We have shown, by PCR using species-specific primers, that *C. jejuni* is the predominant *Campylobacter* species in duck faeces collected from around three village ponds in East Yorkshire, England (unpublished data). The literature cited above clearly indicates that waterfowl are a significant environmental reservoir of *Campylobacter* species, particularly *C. jejuni*.

The frequent presence of campylobacters in waterfowl has been related to their feeding habits (Luechtefeld *et al.*, 1980; Waldenström *et al.*, 2003a). For example, the frequency of occurrence of *Campylobacter jejuni* in the intestinal tract of Green-Winged Teal (*Anas acuta*) was much lower than in the Shoveler Duck (*Spatula clypeata*).

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The former feed exclusively on vegetable matter, while the latter usually strain mud from ponds to extract molluscs and other invertebrates (Luechtefeld *et al.*, 1980). Similarly, a greater prevalence of *Campylobacter* was found in gulls than pigeons. Gulls often feed on refuse tips and sewage sludge, whereas pigeons are herbivorous (Kapperud, 1983; Ito *et al.*, 1988; Whelan *et al.*, 1988; Waldenström *et al.*, 2003a).

*Campylobacter*s were readily recovered from water and sediment from ponds in villages and parks and also from rivers and bathing beaches that harboured dense populations of waterfowl (Mawer, 1988; Obiri-Danso, 1999, 2000; Abulreesh *et al.*, 2005). This suggests that waterfowl play a significant role in the dissemination of these pathogens through the contamination of surface water.

Escherichia coli and coliform bacteria

Waterfowl excrete large numbers of faecal coliforms and *E. coli*. A gram dry weight of Mallard (*Anas platyrhynchos*) faeces may contain 7.8×10^{10} faecal coliforms and 3.3×10^7 *E. coli* (Jones, 2002; Taylor, 2003). Deterioration of bacteriological quality of water at bathing and non-bathing amenity sites, in terms of abundance of *E. coli*, has been related to large numbers of waterfowl on these water bodies (Hussong *et al.*, 1979; Standridge *et al.*, 1979; Abulreesh *et al.*, 2004). For example, the positive relationship between *E. coli* in amenity village ponds in East Yorkshire, England and waterfowl abundance is shown in Fig. 1. Skin contact with, and accidental ingestion of, such water potentially has public health implications.

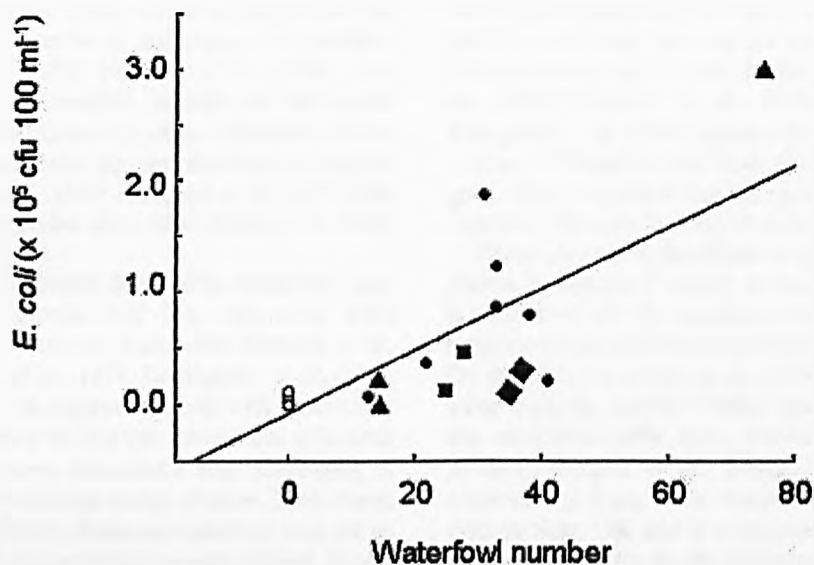


Fig. 1. Relationship between *E. coli* and abundance of waterfowl (Sperman's rank correlation coefficient $r_s = 0.84$, $n = 27$, $p < 0.01$) in seven amenity village ponds in East Yorkshire, England, June 2001 to January 2002. Ponds with waterfowl at: (▲) Brantingham (pond area 240 m²), (■) Garton-on-the-wolds (1,800 m²), (◆) Little Weighton (640 m²), (●) South Dalton (2,410 m²). Ponds without waterfowl at: (○) Bentley (220 m²), (◐) Holme-on-Spalding-Moor (1,530 m²) and (◑) Sancton (85 m²). A total of 13 samples represented ponds without waterfowl: some of the symbols are superimposed. See Abulreesh *et al.* (2004) for methods used.

Studies that have addressed the presence of pathogenic or toxigenic strains of *E. coli* in the faeces of waterfowl suggest that ducks and geese are a potential reservoir of such strains and may play a role in their epidemiology. (Hussong, *et al.* 1979) found that seven out of 75 random isolates of *E. coli* recovered from the faeces of Canada Geese (*Branta canadensis*) and Whistling Swans (*Cygnus columbianus*) in Maryland, USA, were entero-toxin producers. Likewise, in the UK, toxigenic strains of *E. coli* (*E. coli* class I) were found to be prevalent in the droppings of Canada Geese as well as in those of diverse species of ducks (Feare *et al.*, 1999). The percentage of the faeces of Canada Geese, collected from 12 sites around England, that contained *E. coli* class I ranged from 4–100%. An extensive search for

pathogenic *E. coli* serogroups in the faeces of Canada Geese in Colorado, USA was reported by Kullas, *et al.* (2002). This showed that the most prevalent serogroups were enterotoxigenic *E. coli* (ETEC) at 13% of *E. coli* isolates, followed by enterohemorrhagic *E. coli* (EHEC) at 6%. Although *E. coli* O157 has been reported in the faeces of gulls (Wallace *et al.*, 1997) it has apparently not yet been reported in waterfowl (Feare *et al.*, 1999; Fallacara *et al.*, 2001, 2004; Kullas *et al.*, 2002).

Salmonella

Salmonella is an intestinal pathogen of humans and diverse animals, including wild birds and domestic pets (Lightfoot, 2004). The carriage of salmonellae by wild birds and their role in the spread of the organism in the environment is well documented, with much attention

given to gulls. Free-living, apparently healthy pigeons, gulls and crows, were found to be positive for various *Salmonella* serotypes (Kapperud, 1983; Hubálek *et al.*, 1995; Ferns, 2000; Kirk *et al.*, 2002; Refsum *et al.*, 2002; Dovic *et al.*, 2004).

Various *Salmonella* serotypes have been isolated from the faeces of free-living ducks and geese in England (Feare *et al.*, 1999), the US (Fallacara *et al.*, 2001, 2004), the Czech Republic (Hubálek *et al.*, 1995) and Norway (Refsum *et al.*, 2002). While campylobacters are frequently found in the intestinal tract of waterfowl, the incidence of salmonellae seems to be lower (Mitchell, 1971; Hubálek *et al.*, 1995). Fallacara, *et al.* (2001) found only one *Salmonella* isolate in 82 faecal droppings of Mallard while the bacterium was completely absent from 357 faecal samples of Canada Geese. Other studies report the absence of *Salmonella* in the faeces of waterfowl (Damaré *et al.*, 1979; Hussong *et al.*, 1979). Low incidence and/or complete absence of *Salmonella* carriage was also observed in other wild birds, such as gulls, passerines, owls, pigeons, thrushes and eagles (Brittingham *et al.*, 1988; Palmgren *et al.*, 1997; Kirk *et al.*, 2002; Hernandez *et al.*, 2003; Reche *et al.*, 2003; Dovic *et al.*, 2004).

It has been suggested that healthy waterfowl, and wild birds in general, that live well away from pollution do not harbour *Salmonella* (Damaré *et al.*, 1979; Hussong *et al.*, 1979; Brittingham *et al.*, 1988; Tizard, 2004). This suggestion agrees with conclusions drawn from several studies that showed that wild birds (i.e. gulls) acquired *Salmonella* after scavenging at refuse tips and on sewage sludge (Fricker, 1984; Ferns, 2000; Murray, 2000). Although waterfowl tend not to scavenge, gull-like, at insanitary sites (Tizard, 2004), they might acquire salmonellae from contaminated water at roosting sites and recycle them into the water (Mitchell, 1971). Thus, Abulreesh, *et al.* (2004) found *Salmonella* in the sediment of a pond that harboured ducks and geese, in the village of South Dalton in East Yorkshire, England, but not in other East Yorkshire village ponds where waterfowl were absent. In the pond at South Dalton, potential sources of contamination were the faeces of waterfowl and run-off from adjacent roads. We were, however, unable to isolate *Salmonella* from run-off water (unpublished data). We suggest, therefore, that the likeliest source of salmonellae was the dense population of waterfowl.

Other bacteria

Helicobacters are a major cause of peptic ulcers and are closely associated with chronic active gastritis in humans and mammals (Hunter, 1997; Timbury *et al.*, 2002; Percival *et al.*, 2004). People appear to be the main host of helicobacters, however, the detection of *Helicobacter pylori* in domestic cats and dogs raises questions over the zoonotic transmission of the bacterium to humans (Hunter, 1997; Jalava *et al.*, 1998;

Cattoli *et al.*, 1999; Percival *et al.*, 2004). It is unclear whether wild birds, particularly waterfowl, constitute a reservoir for *Helicobacter* species. The isolation of *H. pylori* from the faeces of wild birds (gulls) was reported by Seymour, *et al.* (1994). Recently, *H. canadensis*, a newly emerged human pathogen was isolated from the faeces of Canada Geese and Barnacle Geese (*Branta leucopsis*) (Waldenström *et al.*, 2003b). These findings call for further work on the avian reservoir and the potential for zoonotic transmission of helicobacters.

Shigella species are the aetiological agents for bacillary dysentery or shigellosis in humans (Timbury *et al.*, 2002). The principal recognised habitat for this organism is the intestinal tract of humans (Hunter, 1997). Extensive searches for *Shigella* in waterfowl were unsuccessful, and have led to the conclusion that healthy waterfowl, that are not exposed to polluted environments, may not carry this bacterium (Damaré *et al.*, 1979; Hussong *et al.*, 1979; Roscoe, 1999). Karagüzel, *et al.* (1993) reported the recovery of four isolates of *Shigella sonnei* from 616 faecal samples of gulls. They suggested that the gulls had acquired the organism through feeding on refuse tips.

Vibrio cholerae is the causative agent for pandemic cholera in humans (Timbury *et al.*, 2002). *V. cholerae* is subdivided into 139 serogroups on the basis of its O antigens; pandemic cholera is attributed to serogroups O1 and O139 (Percival *et al.*, 2004). The remaining serogroups, the non-O1 vibrios (non-cholera vibrios), are associated with mild diarrhoea infections in humans (Percival *et al.*, 2004). In wild birds, *V. cholerae* was found in the faeces of apparently healthy gulls in Kent, UK, and it is suggested that wild birds may be a vector for the dissemination of the bacterium (Lee *et al.*, 1982). Similarly, searches for vibrios in the faeces of ducks and geese showed that *V. cholerae* both O1 and non-O1 serogroups are prevalent in apparently healthy waterfowl, and suggested that ducks and geese may be a reservoir and serve as vectors for the transmission of vibrios in the environment (Bisgaard *et al.*, 1978; Schlater *et al.*, 1981; Ogg *et al.*, 1989; Buck, 1990). The sources of vibrios to waterfowl remain unclear; it is possible that the birds may have fed on contaminated material (Ogg *et al.*, 1989).

Yersinia enterocolitica is an important pathogen that causes enteritis in humans (Anon., 1980). Extensive research has been carried out to determine the natural habitat of the bacterium and it is believed that *Y. enterocolitica*, and other species such as *Y. kristensennii*, and *Y. pseudotuberculosis*, are present in animals, particularly wild birds (Kaneko, 1981; Kapperud, 1983; Kato *et al.*, 1985; Fukushima, 1991; Niskanen *et al.*, 2003). The faeces of ducks and geese including migratory birds have been shown to be positive for *Yersinia* species including *Y. enterocolitica*. Waterfowl may, therefore, constitute a reservoir of this

pathogen (Kawaoka *et al.*, 1984; Shayegani *et al.*, 1986; Niskanen *et al.*, 2003).

Infection or commensalism

Are bacteria, which are pathogenic in humans, living as commensals when found in the digestive tract of waterfowl? The answer largely seems to be yes, although there are obvious avian pathogens, such as *Pasteurella multocida* the causative agent of avian cholera (Pedersen *et al.*, 2003), which can also cause infection in humans (Timbury *et al.*, 2002).

The optimal growth temperature of thermophilic campylobacters (i.e. *Campylobacter jejuni*, *C. coli* and *C. lari*) is 42 °C, which is around the body temperature of waterfowl and birds in general (Luechtefeld *et al.*, 1980). High prevalence of thermophilic campylobacters has been found in waterfowl without obvious clinical manifestations, including the absence of the gut lesions associated with *Campylobacter* infections (Luechtefeld *et al.*, 1980; Oyarzabal *et al.*, 1995). This is clear evidence of non-harmful coexistence between thermophilic campylobacters and their bird hosts (Luechtefeld *et al.*, 1980; Kapperud and Rosef, 1983; Oyarzabal *et al.*, 1995; Jones, 2001; Waldenström *et al.*, 2003a).

Similarly, *Salmonella* may be regarded as part of the normal intestinal flora of many animals, including waterfowl (Murray 2000). Waldrup and Kocan (1985) suggested that *Salmonella* infections are not frequent in wild-living waterfowl, e.g. Mallard, American Wigeon (*Anas americana*) and Blue-Winged Teal (*Anas discors*). Ducks may, rarely, be diseased with septicaemia that is attributable to *Salmonella* (Henry, 2000; Tizard, 2004). Such infection may be more common in cultivated ducklings that have been exposed to poor hatchery hygiene (Henry, 2000). In the case of other wild birds (e.g. gulls) there is clear evidence that carriage of salmonellae is passive (i.e. without clinical manifestation) and lasts only a few days (Murray, 1991, 2000).

Escherichia coli is part of the normal flora of the intestinal tract of waterfowl. Although serotypes that are pathogenic to humans are found in the faeces of waterfowl, they may be harmless to their host. Thus, pathogenic strains of *E. coli* in waterfowl were isolated from apparently healthy birds (Hussong *et al.*, 1979; Feare *et al.*, 1999; Kullas *et al.*, 2002). However, there is some evidence to suggest that *E. coli* can be an opportunistic pathogen to avian species. It causes deadly septicaemia in farmed ducklings (2-8 weeks old), due to contaminated ponds and poor husbandry (Miller *et al.*, 2004).

The public-health relevance of pathogenic bacteria in waterfowl

Although waterfowl are a reservoir of bacteria that are potential human pathogens, the relevance of this to public health needs further research.

Two water-borne outbreaks of *Campylobacter*

enteritis in Norway were attributed to Pink-Footed Geese (*Anser brachyrhynchus*) (Varslot *et al.*, 1996). It was suggested that drinking water became contaminated by *C. jejuni* from goose faeces. Similarly, in Florida, an outbreak of water-borne campylobacteriosis was attributed to contamination of water supply by grackles (*Quiscalus quiscalua*) (Sacks *et al.*, 1986). In an attempt to establish a link between the birds and the outbreak, *Campylobacter* serotypes derived from infected people and from faeces of birds were compared. Although different serotypes were found, this might be because the serotypes from birds were obtained seven weeks following the outbreak, during the migration season. At the time of the outbreak, the birds may have carried the outbreak-related serotypes (Sacks *et al.*, 1986). A study of milk-associated *Campylobacter* infections in south Wales showed that there was strong association between *Campylobacter* infection and doorstep delivery of bottled milk (Southern *et al.*, 1990). The source of *Campylobacter* contamination was perhaps wild birds, magpies (*Pica pica*) and jackdaws (*Corvus monedula*), piercing foil milk-bottle tops. This hypothesis was, however, based only on eyewitness accounts of birds attacking milk bottles (Southern *et al.*, 1990). In northwest England, similar *Campylobacter* strains were found in the faeces of diarrhoea patients, in the faeces of waterfowl and other wild birds, and also in surface waters. However, no clear route for the transfer of campylobacters from birds to the community was established, apart from the potential for human consumption of contaminated food or water (Jones, 2001). In general the lack of routine parallel typing of clinically and environmentally derived pathogenic bacteria may enhance the difficulty in establishing a definite link between waterfowl and bacterial infection in the community. Nevertheless, waterfowl roosting at amenity sites or near drinking water supplies constitute a potential human health hazard.

Multi-drug resistant bacterial pathogens in the environment are a concern to public health authorities. Information on antibiotic resistant pathogenic bacteria in free-living waterfowl is sparse. Aydin, *et al.* (2001) tested 12 isolates of *C. jejuni* recovered from the faeces of Domestic Geese (*Anser anser*) for susceptibility to antimicrobial agents. The isolates showed varying degrees of resistance to eight out of 17 antibiotics. All 12 isolates were resistant to penicillin G and cephalothin. Eleven isolates were resistant to sodium cefuroxime and eight to cloxacillin, ampicillin and colistin sulphate. Fewer isolates were resistance to tetracycline, sulfamethoxazole/trimethoprim and kanamycin. Multidrug resistance was also observed in *C. jejuni* and *E. coli* isolated from Mallard and Canada Geese (Fallacara *et al.*, 2001, 2004; Cole *et al.*, 2005; Middleton and Ambrose, 2005). Similarly, S.

typhimurium and *C. jejuni* from the faeces of other wild birds, e.g. gulls and Starlings (*Sturnus vulgaris*) showed a high degree of antibiotic resistance (Palmgren *et al.*, 1997). It follows that waterfowl are potentially a reservoir for multidrug resistant bacterial pathogens and may serve as vectors for their distribution in the environment.

Large numbers of waterfowl at amenity sites are not necessarily a benign presence. They are a potential source of pathogenic bacteria, including antibiotic resistant strains. The problem may be compounded by migration. Luechtefeld, *et al.* (1980) and Pacha, *et al.* (1988) found that migratory ducks and geese arriving in northern Colorado and central Washington harboured *C. jejuni*. These birds migrate annually between Alaska, north and central Canada and northern and central USA, and visit amenity locations such as Lake Onalaska on the Mississippi River where they contribute to contamination of surface water (Pacha *et al.*, 1988). The role of migratory waterfowl in the spread of pathogens in the environment includes the introduction of new strains or species into new locations. Ogg, *et al.* (1989) isolated *Vibrio cholerae*, both O1 and non-O1 strains, from the faeces of migratory waterfowl and in environmental waters in Colorado and Utah. They found no local source of the bacterium and suggested that waterfowl may have brought the organism from Chesapeake Bay in the eastern USA, where *V. cholerae* is prevalent.

Conclusions

Large populations of waterfowl at amenity sites can foul lawns and gardens, destroy vegetation, cause physical damage and become a public nuisance. Waterfowl are also a potential reservoir of bacterial pathogens, including antibiotic resistant strains, and their excreta may constitute a public health hazard. The incidence of *Campylobacter*, *Salmonella* and other human pathogens in the intestinal tract of waterfowl may reflect commensalism rather than infection of the birds. Water-borne enteritis outbreaks have been associated with contamination of drinking water by waterfowl. The lack of routine parallel typing of infection-associated strains and those derived from waterfowl and the environment make it difficult to establish direct links between waterfowl and enteric infection in the human community. Migratory waterfowl may carry pathogenic bacteria between geographic areas, including multidrug resistant strains. For these reasons it is suggested that authorities with local responsibility should review their policies towards the presence of large populations of waterfowl at civic parks and other amenity sites.

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