PREVALENCE OF *CAMPYLOBACTER* IN DUTCH SEWAGE PURIFICATION PLANTS

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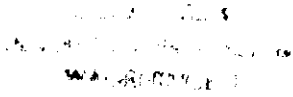

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Proefschrift

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in de landbouw- en milieuwetenschappen,
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STELLINGEN

1. De braakreactie na het nuttigen van een grote hoeveelheid bier zou het gevolg kunnen zijn van de aanwezigheid van het *Fusarium* mycotoxine vomitoxine (deoxynivalenol) in het bier.
   Scott, P.M., Kanhere, S.R. and D. Weber (1993) *Food Addit. Contam.* **10**, 381-389.

2. De species *Campylobacter jejuni, Campylobacter coli* en *Campylobacter lari* worden ten onrechte aangeduid met 'thermofiele' campylobacters.
   Brock, T.D. (ed.). *Thermophiles* (1986) John Wiley & Sons, New York, p. 40.

3. Zolang onbewezen is dat niet-kweekbare coccoïde *Helicobacter* cellen niet infectieus en/of niet levensvatbaar zijn, wordt in publikaties over de verspreiding van *Helicobacter* de eventuele rol van deze coccoïde vorm ten onrechte niet vermeld.
   Cellini, L., Allocati, N., Angelucci, D., Iezzi, T., Dicapli, E., Marzio, L. and B. Dainelli (1994) *Microbiol. Immunol.* **38**, 843-850.

4. Het feit dat de verspreiding van *Campylobacter* in pluimveestallen moeilijk is te beheersen, is in strijd met de geringe overlevingskansen van dit micro-organisme in het externe milieu.
   Doyle, M.P. (1990) *Campylobacter jejuni*. In: Cliver, D.O. (ed.). *Foodborne diseases*. Academic Press Inc., San Diego, p. 217-222.

5. De conclusie van Park en Krol dat cellobiose mogelijk gebruikt kan worden voor preventie van ziektes veroorzaakt door *Listeria* wordt niet ondersteund door de beschreven experimenten.
   Park, S.F. and R.G. Kroll (1993) *Mol. Microbiol.* **8**, 653-661.

6. Chuma *et al.* concluderen ten onrechte dat de beschreven DNA-DNA hybridisatie een gevoeligere detectiemethode voor *Campylobacter* is dan de selectieve ophoping in Preston bouillon.
   Chuma, T., Yamada, T., Yano, K., Okamoto, K. and H. Yugi (1994) *J. Vet. Med. Sci.* **56**, 697-700.

7. Het belang van het standaardiseren van de ontwikkelingstijd van foto's ten behoeve van het vastleggen van DNA gels wordt onderschat.

8. Bij de toetsing van de effectiviteit van vrouwvriendelijke maatregelen die het doorstromen van vrouwen naar hogere functies bevorderen, moet gecorrigeerd worden voor het aantal dames dat een promotiekans heeft laten liggen.
   Vrouwen in hogere functies; Lessen uit de praktijk (1993) Commissie Ontwikkeling Bedrijven, Sociaal-Economische Raad.

9. Door de voorlichtingscampagne rond AIDS heeft het medische begrip 'seropositief' een beladen, stigmatiserende betekenis gekregen.

10. Uit het oogpunt van voorlichting en bewustwording van de consument zou gebruik van de term 'milieuvriendelijk' vermeden moeten worden en moet de omschrijving 'milieuvijandig' in voorkomende gevallen worden aanbevolen.

Petra M.F.J. Koenraad, Wageningen, 20 september 1995.

Stellingen behorende bij het proefschrift 'Prevalence of *Campylobacter* in Dutch sewage purification plants'.

ABSTRACT

Koenraad, P.M.F.J. (1995) *Prevalence of* Campylobacter *in Dutch sewage purification plants*. PhD-thesis, Wageningen Agricultural University, The Netherlands (149 pp., English and Dutch summaries).

Campylobacter bacteria are an important cause of bacterial gastro-enteritis in man. Although food of animal origin is the main source of human infection, a case-control study in the United States of America showed that 8% of all campylobacteriosis cases could be attributed to consumption of contaminated surface water. In this thesis the prevalence of *Campylobacter* in sewage purification plants was investigated in order to obtain more information on the survival of this pathogen in aquatic environments. A survey carried out on three municipal plants showed that sewage and surface waters are frequently contaminated with *Campylobacter*. The contamination of sewage was higher when meat-processing industries were present in the drainage area of these plants. Indeed, drain water of a poultry abattoir contained high numbers of *Campylobacter*. It is clear that other sources contribute far lower numbers to sewage. Furthermore, the aquatic *Campylobacter* isolates were more resistant to quinolones and to ampicillin when a meat-processing industry was draining its waste on the sewerage.

The purification process reduced the numbers of *Campylobacter*, but this pathogen was not eliminated completely. The prevalence and reduction were not correlated with environmental parameters, such as water temperature and oxygen pressure.

The phenomenon of the transformation of spiral *Campylobacter* cells to coccoid cells was also investigated. Determination of several physiological parameters indicated that the contribution of the nonculturable, coccoid *Campylobacter* cells is to infection routes probably negligible.

Considering the observed occurrence of *Campylobacter* in surface waters, in the context of the reported dose-response model for this pathogen, it can be concluded that the role of surface waters in the epidemiology of *Campylobacter* may be underestimated.

Keywords: *Campylobacter*, sewage, purification, sources, coccoid cells.

CONTENTS

Chapter		Page
	Abstract	v
1.	General introduction	1
2.	Methods for the detection of *Campylobacter* in sewage: evaluation of efficacy of enrichment and isolation media, applicability of polymerase chain reaction and latex agglutination assay	25
3.	Survey of *Campylobacter* in sewage purification plants in the Netherlands	39
4.	A short-term evidence of *Campylobacter* in a sewage purification plant and in the drain water of an associated poultry abattoir	53
5.	Speciation and subtyping of *Campylobacter* isolates from sewage purification plants and drain water from an associated poultry abattoir using molecular techniques	69
6.	Antibiotic susceptibility of *Campylobacter* isolates from sewage and poultry abattoir drain water	83
7.	Temperature-dependent membrane fatty acid and cell physiology changes in coccoid forms of *Campylobacter jejuni*	95
8.	An estimation of water-borne human *Campylobacter* infections: recreation water as potential source of human campylobacteriosis	113
9.	General discussion	123

Summary 137

Samenvatting 140

Nawoord 145

Curriculum vitae 147

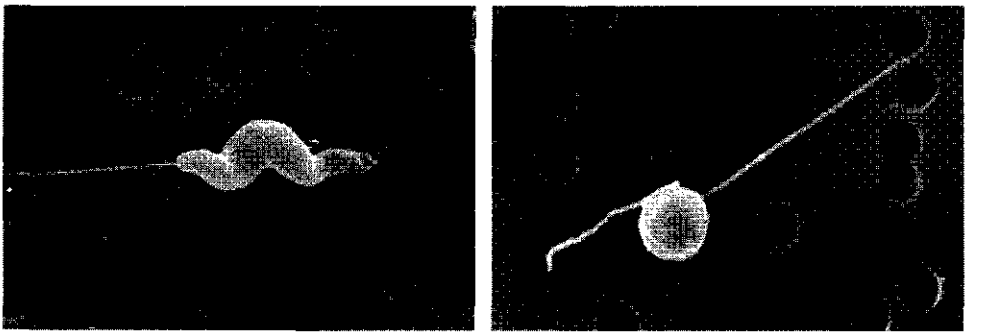
List of publications 148

CHAPTER 1

GENERAL INTRODUCTION[1]

THE GENUS *CAMPYLOBACTER*

The bacteria now known as campylobacters were first isolated at the beginning of this century (McFadyean and Stockman, 1913) and they were considered to be a member of the genus *Vibrio*. The genus name of *Campylobacter* was introduced by Sebald and Véron (1963). Based on their morphological features, the genera *Campylobacter* and *Spirillum* were grouped in the family of *Spirillaceae* (Smibert, 1974). However, the phylogenetic relationships of these organisms were unknown, so the family name *Spirillaceae* was no longer current (Krieg, 1984). Additionally, during the past decade several new *Campylobacter* species have been described and Vandamme and De Ley (1991) pro-



(A) (B)

Fig. 1. Electron micrographs presenting the spiral and coccoid morphology of *Campylobacter*. A: Spiral form (magnification 8,800 ×); B: Coccoid form (magnification 14,400 ×) (TFDL, Wageningen, The Netherlands).

[1] Part of this chapter is submitted as a review. Authors: P.M.F.J. Koenraad, F.M. Rombouts, and S.H.W. Notermans.

posed a new family, *Campylobacteraceae,* to include the 11 presently described species of *Campylobacter* (Table 1) and also the genus *Arcobacter*. The Gram-negative, non-sporeforming, oxidase and catalase positive *Campylobacter* cells are small, curved, spiral-shaped cells with corkscrew-like, darting motility. *Campylobacter* does not ferment or oxidize carbohydrates and has in general a micro-aerophilic character. Occasionally, some species grow aerobically and others can grow under anaerobic conditions with only fumarate as electron acceptor or with fumarate in combination with H_2 or formate as electron donors (Holt *et al.*, 1993). Under unfavourable conditions the spiral cells transform into a coccoid nonculturable stage (Fig. 1, B) (Rollins and Colwell, 1986).

Table 1. *Campylobacter* species with their hosts and medical associations for humans.

Species	Hosts	Disease association	References
C. jejuni	Man, gulls, dogs, cats, poultry, cattle, goats, sheep, pigs	Fever, enteritis, bacteraemia, Guillain-Barré syndrome	Ladrón De Guevara *et al.* (1994), Skirrow *et al.* (1993), Kuroki *et al.* (1991)
C. coli	Man, gulls, dogs, cats, poultry, pigs	Enteritis, bacteraemia	Skirrow (1994), Ladrón de Guevara *et al.* (1994)
C. lari	Man, gulls, dogs, cats, poultry, monkeys, fur seal	Enteritis	Simor and Wilcox (1987)
C. fetus	Man, cattle, sheep	Systematic illness	Skirrow *et al.* (1993), Cover and Blaser (1989)
C. hyointestinalis	Man, pigs, cattle, hamsters	Enteritis	Edmonds *et al.* (1987)
C. upsaliensis	Man, dogs, cats	Bacteraemia, enteritis	Skirrow *et al.* (1993)
C. concisus	Man	Gingivitis, periodontotis	Smibert (1984)
C. sputorum	Man, cattle	Gingival crevices	Skirrow (1994)
C. curvus	Man	Gingival crevices, periodontotis	Smibert (1984)
C. rectus	Man	Gingival crevices, periodontotis	Skirrow (1994)
C. mucosalis	Pig	Not associated with human diseases	Skirrow (1994)

C. jejuni, *C. coli*. and *C. lari*, generally referred to as the thermophilic species, are the most important species causing human diarrhoeal diseases (Mishu *et al.*, 1991). These organisms do not proliferate at temperatures below 30°C. Most recently, there has been growing understanding that many *Campylobacter* species besides *C. jejuni*, *C. coli*, and *C. lari* may also be human pathogens in immunocompromised persons, homosexual men, and the population at large. However, these species are rarely isolated, and represent only 1% of the human *Campylobacter* isolations reported in the United States from 1987 to 1989 (US Department of Health and Human Services, Public Health Service, 1990). Hosts and medical importance for humans of these 11 species are presented in Table 1.

The three species *C. jejuni*, *C. coli*, and *C. lari* are generally identified by several biochemical tests, as summarized in Table 2. In the species *C. jejuni*, two subspecies can be distinguished, mainly based on nitrate reduction and cephalothin susceptibility. Within the *C. jejuni* species, only *C. jejuni* subsp. *jejuni* is thermophilic and usually this subspecies is described as *C. jejuni*. The biochemical tests for *Campylobacter* identification, as presented in Table 2, are regarded as unsatisfactory. Therefore, various methods have been developed for more rapid identification, such as agglutination assays (Nachamkin and Barbagallo, 1990), DNA hybridization tests (Cudjoe *et al.*, 1991, Giesendorf *et al.*, 1993) and polymerase chain reaction tests (Eyers *et al.*, 1993).

THERMOPHILIC *CAMPYLOBACTER* IN HUMANS

Clinical features of human enteritis

Manifestations of illness due to *Campylobacter* infections are often set out by a prodrome with fever, headache, myalgia, and malaise. They mostly start 24-72 hours after ingestion, but may extend up to 7 days (Skirrow, 1994). After 12-24 hours this prodrome is followed by intestinal manifestations: diarrhoea and abdominal pain. Fever usually persists but nausea and vomiting rarely occur (Cover and Blaser, 1989). Although the clinical picture may resemble that of other forms of acute enteritis, the severity of symptoms, the prodromal period, and the severe pain, which sometimes mimic acute

appendicitis, are characteristic features. Furthermore, abdominal cramps reflect the severity of the diarrhoea (Skirrow, 1994). For up to 2-3 weeks, 10^6-10^8 *Campylobacter* bacteria can be isolated per gram human patient's faeces (Taylor *et al.*, 1993).

Most of the *Campylobacter* infections are self limiting and only incidentally treat-

Table 2. Biochemical identification of thermophilic *Campylobacter* species (Smibert, 1984).

Reaction or growth condition	*Campylobacter* species			
	jejuni subsp. *jejuni*	*jejuni* subsp. *doylei*	*coli*	*lari*
Catalase	+[a]	V[b]	+	+
Nitrate reduction	+	−[a]	+	+
Nitrite reduction	−	−	−	−
H_2 requirement	−	−	−	−
Urease	−	−	−	V
H_2S in Triple Sugar Iron Agar	−	−	−	−
Hippurate hydrolysis	+	V	−	−
Indoxyl acetate hydrolysis	+	+	+	−
Growth at 25°C	−	−	−	−
Growth at 42°C	+	−	+	+
Growth in the presence of 3.5% NaCl	−	−	−	−
Growth in the presence of 1% glycine	+	+	+	+
Growth in the presence of 0.1% Trimethylamine-N-oxide HCl	−	−	−	+
Nalidixic acid susceptibility	S[c]	S	S	R[c]
Cephalothin susceptibility	R	S	R	R

[a] + = Positive reaction, − = Negative reaction.

[b] V = Variable reaction.

[c] R = Resistant, S = Sensitive.

ment with antimicrobial agents is indicated. Erythromycin is still the drug of choice (Skirrow and Blaser, 1992) but nowadays, fluoroquinolones are considered to be good, safe but expensive alternatives for the treatment of *Campylobacter* enteritis (Mattila *et al.*, 1993). However, the rapid increase of quinolone resistance of *Campylobacter* strains isolated from human stools and poultry may have implications for the treatment of human diarrhoeal diseases (Endtz *et al.*, 1991; Jacobs-Reitsma *et al.*, 1994b).

Patients suffering from *Campylobacter* enteritis are at risk for bacteraemia, but its actual incidence remains unknown (Ladrón De Guevara *et al.*, 1994). Furthermore, *C. jejuni* enteritis may incidentally preceed Guillain-Barré syndrome (Kuroki *et al.*, 1991), which is a polyneuritis attended with severe and sometimes fatal paralysis (Kaldor and Speed, 1984).

Infectivity and immunity

The infectious dose of *Campylobacter* for humans appears to be low (Black *et al.*, 1988). Human volunteer studies revealed that illness was caused in 10% of the individuals who were exposed to approximately 8×10^2 *Campylobacter* cells (Black *et al.*, 1992). Based on the dose-response relationship, Rose and Gerba (1991) estimated the probability of contracting an infection from exposure to one *Campylobacter* organism to be 7×10^{-3}. Some studies indicated that regular exposure to a low dose of *Campylobacter* cells leads to protection. Sibbald and Sharpe (1985) found that the incidence rate in rural areas was lower than in urban areas. Blaser *et al.* (1987) observed that in individuals with a regular consumption of raw milk, no illness occurred, which may indicate immunity. Black *et al.* (1992) carried out volunteer studies in order to evaluate the immune responses following infection and the extent of protective immunity. Volunteers were challenged with two strains at different doses (10^2-10^9 *Campylobacter* cells) and the extent of infections, defined as *Campylobacter*-positive stools, and illness, defined as the presence of diarrhoea or fever, were determined. Although infection and illness were not clearly correlated with the challenge dose, rechallenging of human volunteers within 28 days after they had suffered from campylobacteriosis, resulted in a significantly lower rate of infection and/or illness compared to a control group of volunteers, who had not previously participated in challenge studies. These results indicate that homologous immunity can be induced, at least for the short term, by symptomatic *C. jejuni* infection.

However, the predictive value of such human volunteer experiments with single strains is disputable because of differences in virulence between the various *Campylobacter* phenotypes (Newell *et al.*, 1985; Mawer, 1988).

In developing countries, where *Campylobacter* is hyperendemic, the highest rate of infections is observed in very young children (Isselbacher, 1994) and with increasing age, the illness-to-infection rates fall by acquisition of immunity (Skirrow and Blaser, 1992). Contrarily, investigations on the specific antibody level against *C. jejuni* among Danish slaughterhouse workers showed no increased level in comparison with the control group of greenhouse workers (Lings *et al.*, 1994).

In summary, challenge studies and epidemiological investigations indicate that natural infections with *Campylobacter* confer protective immunity. This may explain the age-related decrease in the illness-to-infection rate for *C. jejuni* in developing countries (Calva *et al.*, 1988). The extent and mechanism of heterologous immunity remain to be determined. Furthermore, the applicability of artificial immunogens, has to be evaluated in the context of the great variety of phenotypes (Report on a WHO Consultation on Epidemiology and Control of Campylobacteriosis, 1994).

Sources of infection and incidence rates

Campylobacter is recognised as one of the most important causes of human diarrhoeal disease. It is now clear that the number of *Campylobacter* infections, which are mostly so-called single cases, exceeds that of *Salmonella* (Notermans and Van De Giessen, 1993). From sentinel and population studies carried out in the Netherlands, it can be estimated that about 2,000 per 100,000 people suffer annually from campylobacteriosis and for *Salmonella* the incidence rate was estimated to be 800 cases per 100,000 people (Notermans and Van De Giessen, 1993). These results are in agreement with estimates from the United Kingdom and the United States of America (Johnson and Nolan, 1985; Kendall and Tanner, 1982; Sacks *et al.*, 1986).

The incidence of *Campylobacter* infections in developing countries is much higher compared to that in developed countries. For example, the incidence rate of *Campylobacter* enteritis in Mexico and Thailand is estimated to be 40,000 per 100,000 for children younger than five years old. Although the occurrence of diarrhoea in young children in developing countries is over 100 times more frequent than that in the United Kingdom,

the rates of symptomatic illness in school-age children and adults in developing countries are lower than in the United Kingdom (Taylor, 1992). Opposed to this, in developed countries persons of all ages may be affected and the male group aged 20-28 years has a greater risk to become diseased (Tauxe, 1992).

A seasonal distribution among human infections, consisting of peaks in springtime and autumn (Hoogenboom-Verdegaal *et al.*, 1990) has been reported for the temperate climates. Seasonal trends are much less evident in tropical and subtropical countries, although in general higher incidences have been observed during rainy seasons (Skirrow and Blaser, 1992). Outbreaks of campylobacteriosis mostly occur in May and October, however, reported isolates which are largely sporadic cases, peak in the summer months. This contrast in seasonal distribution indicates different vehicles of transmission (Tauxe, 1992). An overview of reported outbreaks and their indicated sources is presented in Table 3.

Sporadic cases of *Campylobacter* enteritis are far more common than outbreak-associated cases. In many countries single cases are not reported in surveillance programmes and therefore little information is available concerning the types of foods involved in campylobacteriosis. However, several case-control studies revealed that eating or handling poultry products, drinking raw milk or untreated surface water, and to a lesser degree, living in a household with a cat or dog are considered as the most important risk factors (Deming *et al.*, 1987; Hopkins *et al.*, 1984; Kapperud *et al.*, 1992; Wood *et al.*, 1992). One case-control study indicated that consumption of curd or cottage cheese was identified as being protective (Schorr *et al.*, 1994). A case-control study in the United States of America showed that 50% of all cases is caused by consumption of poultry meat, and 8% could be attributed to drinking contaminated surface water (Tauxe, 1992). Even purified drinking water may contribute to infections with *Campylobacter*. A risk analysis carried out by Medema and Havelaar (1994), based on the dose-respons relationship as deduced by Rose and Gerba (1991), indicated that purified drinking water may be the cause of 0.2-0.5% of all *Campylobacter* infections registered in the Netherlands. Since cases and controls are generally matched by geographic region, the importance of water as a risk factor may be underestimated.

Reviewing the reported common-source outbreaks of human infections caused by *C. jejuni, C. coli*, and/or *C. lari* (Table 3) it becomes clear that especially untreated

water or contaminated surface water are indicated sources in causing infections among

Table 3. Reported outbreaks of human *Campylobacter* enteritis.

Country	Year of outbreak	Number of persons involved	Indicated source	References
USA	1978	3000	Contaminated drinking water	Vogt *et al.* (1982)
USA	1980	28	Raw clams	Griffin *et al.* (1983)
USA	1981	>250	Raw milk	Kornblatt *et al.* (1985)
USA	1982	11	Barbecued chicken	Istre *et al.* (1984)
USA	1983	6	Raw goat's milk	Harris *et al.* (1987)
USA	1983	865	Contaminated drinking water	Sacks *et al.* (1986)
USA	1985	22	Raw milk	Korlath *et al.* (1985)
USA	1986	15	Raw milk	Klein *et al.* (1986)
Canada	1985	162	Contaminated drinking water	Broczyk *et al.* (1987)
Canada	1987	13	Community water supply	Alary and Nadeau (1990)
Canada	1991	241	Drinking water contaminated by meltwater	Millson *et al.* (1991)
UK	1985	7	Chicken casserole	Rosenfield *et al.* (1985)
UK	1990	?	Bird-pecked milk	Hudson *et al.* (1991)
UK	1991	11	Bird-pecked milk	Riordan *et al.* (1993)
UK	1992	72	Unpasteurised milk	Morgan *et al.* (1994)
New Zealand	1984	80	Raw milk	Brieseman (1984)
New Zealand	1987	19	Town water supply	Brieseman (1987)
Finland	1986	94	Contaminated drinking water	Rautelin *et al.* (1990)
Finland	1989	75	Surface water	Aho *et al.* (1989)
Norway	1991	680	Non-chlorinated drinking water	Melby *et al.* (1991)
Germany	1986	556	Cabbage stew with beef	Steffen *et al.* (1986)

considerably large groups of persons. These findings indicate that activities such as swimming or sailing in recreational waters may be a risk as well. Also sewage may be involved in *Campylobacter* infections; Sumathipala and Morrison (1983) reported a peculiar case of campylobacteriosis after a sewage worker had fallen into a tank of untreated sewage sediment.

ANIMAL SOURCES OF *CAMPYLOBACTER*

The natural habitat of *Campylobacter* is the intestinal tract of warm-blooded animals. These animals excrete the organism in large numbers and provide a constant flow of *Campylobacter* into the external environment.

Poultry can be highly contaminated with *Campylobacter*; 82% of the investigated flocks were *Campylobacter* positive (Jacobs-Reitsma *et al.*, 1994a). Most of the broiler flocks become colonised at about three to four weeks of age and remain carriers up to slaughter (Jacobs-Reitsma *et al.*, 1995). It is increasingly recognized that horizontal transmission, which includes many potential sources of infection, like other livestock, flies, wild birds and vermin, is the main route of contamination (Evans, 1992; Jacobs-Reitsma, 1994). Kapperud *et al.* (1993) has identified the use of untreated well water and pig breeding on the same farm as factors increasing the risk of broiler contamination.

An epidemiological study on the prevalence of *Campylobacter* in pigs showed that more than 85% of the pigs were intestinal carriers during all stages of fattening. It was suggested that piglets were already infected at a young age on the breeding farm before weaning (Weijtens *et al.*, 1993).

Cattle can also harbour *Campylobacter* in the intestines, although a lower contamination level, approximately 23%, was observed (Humphrey and Beckett, 1987). Faeces may contaminate milk (Beumer *et al.*, 1988) and raw milk is frequently documented as source of human campylobacteriosis (Wood *et al.*, 1992) (Table 3).

Wild birds are a major animal reservoir of *C. jejuni*; especially water-fowl, such as gulls, are frequently carriers of *Campylobacter* (Luechtefeld *et al.*, 1980). Moreover, this organism has also been recovered from many other wild bird species such as geese (Yogasundram *et al.*, 1989), blue magpies and pigeons (Ito *et al.*, 1988).

Furthermore, *Campylobacter* has been isolated from faeces of household pets and kennel animals (Blaser *et al.*, 1980), rodents (Fox *et al.*, 1981), flies (Rosef and Kapperud, 1983; Shane *et al.*, 1985) and shellfish (Abeyta *et al.*, 1993).

CAMPYLOBACTER IN AQUATIC ENVIRONMENTS

Prevalence

Patients suffering from campylobacteriosis and healthy animal carriers of *Campylobacter* provide a constant flow of this micro-organism into the environment. The external environment may become contaminated either directly, via livestock or wild-living animals, or via its waste, which is carried off as sewage. Although *Campylobacter* cannot multiply outside its host, it may survive fairly well in the external environment, especially in aquatic niches (Blaser *et al.*, 1980; Stelzer *et al.*, 1991). As a consequence of faecal pollution, sewage is regularly contaminated with various pathogens including *Salmonella*, *Helicobacter*, *Listeria*, *Arcobacter* (Kearney *et al.*, 1993) and *Campylobacter*. In various studies in the United Kingdom, Germany and Italy on the occurrence of *Campylobacter* in sewage, numbers of *Campylobacter* from 1.7 $\log_{10}$ (MPN/100 ml) up

Table 4. Occurrence of *Campylobacter* in various sewage waters and the effects of various purification treatments.

Purification system	Numbers in sewage ($\log_{10}$(MPN/100 ml))	Reduction ($\log_{10}$ units)	References
Oxidation pond	1.7	1.7	Stelzer and Jacob (1991)
Activated sludge	3	0.9	Stelzer and Jacob (1991)
Activated sludge	3.6	2.3	Höller (1988)
Activated sludge	3.2	2	Stampi *et al.* (1992)
Trickling filter	4.7	2.3	Arimi *et al.* (1988)

to 4.7 $\log_{10}$(MPN/100 ml) are reported (Table 4). Seasonal variations have often been observed and could be explained by variations in human infection rates and/or animal carrier state (Jones *et al.*, 1990).

From various investigations, it has become clear that purification of sewage does not result in a complete elimination of *Campylobacter* (Table 4). Efflux, if not disinfected, may be therefore a source of contamination of surface waters. The numbers of *Campylobacter* reported in surface water vary considerably between the various investigations. In Weiße Elster (Germany) *Campylobacter* was detected in 82% of the river water samples, which contained usually less than 1 $\log_{10}$(MPN/100 ml). The presence of water-fowl and faecal contamination from a poultry farm resulted in considerable higher *Campylobacter* contents (2.4 $\log_{10}$(MPN/100 ml)) (Stelzer and Jacob, 1991). So, infections with *Campylobacter* due to recreational activities on surface water are quite likely to occur.

The reduction of numbers of *Campylobacter* in sewage purification plants (Table 4) might be explained by attachment of the bacteria to sludge particles. Bearing in mind the disposal practices of sewage sludge and the use of surface water on arable land and pastures, it is important to determine the hazards of these activities to the health of man and animals (Park and Sanders *et al.*, 1992).

Sources of aquatic *Campylobacter* strains

Typing of aquatic strains may provide information about the origin of *Campylobacter* strains and their possible significance in human infections. Until now, investigations on the transmission of *Campylobacter* in aquatic environments are rare. The number of phenotypes of *Campylobacter* isolated from sewage and surface water is considerable. Many investigators used sero- and biotyping (Jones *et al.*, 1984) as well as phagetyping, allowing for limited conclusions only because of the high numbers of nontypable strains. DNA-based typing methods overcome this problem. They do not depend on the variable environment-dependent phenotypic charateristics of bacterial cells. Especially, the recently developed molecular typing scheme called polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) is suitable for epidemiological studies. However, up to now this scheme has not been applied for tracing the origin of *Campylobacter* in water-related environments.

VIABLE BUT NONCULTURABLE STAGE OF *CAMPYLOBACTER*

In an aquatic environment, bacteria which are capable of growing in warm-blooded hosts, may be stressed by starvation or by exposure to suboptimal temperature, salinity or toxic chemicals. As a consequence, these cells may enter into a viable but nonculturable stage as a possible strategy for survival until environmental conditions change (Kjelleberg *et al.*, 1987). Oliver (1993) defined these viable but nonculturable cells as bacteria which are metabolically active, while being incapable of undergoing the cellular division required for growth. This useful definition for this viable but nonculturable stage will be used in this thesis. Also for *Campylobacter* such a viable but nonculturable stage has been observed. During the decline of culturability, *Campylobacter* cells are transformed from the spiral form into a coccoid form (Fig. 1, B) (Rollins and Colwell, 1986).

Infectivity of coccoid cells

The ability of viable but nonculturable enteropathogenic bacteria to regain their culturability when conditions become favourable is essential for their significance as causal organisms. Moreover, if nonculturable cells could provoke carriership in animals, they would be of significance in environmental contamination cycles of these pathogenic micro-organisms. Despite their potential importance, reports of challenge experiments with nonculturable *Campylobacter* cells are rare, poorly documented and controversary. Differences in opinions exist regarding the capacity of nonculturable *Campylobacter* cells to colonize warm-blooded animals and humans. From challenge experiments, Medema *et al.* (1992) and Fearnley *et al.* (1994) reported that nonculturable *C. jejuni* were not able to colonize chickens. Beumer *et al.* (1992) administered *Campylobacter* coccoid cells to laboratory animals and human volunteers. In these challenge experiments no symptoms of campylobacteriosis and/or serum antibodies against *C. jejuni* were observed.

However, Saha *et al.* (1991) reported that nonculturable *C. jejuni* could be converted to a resuscitated form after passage through the gastro-intestinal tract of a rat and Jones *et al.* (1991) was able to colonize mice after challenging with nonculturable forms of *C. jejuni*. Stern *et al.* (1994) found that coccoid *Campylobacter* cells of four different *Campylobacter* isolates could colonize one-week-old chicks. These contradicting results might be explained by the different conditions under which cocci were formed and also

by differences in the reversion experiments or viability assays, or by the presence of a few culturable *Campylobacter* cells in the preparation of coccoid cells. Nevertheless, the observation of nonculturable *Campylobacter* forms in water supplies by direct immunofluorescence microscopy and the subsequent colonization of originally *Campylobacter*-free chickens (Pearson *et al.*, 1993), could imply a role for coccoid *Campylobacter* cells in transmission routes.

If these *Campylobacter* coccoid cells are still infective, the contribution of the cocci in the contamination cycle could be substantial. Therefore, their role in transmission should be evaluated by investigating the physiological status of coccoid cells and/or determining the colonization features of these nonculturable stages of *Campylobacter*.

Factors influencing the transformation from culturable to nonculturable cells

The loss of culturability is influenced by many natural environmental stresses, which could normally be encountered by bacteria in their natural environment (Oliver, 1993). The decline of culturability of *Campylobacter* is dependent on parameters such as gas atmosphere, temperature and availability of nutrients.

It is known that *Campylobacter* is sensitive to oxygen and therefore, it is possible that the presence of oxygen enhances the transformation rate of spirals into coccoid cells. The formation of coccoid cells was minimized after the addition of detoxifying, oxygen-scavenging agents. Antibiotic formulations used in the selective media did not influence production of coccoid forms (Moran and Upton, 1987). Microcosms, aerated by shaking, exhibited logarithmic decline in culturable *C. jejuni* cells, while stationary systems underwent a more moderate rate of decrease into the nonculturable stage (Rollins and Colwell, 1986).

Physiology of coccoid cells

Some investigations have been carried out to determine the physiological status of viable but nonculturable *Campylobacter* forms, which were formed under different conditions. It is likely that these different conditions yield cocci with distinct physiological features, which hampers comparison of results from different investigations.

Various investigations have established that cocci contain less low molecular weight cytoplasmic components and that the DNA and RNA content was also lower than

in spirals (Moran and Upton, 1986; Hazeleger *et al.*, 1994). However, Jacob *et al.* (1993) observed similar whole-cell protein and lipooligosaccharide patterns for cocci transformed under different conditions and spirals. Furthermore, the ATP level of *Campylobacter* cocci suspensions remained constant during a period of at least three weeks (Beumer *et al.*, 1992).

The cell wall integrity and composition also provide information on the survival abilities of the nonculturable stage of *Campylobacter* cells. Observed differences in the Gram stain reaction of coccoid forms compared with spirals, and lack of cell integrity investigated by electron microscopy indicate that changes occur in the cell membrane during conversion. The *Campylobacter* cocci are generally not motile, although flagella can be observed with these forms. This indicates that the coccoid cells may be in a degenerative stage and unable to supply the flagella with sufficient energy to cause motility, or that the flagella apparatus itself is degenerative (Moran and Upton, 1986).

More and more evidence is accumulated from the various reports that the viable but nonculturable stage of *Campylobacter* is a degenerative form. However, the vital question remains if these cocci occurring in the environment can revert to normal cells and thereby contribute to contamination cycles.

SCOPE OF THIS THESIS

Most outbreak-associated cases of campylobacteriosis are related to the consumption of raw milk or untreated water. Due to these observations, more knowledge on the significance of surface waters in the epidemiology of *Campylobacter* should be generated. Therefore, the overall aim of the investigations described in this thesis is to obtain a better understanding of *Campylobacter* epidemiology in sewage and surface waters. This will be of benefit to estimate the public health risk of recreational exposure to surface water.

Various combinations of isolation and enrichment media were evaluated for their efficacy in detection of *Campylobacter* in sewage. The polymerase chain reaction (PCR) technique and the latex agglutination assay were tested on their applicability as tools for rapid detection of *Campylobacter* in enrichments. These evaluations of methodology are

described in **Chapter 2**.

Chapter 3 deals with the prevalence of *Campylobacter* in various municipal sewage purification plants related to the presence of meat-processing industries in the drainage area of these plants. During a two-year surveillance programme the occurrence of *Campylobacter* was determined and the influence of several auxiliary parameters on the reduction was studied. As a sequel to this surveillance programme, a short-term study on the prevalence of *Campylobacter* in an activated sludge system and the waste water of an associated poultry abattoir was carried out, as described in **Chapter 4**. The aim of this short-term investigation was to deduce a transmission route from serotyping and antibiotic susceptibility results. However, it became clear that a disadvantage of the serotyping system applied was the relatively high percentage of nontypable strains. Therefore, in **Chapter 5** the recently developed polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) technique was used for subtyping of *Campylobacter* isolates. The PCR-RFLP technique was based on the variety of the flagellin gene of *Campylobacter* (Ayling *et al.*, 1995). The isolates obtained from the activated sludge system and poultry abattoir waste water during the two-year monitoring programme were subtyped and compared. A screening for antibiotic resistance, especially to quinolones, was included in the two-year study on the prevalence of *Campylobacter* in sewage purification plants. These results on antibiotic susceptibility are presented in **Chapter 6**.

In **Chapter 7** the physiological changes during the transformation from *Campylobacter* spirals to cocci are presented as function of the incubation temperature and media composition. From the results on the ATP measurements, protein profiles and fatty acid compositions, the possible role of the coccoid *Campylobacter* cells in transmission routes, could be deduced.

As surface waters are frequently used for recreational purposes, it is important to estimate possible health risks caused by exposure to water contaminated with *Campylobacter*. Therefore, the annual incidence of *Campylobacter* enteritis caused by exposure to contaminated surface water and the consequences for public health has been estimated, based on the dose-response relationship as deduced by Rose and Gerba (1991). The results of this exercise are presented in **Chapter 8.** Finally, a general discussion of the work presented in this thesis is given in **Chapter 9**.

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CHAPTER 2

METHODS FOR THE DETECTION OF *CAMPYLOBACTER* IN SEWAGE: EVALUATION OF EFFICACY OF ENRICHMENT AND ISOLATION MEDIA, APPLICABILITY OF POLYMERASE CHAIN REACTION AND LATEX AGGLUTINATION ASSAY

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In this study several combinations of enrichment and isolation media are compared in the conventional most probable number procedure for the isolation of *Campylobacter* from sewage. Furthermore, the potential of the polymerase chain reaction (PCR) technique and the latex agglutination assay (LAA) is assessed for rapid detection and identification of *Campylobacter* in the culturing procedure. No significant difference in efficacy was observed for the various blood- and charcoal-based enrichment broths and isolation media tested.

The LAA had a positive predictive value of 39% and therefore, this assay should not be used as a rapid method for detection of *Campylobacter* in sewage enrichments. The PCR culture assay is suitable for the detection of *Campylobacter* in sewage enrichments; the method is faster and has a higher detection ability than the conventional culturing procedure, also when including four isolation media. For a qualitative analysis by conventional culturing 1 ml of the 10^{-1} dilution of sewage is preferably enriched, and for the PCR assay raw sewage can be enriched for qualitatively detecting *Campylobacter*.

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INTRODUCTION

In developed countries *Campylobacter* is now well recognized as an important cause of human enteritis. Poultry products, unpasteurized milk, and non-chlorinated drinking water were shown to be the main vehicles of transmission to man. Campylobacteriosis patients provide a constant flow of this pathogen into the environment. These and other sources, contribute to the *Campylobacter* contamination of sewage, sewage sludge, and surface water (Koenraad *et al.*, 1994; Stelzer *et al.*, 1991).

A great variety of broths and media has been proposed for the isolation of *Campylobacter* from clinical, veterinary, and food samples. These media can be categorized in blood-based or charcoal-based types and they differ in the mix of selective agents and the choice of the basal medium (Goossens *et al.*, 1986; Park and Sanders, 1991; Scotter *et al.*, 1993). For the analysis of artificially-contaminated chicken livers, Peterz (1991) did not observe a difference in yield between the blood-based Preston agar and charcoal-based charcoal cefoperazone deoxycholate broth (CCDB), although the selectivity of the CCDB was higher. Goossens *et al.* (1986) and Gun-Munro and coworkers (1987) both recommended CCDA for the isolation of *Campylobacter* from faecal samples. Korhonen and Martikainen (1990) recommended a combination of Oosterom broth/CCDA or CCDB/CCDA for the isolation of *Campylobacter* from surface water samples and they suggested that CCDB was better applicable to polluted waters than Preston broth (PB). Höller (1991) analysed 15 sewage samples in parallel and examined the best broth/agar combination from Preston and CCD media. No significant differences between the several combinations could be ascertained. Despite differences in incubation temperature and/or time, *Campylobacter* contamination levels of the analysed samples, levels of competing flora, the use of artificially or naturally-contaminated samples, the CCD media are slightly preferred. It is advisory to evaluate the optimal medium combination for each specific application, which was the isolation of *Campylobacter* from sewage samples in this study.

Enrichment procedures are necessary to isolate *Campylobacter* from samples with low contamination levels, such as surface water or sewage. These conventional procedures are laborious, mainly because of the requirement of a non-selective pre-enrichment step (Fricker, 1987). The selective enrichment requires two days while isolation and subsequent identification of *Campylobacter* by biochemical tests lasts three days. The

identification can be shortened in time by applying rapid methods, such as an immunological agglutination assay (Nachamkin and Barbagallo, 1990), or a DNA probe (Cudjoe *et al.*, 1991; Tenover *et al.*, 1990). Griffiths *et al.* (1989) and Hazeleger *et al.* (1992) reported that a latex agglutination assay (LAA) could be applied to enrichments of faeces from campylobacteriosis patients and to enrichments of chicken products, respectively.

Oyofo and Rollins (1993) reported that the application of the polymerase chain reaction (PCR) is a possibility to shorten the detection procedure of *Campylobacter* in water samples. These authors developed a filtration PCR method with a high specificity and a sensitivity of 10 to 100 viable *Campylobacter* cells per 100 ml filtered water for the detection of *C. jejuni* and *C. coli* in environmental waters. Sewage has a high degree of turbidity and therefore, the PCR culture assay, developed by Giesendorf *et al.* (1992) was evaluated as a rapid detection method for *Campylobacter* in sewage enrichments.

In order to obtain the optimal detection procedure for *Campylobacter* from sewage, a comparison was made between enrichment broths and isolation media, applied within a conventional most probable number (MPN) procedure. Furthermore, the potential of the PCR assay, described by Giesendorf *et al.* (1992), and the LAA are assessed for rapid detection and identification of *Campylobacter* in the MPN procedure for sewage samples.

MATERIALS AND METHODS

Sample collection

The influx and the efflux of an activated sludge system were sampled during 8 consecutive weeks in the period 26 May - 28 July 1993. The activated sludge system, with a capacity of 46,000 citizen equivalents receives approximately 15,000 m^3 sewage daily, including sewage from approximately 30,000 inhabitants, a poultry abattoir and other small industries. The samples were transferred to the laboratory in a cold storage container on ice and analysed for *Campylobacter* within 2 h of collection.

Table 1. Basic compositions of enrichment and isolation media.

Enrichment media	**Antibiotics**	**Supplements**
Preston broth **(PB)** (Bolton *et al.*, 1983)	5,000 IU/l polymyxin-B, 10 mg/l rifampicin, 10 mg/l trimethoprim lactate, and 100 mg/l cycloheximide (SR117, Oxoid, Basingstoke, UK)	5% (v/v) defibrinated, lysed horse blood
Charcoal cefoperazone deoxycholate broth **(CCDB)** (Bolton *et al.*, 1984)	32 mg/l cefoperazone and 10 mg/l amphotericin-B (SR155E, Oxoid)	0.025% (w/v) ferrous sulphate, sodium metabisulphite and sodium pyruvate (Humphrey, 1990)
Isolation media		
Columbia agar base **(CAB^+)** (CM331, Oxoid)	32 mg/l cefoperazone and 10 mg/l amphotericin-B (SR155E, Oxoid)	5% (v/v) defibrinated, lysed horse blood
Campylobacter blood-free selective medium-modified CCDA-Preston **(CCDA)** (CM739, Oxoid)	32 mg/l cefoperazone and 10 mg/l amphotericin-B (SR155E, Oxoid)	
Campylobacter blood-free selective medium-modified CCDA-improved **($CCDA^+$)** (112, LabM, Amersham, Bury, UK)	32 mg/l cefoperazone and 10 mg/l amphotericin-B (SR155E, Oxoid)	
Campylobacter agar base Karmali **(KAR)** (CM908, Oxoid)	32 mg/l hemine, 100 mg/l sodium pyruvate, 16 mg/l cefoperazone, 20 mg/l vancomycin, and 100 mg/l cycloheximide (Karmali Selective Supplement, SR139, Oxoid)	

Conventional culturing procedure of *Campylobacter*

The most probable number technique, described by Koenraad *et al.* (1994) was used to determine the numbers of *Campylobacter* in the sewage samples. Eight broth/ agar combinations, whose compositions are summarized in Table 1, were compared. The basic compositions of the KAR and $CCDA^+$ are very similar to the composition of the CCDA, however according to the manufacturer's instructions *C. jejuni*, *C. coli*, and *C. lari* should be distinguishable from their colony morphology on $CCDA^+$. All incubations were under micro-aerobic conditions and checked by incubating a positive control, consisting of a brain heart infusion broth culture (BHI, 0037-01-6, Difco Laboratories, Detroit, USA). Enrichment included a non-selective step of 4 h at 37°C. After 2 days of incubation at 42°C, 0.1 ml from each enrichment tube were streaked on plates of the various isolation media (Table 1). At least 5 suspected colonies per plate were purified by subculturing (42°C) on Columbia agar base plates containing 5% (v/v) defibrinated, lysed horse blood (CAB). Confirmation was based on microscopic appearance (curved or spiral-shaped cells with darting corkscrew-like motility), ability of growth under micro-aerobic conditions in BHI at 42°C (+) and 25°C (—), oxidase activity (+) and catalase activity (+). Plates confirmed for *Campylobacter* were used in calculating the logarithm$_{10}$ of the most probable number per 100 ml ($\log_{10}$ (MPN/100 ml)).

Detection of *Campylobacter* in enrichments by using PCR

After 18 h enrichment at 42°C in PB and CCDB, samples were taken from the enrichments of the influx samples and stored at -20°C for the PCR. Extraction of nucleic acids, based on guanidinium isothiocyanate and PCR were performed as described by Giesendorf *et al.* (1992). Briefly, a standard PCR reaction mixture (100 µl) contained 25 µl of the test samples and 75 µl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.1% (w/v) Triton X-100, 200 µM each deoxyribonucleotide triphosphate, 0.25 U of SuperTaq DNA polymerase (1865, Promega, Madison, USA), and 50 pmol of each primer. The PCR programme consisted of 40 cycles of amplification in a thermocycler (Biomed model 60, Alkmaar, The Netherlands) with 1 min at 94°C, 1 min at 52°C, and 1 min at 74°C. Primers based on the 16S rRNA gene (Giesendorf *et al.*, 1992) specific for *Campylobacter*, were used: C442: 5'-GGAGGATGACACTTTTCGGAGC-3' and C490: 5'-ATTACTGAGATGACTAGCACCCC-3'. In

each PCR experiment, distilled water was used as negative control and *C. jejuni* DNA as positive control. PCR fragments were confirmed by hybridization with the oligonucleotide probe C631: 5'-GGAAGAATTCTGACGGTACCT-3'. Enrichments confirmed for the presence of *Campylobacter* by PCR were used in calculating the logarithm$_{10}$ of the most probable number per 100 ml sewage ($(\log_{10}$(MPN/100 ml)).

Detection of *Campylobacter* in enrichments by using LAA

After 48 h incubation in PB and CCDB, the enrichments were screened for the presence of *Campylobacter* by applying LAA and by streaking 0.1 ml of the suspensions on selective plates. Fifty microliter suspension were used in the LAA immunotest, according to the manufacturer's directions (M46, Microscreen, Mercia Diagnostics Limited, Guildford, UK). The assay includes a test latex reagent and a control reagent consisting of uncoated latex particles. When both the test and control reagent agglutinate with the sample, the agglutination was considered nonspecific. The detection limit of the Microscreen agglutination assay was determined by applying the test on dilutions of a two days BHI culture of the laboratory strain WAU080193-C50, isolated from sewage. It appeared that the minimum cell concentration for specific agglutination was 6 $\log_{10}$(colony forming units(CFU))/ml).

Statistical analyses

The isolation rates of the various broth/agar combinations were compared by variance analysis. Logarithmic transformations were carried out prior to statistical analysis in order to obtain normal distribution in the most probable numbers. For each enrichment, a presence/absence result was obtained by both PCR and conventional culturing. The McNemar's test, a non-parametric, matched-pair test (O'Mahony, 1986) which compares the numbers of enrichments registering presence/absence discrepancies, was used to evaluate the null hypothesis that the PCR and conventional culturing were similar in the ability to detect *Campylobacter*. P values ≤ 0.05 were regarded to be significant.

RESULTS AND DISCUSSION

Eight influx and eight efflux samples from successive weeks were quantitatively analysed for the presence of *Campylobacter* in order to compare the isolation rate of different combinations of enrichment/isolation media. The results are presented as $\log_{10}$ (MPN/100 ml) in Tables 2 and 3. Statistical analysis did not indicate an optimal combination of the enrichment broths (PB and CCDB) and the isolation media tested (CAB^+,

Table 2. Comparative efficacy of the various enrichment/isolation media combinations and PCR for the detection of *Campylobacter* in influx enrichments.

Week no.	Enrichment in PB[a]					Enrichment in CCDB				
	Numbers of *Campylobacter* $\log_{10}$(MPN[b]/100 ml)					Numbers of *Campylobacter* $\log_{10}$(MPN/100 ml)				
	CAB^{+c}	CCDA	$CCDA^+$	KAR	PCR	CAB^+	CCDA	$CCDA^+$	KAR	PCR
1	3.4	3.2	3.2	1.5	2.2	3.2	1.8	1.5	<1.5	4.0
2	2.5	2.5	<1.5	2.5	2.2	1.9	3.2	1.5	<1.5	4.2
3	3.5	4.4	4.4	4.4	4.4	3.2	3.2	3.0	3.2	4.7
4	4.4	4.4	4.4	3.5	3.2	2.5	3.0	2.6	2.8	5.0
5	<1.5	<1.5	<1.5	<1.5	3.7	1.5	1.5	1.8	1.5	3.7
6	2.9	3.2	2.9	2.5	3.4	2.8	3.0	2.8	3.0	3.2
7	3.2	3.3	3.3	3.3	>5	2.3	2.4	3.3	3.3	3.7
8	3.3	4.4	3.6	3.2	4.4	3.2	3.0	3.0	2.8	4.2

[a] PB = Preston broth.
CCDB = Charcoal cefoperazone deoxycholate broth.
[b] MPN = Most probable number.
[c] CAB^+ = Columbia agar base with 5% defibrinated, lysed horse blood, cefoperazone, and amphotericin-B.
CCDA = *Campylobacter* blood-free selective medium-modified CCDA-Preston.
$CCDA^+$ = *Campylobacter* blood-free selective medium-modified CCDA improved.
KAR = *Campylobacter* agar base Karmali with Karmali selective supplement.
PCR = Polymerase chain reaction.

CCDA, CCDA$^+$ and KAR) ($P \geq 0.05$). This agrees with the observations of Höller (1991), but is in contrast with the results of Korhonen and Martikainen (1990). Dousse *et al.* (1993) compared the isolation efficacy of KAR plates with CCDA plates for sewage samples and, as in our study, they did not observe a significant difference in performance of these two charcoal-based media.

CAB$^+$ was less selective than the charcoal-based media CCDA, CCDA$^+$, and KAR. However, competing flora, identified to be *Escherichia coli*, *Proteus mirabilis*, *Bacillus*, and *Enterococcus faecalis*, was present on the four isolation media. With all types of isolation media, several subculturings were required to obtain pure colonies for *Campylobacter* confirmation, but subculturing of suspected colonies from CAB$^+$ plates was easiest. CCDA$^+$ plates inoculated with *C. jejuni*, *C. coli*, and *C. lari* BHI suspensions, did not show differences in colony morphology, which is in contrast with the manufacturer's claim. Based on practical aspects, like blood availability, costs, and con-

Table 3. Comparative efficacy of the various enrichment/isolation media combinations for the detection of *Campylobacter* in efflux samples.

Week no.	Enrichment in PB[a]				Enrichment in CCDB			
	Numbers of *Campylobacter* $\log_{10}$(MPN[a]/100 ml)				Numbers of *Campylobacter* $\log_{10}$(MPN/100 ml)			
	CAB^{+a}	CCDA	CCDA$^+$	KAR	CAB$^+$	CCDA	CCDA$^+$	KAR
1	4.2	4.0	4.0	4.0	4.5	3.4	2.8	3.3
2	2.3	3.4	3.4	2.2	3.6	2.0	1.5	1.5
3	3.0	3.0	3.0	3.0	3.6	3.6	4.0	3.6
4	2.8	2.8	2.8	2.8	2.5	2.4	2.5	1.6
5	2.6	2.6	3.2	3.7	2.0	2.0	3.1	3.2
6	3.1	3.1	3.3	3.3	1.5	1.9	1.5	1.5
7	2.8	2.8	2.8	2.8	3.0	2.9	3.2	4.0
8	2.6	2.6	2.6	2.2	—[b]	—	—	—

[a] For abbreviations see Table 2.
[b] — = Not determined.

venience of preparation, the charcoal-based media could be recommended.

PCR was performed on the enrichments of influx samples and the PCR assay was found to be suitable for the detection of *Campylobacter* in sewage enrichments, in both PB and CCDB. Since the efflux of a sewage purification plant contains less competing flora than the influx, a similar performance of the PCR can be expected for the efflux enrichments.

The performance of the enrichment media PB and CCDB without effects of isolation media can be evaluated by confirming the presence of *Campylobacter* in the influx

Table 4. Statistical analysis of *Campylobacter* presence/absence results for individual enrichments determined by PCR and by conventional isolation on various media.

Isolation media	Growth of *Campylobacter*	PCR[a]		P[b]
		PCR$^+$	PCR$^-$	
CAB$^+$[c]	+[d]	38	12	<0.001
	−	74	67	
CAB$^+$ or CCDA	+	48	15	<0.001
	−	64	64	
CAB$^+$, CCDA, CCDA$^+$ or KAR	+	52	16	<0.001
	−	60	63	

Data from eight influx samples, enriched in both PB and CCDB[c].

[a] PCR$^+$ = Enrichment giving positive PCR signal.
PCR$^-$ = Enrichment giving negative PCR signal.

[b] McNemars's test.

[c] For abbreviations see Table 2.

[d] + = Growth of *Campylobacter* on isolation media.
− = No growth of *Campylobacter* on isolation media.

enrichments by PCR and then comparing the MPN enumeration (Table 2). Statistical analysis did not indicate a significant difference in the MPN enumerations of PB and CCDB ($P > 0.05$).

In Table 4, the enrichments are categorized according to presence/absence determined by PCR and selective culturing on isolation media. Growth on only CAB^+, or at least CAB^+ or CCDA, or on at least one out of four media tested (CAB^+, CCDA, $CCDA^+$ or KAR) was taken into account. Evaluating growth on the four isolation media tested, agreement in presence/absence results was observed for 115 of the 191 enrichments, with 52 $PCR^+/growth^+$ and 63 $PCR^-/growth^-$ combinations. Discrepancies in presence/absence results were observed in 76 enrichments; 16 registering $PCR^-/growth^+$ and 60 $PCR^+/growth^-$. From McNemar's test, it can be concluded that when the enrichments are considered individually, the PCR culture assay has a significantly higher detection ability than conventional isolation on CAB^+ only ($P < 0.001$), or on CAB^+ or CCDA ($P < 0.001$) (Table 4). Isolation on at least one of the four isolation media tested had also a lower detection ability than PCR ($P < 0.001$) (Table 4). Since enrichment lasted 48 h, numbers of *Campylobacter* cells after 18 h incubation being below the detection limit of the PCR assay, which is 2.7 $\log_{10}$(CFU/ml) (Giesendorf *et al.*, 1992), can explain the discrepancy $PCR^-/growth^+$. The absence of growth

Table 5. Number of *Campylobacter*-positive enrichments as a function of the amount of enriched sewage.

Detection method	Volume of enriched sewage			
	1 ml	0.1 ml	0.01 ml	0.001 ml
Conventional enrichment[a]	9	25	26	4
PCR[b]	36	38	29	8
Total	45	63	55	12

Data from eight influx samples, enriched in both PB and CCDB[b].

[a] Enrichments confirmed for the presence of *Campylobacter* by PCR and by conventional isolation on at least one of the tested media CAB^+, CCDA, $CCDA^+$ or KAR.

[b] For abbreviations see Table 2.

on the isolation media together with a positive PCR signal can be due to a too large selectivity of the enrichment media or *Campylobacter* could be outcompeted in a later phase of the enrichment, as suggested by Rosef *et al.* (1987). Wegmüller *et al.* (1993) also reported 6 out of 54 dairy samples giving a positive PCR result whereas none of these samples were *Campylobacter* positive in the conventional culture procedure. Hazeleger *et al.* (1994) observed that nonculturable cells formed at 12°C can be determined by PCR with a similar detection limit as spirals (2×10^3 cells/PCR within 30 cycles). The detection limit of the PCR used in this investigation is even lower (13 cells/PCR within 40 cycles), so the presence of nonculturable forms in the enrichments, especially the low dilution enrichments, cannot completely be ruled out. The extent of possible interfering effects of these nonculturable forms cannot be quantified from these experiments.

In Table 5, the *Campylobacter*-positive enrichments, determined by PCR and by conventional isolation, are represented as function of the amount of enriched sample. In

Table 6. The results of the LAA performed on the influx en efflux enrichments, related to confirmed growth on the isolation media.

Sample type	*Campylobacter*-positive enrichments			*Campylobacter*-negative enrichments		
	n[a]	LAA^{+}[b] (%)	LAA^{-} (%)	n	LAA^{+} (%)	LAA^{-} (%)
All	133	39	61	199	12	88
Influx	58	33	67	111	9	91
Efflux	75	44	56	88	16	84
PB[c]	69	4	96	90	1	99
CCDB	64	77	23	109	21	79

[a] n = Number of analysed enrichments.

[b] LAA^{+} = Enrichment giving positive Latex Agglutination Assay result.
LAA^{-} = Enrichment giving negative Latex Agglutination Assay result.

[c] For abbreviations see Table 2.

this study, enrichment of 1 ml sewage in CCDB and PB, yielded eight and seven PCR positive samples, respectively. Conventional isolation after enrichment of 0.1 ml (diluted) sewage resulted in seven CCDB-enriched and six PB-enriched positive samples. For enrichment of 0.1 ml sewage, 1 ml of a 10^{-1} dilution of the original sample was added to the enrichment. *Campylobacter* cannot easily compete with the accompanying flora originating from 1 ml sewage during the isolation on selective plates and therefore, it is likely that a dilution step prior to enrichment reduces the influence of competing flora. For detection by conventional isolation, enrichment of 1 ml of 10^{-1} or 10^{-2} dilution of sewage is recommendable, PCR can detect *Campylobacter* quite well in enrichments of undiluted sewage or in 1 ml of 10^{-1} diluted sewage.

In order to determine the sensitivity of the LAA performed on enrichments after two days of incubation, the LAA results were compared with the results obtained by the conventional isolation procedure (Table 6). Both enrichments of influx and efflux samples were tested, a total of 332 tubes. Enrichments were considered *Campylobacter* positive when growth could be confirmed on at least one type of isolation medium. Non-specific agglutination reactions, which were excluded, were observed in an equal frequency (2.4%) in the two types of samples and in the two types of enrichment media. Presence of *Campylobacter* could be confirmed by isolation on selective plates for 133 enrichments and only 39% of the *Campylobacter* containing enrichments were recognized by LAA. Especially for PB enrichments, LAA had a low predictive value for *Campylobacter*-positive enrichments (4%). Twelve percent of enrichments tested had positive LAA's, while growth for *Campylobacter* could not be confirmed on isolation plates. The LAA reacts with *C. jejuni*, *C. coli*, *C. upsaliensis*, *C. lari*, and *C. fetus* subsp. *fetus*, so the low overall predictive value has to be explained by the observed high detection limit of the LAA (6 $\log_{10}$(CFU/ml)). Plate counting on CAB^{+} of several individual enrichments showed that the average counts of PB (4 $\log_{10}$(CFU/ml)) were lower than those of CCDB (5.7 $\log_{10}$(CFU/ml)). Reina and Muñoz (1993) applied the Microscreen LAA directly to faeces and reported a positive predictive value of only 25% and a high rate of false positive reactions.

In conclusion, this investigation did not indicate a significant difference in efficacy of the various enrichment and isolation media tested for the quantitative detection of *Campylobacter* in sewage samples. The Microscreen LAA is not suitable as a

rapid method for the confirmation of the presence of *Campylobacter* in sewage enrichments because of the low sensitivity. The PCR culture assay developed by Giesendorf *et al.* (1992) can be performed rapidly on the sewage enrichments and for individual enrichments, the assay has a higher detection ability than conventional isolation. For qualitative analysis, both the conventional culturing procedure and PCR assay are satisfactory. However within the conventional isolation procedure diluted sewage should be enriched and for a qualitative PCR analysis undiluted sewage can be applied. At present, a quantitative, unambiguous method with a high recovery rate, high selectivity and general applicability for the detection of *Campylobacter* in sewage is not available and the selectivity of the isolation media needs to be further improved.

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CHAPTER 3

SURVEY OF *CAMPYLOBACTER* IN SEWAGE PURIFICATION PLANTS IN THE NETHERLANDS

Food Microbiology[1] (1994) **11**, 65-73.

The prevalence of *Campylobacter* in two trickling filter systems and one activated sludge plant in the Netherlands was monitored. Also the drain water of poultry abattoirs, which are located in the drainage area of the investigated sewage purification plants, was analysed in order to reveal possible sources of *Campylobacter*. The numbers of *Campylobacter* isolated from the different plants could be related to the presence of poultry abattoirs in the drainage areas. There appeared to be a seasonal variation in the numbers observed in the sewage of the activated sludge system; during the end of the summer the numbers were in average 1 $\log_{10}$ unit smaller than during the rest of the year. This variation was not correlated to water temperature, oxygen pressure, pH, intensity of rainfall or of sunlight. Purification in an activated sludge tank reduced the *Campylobacter* counts by 1 $\log_{10}$ unit and in a trickling filter system the decline was 0.6 $\log_{10}$ unit. Since the elimination of *Campylobacter* is far from complete, disinfection of sewage purification plants effluxes should be considered in order to obstruct the environmental cycles of *Campylobacter*.

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INTRODUCTION

During the past decade *Campylobacter jejuni/coli* emerged as a major cause of human enteritis. Sentinel studies in the Netherlands revealed that campylobacteriosis accounts for approximately 12-15% of all cases of acute enteritis (Notermans and Van De Giessen, 1993). Improperly prepared poultry products and unchlorinated or inadequately chlorinated water have been shown to be important sources of infection (Anonymous, 1988; Millson *et al.*, 1991; Rautelin *et al.*, 1990). Many warm-blooded, healthy animals like poultry, pigs, and cattle, harbour this pathogen in their intestinal tract (Anonymous, 1988). People suffering from *Campylobacter* infection excrete the organism in large numbers, up to 10^6-10^8/g faeces (Blaser *et al.*, 1980). These patients and healthy animal carriers of *Campylobacter* provide a constant flow of this bacterium in the environment.

Campylobacter is a micro-aerophilic micro-organism which cannot proliferate in the environment but which can survive fairly well in aquatic environments at low temperatures (Stelzer *et al.*, 1991). Several studies in the United Kingdom, Germany and Italy showed that sewage is regularly heavily contaminated with this pathogen. *Campylobacter* can pass sewage purification plants, so the efflux could be a vehicle for the spread of this pathogen in the environment (Arimi *et al.*, 1988; Höller, 1988; Jacob *et al.*, 1991; Stampi *et al.*, 1992). To become better informed into the survival in aquatic environments, the prevalence of *Campylobacter* in sewage purification plants in the Netherlands was investigated. During two years, two trickling filters and one activated sludge system were studied. Also the drain water of two poultry abattoirs, which are located in the drainage area of the investigated sewage purification plants, was sampled in order to trace the source of *Campylobacter*. This investigation was conducted to study in more detail the occurrence of environmental cycles of *Campylobacter* and their consequences in maintaining infection routes.

MATERIALS AND METHODS

Sample collection

Samples of the influx and the efflux were collected in sterile bottles (30 ml) at

three different municipal sewage purification plants (**spA**, **spB** and **spC**), which are described in Table 1. The municipal plants and poultry abattoirs are all located in the region "De Veluwe", province Gelderland in the Netherlands. Additional sample material is also indicated in Table 1. Furthermore, drain water of a poultry abattoir (**dwA**) in the drainage area of sewage purification plant **spA**, and of a poultry abattoir (**dwC**), located in the drainage area of sewage purification plant **spC** were sampled (Table 1). The drain water of poultry abattoir **dwC** was purified in an activated sludge system and its efflux was also sampled. The samples were transferred to the laboratory in a cold storage container with ice and analysed for *Campylobacter* within two hours of collection. Meteorological data, as well as data concerning the water temperature, the oxygen pressure and the pH of sewage of plant **spA** were available.

Detection of *Campylobacter* in the water samples

The numbers of *Campylobacter* organisms in the water samples were analysed by

Table 1. Survey of the investigated sewage purification plants and poultry abattoirs.

	Sewage purification plants		
	spA	**spB**	**spC**
Purification system	Activated sludge	Trickling filter	Trickling filter
Capacity in citizen equivalents	46,000	130,000	280,000
Sample materials	Influx, efflux, surplus sludge, sedimented undigested sludge, surface water	Influx, efflux, digested sludge, undigested sludge, surface water	Influx, efflux, digested sludge, undigested sludge, surface water
Poultry abattoir	**dwA**	None	**dwC**
Purification system of poultry abattoir	None		Activated sludge
Sample materials of poultry abattoir	Drain water		Drain water abattoir, efflux activated sludge

the most probable number technique (De Man, 1975), using triplicates (one ml sample in nine ml enrichment broth, up to 10^{-3} dilution). All incubations were under micro-aerobic conditions (anaerobic jar; Don Whitley Scientific Limited, West Yorkshire, UK, flushed with 5% O_2, 10% CO_2 and 85% N_2). Charcoal cefoperazone deoxycholate broth was used as enrichment medium according to Bolton *et al.* (1984). After pre-enrichment for 4 h at 37°C (Humphrey, 1989); the antibiotics cefoperazone and amphotericin-B (SR155E, Oxoid, Basingstoke, UK; 32 and 10 mg/l medium, respectively) were added to the tubes, which were further incubated at 42°C. After 2 days of incubation, a loop from each tube was streaked on Columbia agar base plates (CM331, Oxoid) with the same antibiotics in equal concentrations as mentioned above (CAB^+). These CAB^+ plates were incubated at 42°C for 2 days. Suspected *Campylobacter* colonies were identified by checking microscopic appearance (curved or spiral cells with rapid corkscrew-like motility). One suspected colony per CAB^+ plate was purified by subculturing (42°C) on Columbia agar base plates, without antibiotics (CAB). Both CAB and CAB^+ plates contained 5% (v/v) difibrinated, lysed horse blood. Confirmation was based on microscopic appearance, possibility of growth under micro-aerobic conditions in brain heart infusion broth (0037-01-6, Difco Laboratories, Detroit, USA) at 42°C (+) and 25°C (—), oxidase activity (+) and catalase activity (+). Plates positive for confirmed *Campylobacter* were used in calculating the $\log_{10}$ of the most probable number (MPN) of *Campylobacter* per 100 ml ($\log_{10}$(MPN/100 ml)).

RESULTS

Occurrence of *Campylobacter* in sewage purification plants

It was observed that the pre-enrichment procedure (4 h, 37°C, non-selective) increased the isolation rate of *Campylobacter* (data not shown). In preliminary experiments a number of samples were analysed by this enrichment procedure, including a pre-enrichment, in triplicate and the minimum and maximum value of these analyses differed 0.3 $\log_{10}$ units.

Occurrence of *Campylobacter* in the activated sludge system during April 1991 -

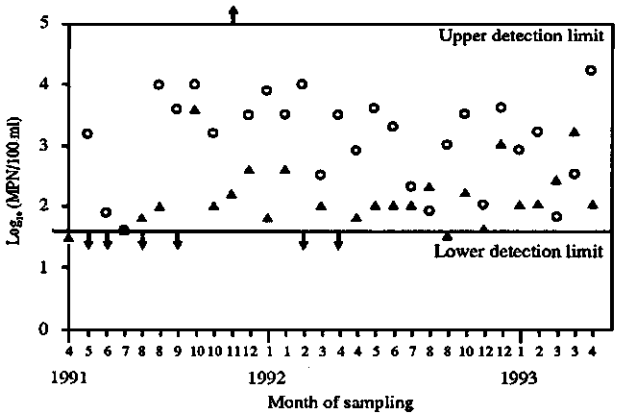

Fig. 1. Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml)) in activated sludge system **spA**, during April 1991 - April 1993. On the horizontal axis, the date of sampling is represented by the number of the month. ○ and ▲ represent an influx and an efflux sample, respectively. The following markers represent samples containing numbers below or above the detection limit: ↓ an influx sample, ↓ an efflux sample, ⇩ an influx as well as an efflux sample.

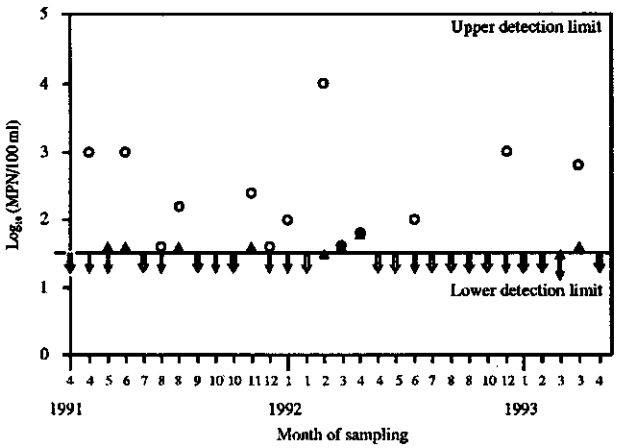

Fig. 2. Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml)) in trickling filter **spB**, during April 1991 - April 1993. On the horizontal axis, the date of sampling is represented by the number of the month. For symbols see Fig. 1.

April 1993 is illustrated in Fig. 1. The data of the trickling filters **spB** (April 1991 - April 1993) and **spC** (February 1992 - April 1993) are shown in Fig. 2 and Fig. 3, respectively. *Campylobacter* could be recovered from the sewage of all three locations. However, the influx of the activated sludge plant **spA** was more contaminated with *Campylobacter* (average $\log_{10}$(MPN/100 ml) = 3) than the influx of plant **spB** (average $\log_{10}$(MPN/100 ml) = 2.3) or plant **spC** (average $\log_{10}$(MPN/100 ml) = 2.6). The numbers in the influx of plant **spA** showed a seasonal variation. In average 1.9 $\log_{10}$(MPN/100 ml) were found in the sewage in the period June - July 1991 and July - August 1992. In the remaining periods 3.2 $\log_{10}$(MPN/100 ml) were found. The observation of the lower numbers was based on only four samples, but the decline in this period was observed both in 1991 and in 1992.

In all three locations the purification processes resulted in a reduction of the numbers of *Campylobacter*. In plant **spA**, using an activated sludge system, the numbers

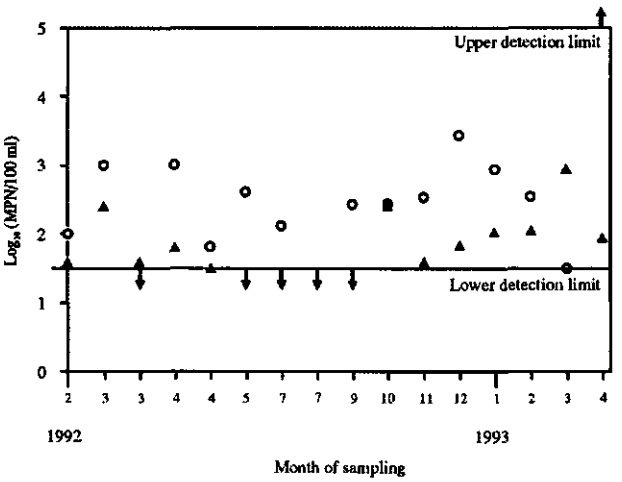

Fig. 3. Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml)) in trickling filter **spC**, during February 1992 - April 1993. On the horizontal axis, the date of sampling is represented by the number of the month. For symbols see Fig. 1.

of *Campylobacter* were on average reduced by 1 $\log_{10}$ unit. The treatment in the trickling filter of plant **spC** decreased the numbers of *Campylobacter* with 0.6 $\log_{10}$ units. The numbers of *Campylobacter* in the efflux of plant **spB** were close to or below the detection limit (< 1.5 $\log_{10}$(MPN/100 ml)). During the period May 1992 - September 1992 the numbers of *Campylobacter* in the efflux of plant **spC** were also below the detection limit. During this period of the year the efflux of this plant was chlorinated (15 min before discharge, 0.7 - 1.7 mg/l).

Campylobacter in digested and undigested sludge

The numbers of *Campylobacter* isolated from the surplus and the sedimented sludge of plant **spA** averaged 2.4 $\log_{10}$(MPN/100 ml). Of the 30 samples of both surplus sludge and sedimented sludge of plant **spA**, 88% were positive. In the surplus sludge, the

Table 2. Numbers of *Campylobacter* in the drain water of poultry abattoir **dwA**, during August 1992 - April 1993.

Date of sampling	Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml))
1992	
August 5	5.0
August 26	2.1
September 16	> 6.0
October 21	2.6
November 11	6.7
1993	
January 27	> 6.0
February 17	6.0
March 10	6.4
March 31	> 7.0
April 21	6.7

numbers ranged from 1.5 to 4.8 $\log_{10}$(MPN/100 ml) and for the sedimented sludge the range was 1.5 to 4.4 $\log_{10}$(MPN/100 ml). *Campylobacter* could not be isolated from the

Table 3. Numbers of *Campylobacter* in the drain water of poultry abattoir **dwC** and in the efflux of its activated sludge system, during December 1991 - April 1993.

Date of sampling	Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml))	
	Drain water poultry abattoir	Efflux activated sludge
1991		
December 12	> 5.0	3.7
1992		
January 15	> 5.0	3.7
March 4	3.7	3.7
March 18	4.2	3.3
April 8	3.5	1.8
April 29	2.8	2.2
May 13	3.5	1.6
July 1	3.1	1.6
July 21	2.2	2.2
September 2	3.8	2.2
October 14	> 4.0	2.0
November 4	3.2	3.0
December 2	5.4	6.0
1993		
January 20	2.8	2.5
February 10	2.5	1.6
March 17	4.0	2.0
April 14	6.0	4.0

digested and undigested sludge of either plant **spB** or **spC** (five samples each).

***Campylobacter* in surface waters near the discharge points of the sewage purification plants**

The numbers of *Campylobacter* in 13 samples of surface water near the discharge point of plant **spA** averaged 2.4 and ranged from 1.5 to 4.2 $\log_{10}$(MPN/100 ml). From five out of eleven samples taken near the discharge point of plant **spB**, *Campylobacter* was isolated in average numbers of 1.6 $\log_{10}$(MPN/100 ml). Also seven out of twelve samples from the surface water of plant **spC** were *Campylobacter* positive. The mean value was 2.1 $\log_{10}$(MPN/100 ml) and ranged from 1.6 to 2.4 $\log_{10}$(MPN/100 ml).

***Campylobacter* in effluxes of poultry abattoirs**

Table 2 illustrates the numbers of *Campylobacter* isolated from the drain water of poultry abattoir **dwA** in the drainage area of plant **spA**. The numbers of *Campylobacter* isolated from the drain water of abattoir **dwC** and its activated sludge efflux are summarized in Table 3. The numbers in the drain water of **dwA** varied enormously; from 2.1 to $>$ 7 $\log_{10}$(MPN/100 ml) and the log-average ($>$ 5.3 $\log_{10}$(MPN/100 ml)) was higher than in the drain water of poultry abattoir **dwC** ($>$ 3.8 $\log_{10}$(MPN/100 ml)). Although, the purification treatment resulted in a decrease of 1 $\log_{10}$ unit, the efflux of the activated sludge system at **dwC** still contained *Campylobacter* (average 2.7 $\log_{10}$(MPN/100 ml)), however in lower numbers than present in the drain water of abattoir **dwA**.

Meteorological data

The meteorological and physical data concerning sewage purification plant **spA** are represented in Table 4. The temperature was measured in the sewage at the moment of sampling and at plant **spA** the oxygen pressure and the pH were measured continuously. Rainfall was determined at the sewage purification plant **spA**. Data on sunlight were provided by the Department of Meteorology of the Wageningen Agricultural University. Sunlight intensity was determined in the spectral range 305-2800 nm by a Solarimeter (CM11 Pyranonmeter, Kipp en Zonen, Delft, The Netherlands). In all three sewage purification plants the sewage temperature varied between 10 and 20°C. In plant **spA**, the pH of the sewage was constantly approximately 7.3 and the oxygen pressure

Table 4. Meteorological and physical data, concerning sewage purification plant spA.

Date of sampling	t (°C)	pO_2 (%)	pH (-)	Rainfall as weekly average (mm)	Intensity sunlight as weekly average (J/cm^2)
1991					
April 24	—[a]	—	—	2.5	1201
May 22	—	—	—	0.0	1472
June 19	—	—	—	8.6	1449
July 10	19.3	18.0	8.0	1.7	2020
August 14	19.8	47.5	7.4	0.9	1502
August 28	20.1	64.0	6.4	0.0	1739
September 18	19.5	48.8	7.6	3.1	899
October 2	16.2	38.2	9.2	3.6	822
October 23	15.6	24.0	7.4	0.3	530
November 13	11.8	66.2	7.0	6.0	300
December 4	12.2	32.8	7.6	0.0	171
1992					
January 8	11.8	45.0	7.4	5.3	137
January 29	10.9	56.9	7.4	0.1	320
February 19	10.7	65.4	7.4	0.7	516
March 11	12.1	44.4	7.3	8.3	595
April 1	11.8	48.7	7.2	3.3	1000
April 22	13.4	45.8	7.4	0.8	1382
May 20	17.4	17.0	7.1	0.0	2607
June 24	19.3	17.0	7.4	0.0	1841
July 15	18.9	22.2	7.1	5.1	1796
August 5	19.9	63.7	7.3	0.2	1420
August 26	20.1	19.3	7.2	4.6	1131
October 21	15.2	24.9	7.4	2.4	1075
December 9	12.0	57.7	7.0	5.6	142
1993					
January 27	10.5	42.5	6.6	2.3	175
March 10	10.7	66.7	7.6	0.1	1052
March 31	12.4	50.6	7.6	0.2	1023
April 21	15.4	36.8	6.5	3.1	1337

[a] — = Not determined.

varied widely.

DISCUSSION

A clear distinction is observed in the average numbers of *Campylobacter* isolated from the sewage influxes of the different plants. Sewage from plant **spB** gave the lowest isolation rate. This observation could be related to the absence of certain industrial activities, like poultry- and swine-processing plants. Presence of *Campylobacter* in the sewage of plant **spB** may be related only to patients suffering from campylobacteriosis. Sentinel studies carried out in the Netherlands, have revealed that 2-3% of the Dutch population suffers annually from *Campylobacter* enteritis (Notermans and Hoogenboom-Verdegaal, 1992). The numbers of *Campylobacter* in the sewage of plants **spA** and **spC** were higher than those of plant **spB**, and are probably related to the presence of poultry abattoirs in both drainage areas. The average *Campylobacter* counts in the sewage of plants **spA** and **spC** were similar to the numbers reported earlier by Höller (1988) and Jacob *et al.* (1991). In both studies poultry abattoirs were also located in the drainage areas of the investigated sewage purification plants. This resemblance strengthens the suspicion that the high average *Campylobacter* concentrations in the sewage of **spA** and **spC** are indeed related to the presence of poultry abattoirs in the drainage areas.

For plant **spA**, there appears to be a seasonal variation in the numbers of *Campylobacter* isolated from the sewage influx. For all the flows of plants **spB** and **spC**, and the other flows of plant **spA**, as well as for the sampled flows of the two abattoirs **dwA** and **dwC**, a seasonal variation could not be observed. Many investigators observed a reversed seasonal variation in the numbers of *Campylobacter* in sewage, with a peak during the summer months (Höller, 1988; Jones *et al.*, 1990; Stampi *et al.*, 1992).

The purification processes in both the activated sludge system of sewage purification plant **spA** and of the abattoir **dwC** reduced the numbers of *Campylobacter* by 1 $\log_{10}$ unit. The trickling filter system is less effective in eliminating *Campylobacter* (0.6 $\log_{10}$ unit reduction) than the activated sludge system. However, the observed reduction in the trickling filter of plant **spC** is far below the 99.9% abatement which was observed by Arimi *et al.* (1988) in a similar system. The decline in the activated sludge

system was similar to the values reported by Betaieb and Jones (1990), Höller (1988) and Jacob *et al.* (1991), but is lower than the decrease reported by Stampi *et al.* (1992).

The prevalence of *Campylobacter* in the sewage of plant **spA** was not correlated with the sewage temperature, sunlight or rainfall intensity. Furthermore, there was no correlation between the reduction of this pathogen and either pH or pO_2 of the sewage (**spA**). These results are in contrast with observations of some other authors. Gondrosen (1986) observed that the survival of this pathogen in aquatic environments was influenced by temperature. Jones and Telford (1991) presumed a lethal effect of sunlight. However, in our case a lethal effect of sunlight is questionable, because of the rather high turbidity of sewage.

The purification in the investigated systems is based on aeration and *Campylobacter* is sensitive to oxygen (Rollins and Colwell, 1986). In both the sewage purification systems and in the sewers *Campylobacter* had already been exposed to oxygen, but this effect is difficult to quantify. From the observed numbers in the surplus sludge and sedimented sludge of plant **spA**, it can be concluded that the reduction by purification could also be explained by attachment of cells to particles, as reported by Arimi *et al.* (1988) and by Höller and Schomakers-Revaka (1994). In summary, the reduction of *Campylobacter* in sewage purification plants may be a result of several factors.

Despite purification of sewage, *Campylobacter* is still detectable in the effluxes of plants **spA** and **spC**. Furthermore, *Campylobacter* could also be isolated from the surface waters near the discharge points of plants **spA**, **spB**, and **spC**. The prevalence of *Campylobacter* in the surface waters of all three locations was not correlated with the season of sampling, which is in contrast with the results of Brennhovd *et al.* (1992), who observed the highest isolation rate in spring. The surface water near plant **spA** contained the highest numbers of *Campylobacter* and the presence of this pathogen in surface water is probably related to its presence in the sewage and in the efflux of the plant. The prevalence of *Campylobacter* in the surface waters near plants **spB** and **spC** could be explained by occasional contamination from the efflux and/or the presence of water-fowl (Kapperud and Rosef, 1983; Luechtefeld *et al.*, 1980). Because some of the sewage purification plants have discharge points located in the neighbourhood of recreation areas, the low minimal infectious dose of *Campylobacter* (Black *et al.*, 1988) should be kept in mind.

Campylobacter could be isolated in equal numbers from the sedimented sludge and from the surplus sludge of plant **spA**. The sedimented, undigested sludge was applied to arable land, making contamination of horticultural products possible as suggested by Park and Sanders (1992). The application of untreated (surface) water for the irrigation of farm lands is another way of direct contamination of agricultural products.

In spite of the reduction in the various sewage purification plants, efflux and raw sludge could play a role in maintaining the contamination cycles of *Campylobacter*. Uptake of surface water near a sewage purification plant could directly infect humans, water-fowl and/or farm animals like poultry and cattle. Disinfection of the effluxes of sewage purification plants and/or the drain waters of poultry abattoirs should be considered in order to obstruct the contamination cycle of *Campylobacter*. Furthermore, it is important to be aware of the possible presence of viable but nonculturable *Campylobacter* cells in water (This thesis, Chapter 7; Rollins and Colwell, 1986), even though the infectivity of these coccoid forms is still a matter of debate (Beumer *et al.*, 1992; Medema *et al.*, 1992).

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CHAPTER 4

A SHORT-TERM EVIDENCE OF *CAMPYLOBACTER* IN A SEWAGE PURIFICATION PLANT AND IN THE DRAIN WATER OF AN ASSOCIATED POULTRY ABATTOIR

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In order to study the prevalence of *Campylobacter* in a sewage purification plant, the numbers of *Campylobacter* in the sewage and the efflux of an activated sludge system and in the drain water of a poultry abattoir, connected with this plant were determined in three 24 h periods. In order to study transmission routes, the *Campylobacter* isolates were identified by Penner serotyping and by antimicrobial susceptibility testing.

The poultry abattoir drain water was considerably contaminated with *Campylobacter* (average > 5.1 $\log_{10}$(MPN/100 ml)). The numbers of *Campylobacter* present in the sewage and the efflux of the activated sludge system did not increase significantly during the sampling period. The purification resulted in a reduction of 1 $\log_{10}$ unit, but *Campylobacter* was still detectable in the activated sludge efflux.

Patterns in the antimicrobial susceptibility and serotypes did not match for the various types of samples, and therefore, no transmission route could be deduced. This incongruity of patterns may be due to growth competition during the enrichment. Therefore, enrichment procedures are not recommendable for studies on transmission routes.

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INTRODUCTION

Campylobacter jejuni/coli is a major cause of human enteritis in the Netherlands. Illness is closely related to the consumption of improperly prepared poultry products (Anonymous, 1988), but also several water-borne campylobacteriosis outbreaks were reported in the last decade (Table 3, Chapter 1). The natural habitat of *Campylobacter* is the intestines of warm-blooded animals such as poultry, water-fowl, and pigs. Until now, the contamination cycle of *Campylobacter* is not completely revealed and for that reason the behaviour of this pathogen in the environment has to be investigated thoroughly. Recent studies in the United Kingdom, Germany, Italy and the Netherlands have shown a high contamination level of sewage with *Campylobacter* (Arimi *et al.*, 1988; Höller, 1988; Jones *et al.*, 1990; Koenraad *et al.*, 1994; Stampi *et al.*, 1992). A Dutch study (Koenraad *et al.*, 1994) showed that the numbers of *Campylobacter* in sewage were related to the presence of poultry abattoirs in the drainage area of the sewage purification plant. During the period April 1991 - April 1993, the prevalence of *Campylobacter* in sewage was studied by monitoring the numbers of *Campylobacter* in an activated sludge plant and in a trickling filter. The sewage of the activated sludge system was more contaminated with *Campylobacter* (average 3.0 $\log_{10}$(MPN/100 ml)) than the sewage of the trickling filter system (average 2.3 $\log_{10}$(MPN/100 ml)). The drainage area of the activated sludge plant included a poultry abattoir, while the trickling filter system did not receive drain water from meat-processing industries. The drain water of the poultry abattoir, connected with the activated sludge system, was also sampled and high numbers were determined (average 5.3 $\log_{10}$(MPN/100 ml)). These results are in agreement with observations of Höller (1988) and Jones *et al.* (1990).

In order to study the prevalence of *Campylobacter* in a sewage purification plant in more detail, sewage and efflux of an activated sludge system and drain water of a poultry abattoir associated with this system, were investigated thoroughly during 24 h periods. In addition, *Campylobacter* contamination of the broiler flocks, which were slaughtered in the abattoir during the time interval of water sampling, was established. The isolates were characterized by antimicrobial susceptibility testing in order to verify the observation of Endtz *et al.* (1991) of a rising resistance against fluoroquinolones among *Campylobacter* isolates from man and poultry. Furthermore, Penner serotyping of *Campylobacter* isolates was used to study transmission routes.

MATERIALS AND METHODS

Sample collection

The surveillance was carried out in three experiments, on Wednesday 16th September 1992, Thursday 12th November 1992, and on Friday 8th January 1993. Samples were collected from caeca of processed broilers and poultry abattoir drain water. Furthermore, sewage and efflux of the municipal sewage purification plant, whose drainage area includes the poultry abattoir were sampled.

The poultry abattoir has a capacity of 6,000 chickens/h and the slaughtering lasts from 6:00 h until 16:00 h. During the whole slaughtering period the processing water passes through a floatation overflow tank, in where the fat particles are collected. Subsequently, the water is drained on the sewerage. Samples of poultry abattoir drain water were collected after passage of the floatation tank. After the slaughtering the factory is cleaned and the cleaning water is also drained on the sewerage. Every Saturday, the floatation overflow tank is emptied by draining the remaining water on the sewerage and by shovelling the fat layer away.

The sewage purification plant consists of an activated sludge system with a capacity of 60,000 citizen equivalents and is located at a distance of approximately 4 km from the poultry abattoir. Data concerning the water temperature, the oxygen pressure and the pH of the sewage were available at the plant and the temperature of the poultry abattoir drain water was determined at the moment of sampling. The transport of the poultry abattoir drain water to the activated sludge system takes approximately 1.5 h. The sewage purification plant receives sewage from 30,000 inhabitants and from various small industries, but no meat-processing industries, other than the poultry abattoir.

Campylobacter in caecal material

During the slaughtering period, broilers from each transport truck were sampled by collecting 25 caeca per truck. Ten caeca were collected from the following trucks when broilers were from the same farmer. The caeca were analysed on the presence of *Campylobacter* by streaking material on modified charcoal cefoperazone deoxycholate agar - *Campylobacter* blood-free selective plates (CM739, Oxoid, Basingstoke, UK) with 32 mg/l cefoperazone and 10 mg/l amphotericin-B (SR155E, Oxoid) (Jacobs-Reitsma *et*

al., 1994a). After micro-aerobic (BBL 71045, Campy Gaspak, Cokeysville, USA) incubation for 2 days at 37°C, the plates were examined for typical colony shape, and suspected *Campylobacter* colonies were microscopically checked for spiral-shaped cells with rapid motility. Pure *Campylobacter* isolates were confirmed by latex agglutination (M46, Microscreen, Mercia Diagnostics Limited, Guildford, UK) and used in serotyping and antimicrobial susceptibility testing.

***Campylobacter* in water samples**

The number of *Campylobacter* in the water samples were estimated by the most probable number technique (De Man, 1975) in triplicate (1 ml sample in 9 ml enrichment broth) using the charcoal cefoperazone deoxycholate broth (CCDB) (Bolton *et al.*, 1984) and the selective agents cefoperazone and amphotericin-B (SR155E, Oxoid) (Koenraad *et al.*, 1994) in concentrations as described above. Pure *Campylobacter* isolates were confirmed by using a latex agglutination assay (M46, Microscreen) and used in serotyping and antimicrobial susceptibility testing. According to plates on which *Campylobacter* colonies could be confirmed, the *Campylobacter* numbers ($\log_{10}$(MPN/100 ml)) were calculated from the MPN table.

Antimicrobial susceptibility

Antimicrobial susceptibility was determined using the disk diffusion method described by Bauer *et al.* (1970), as recommended by the National Committee Clinical Laboratory Standards, Subcommittee on Antimicrobial Susceptibility Testing. The applied method was adjusted to the characteristics of *Campylobacter* by Jacobs-Reitsma *et al.* (1994b). Susceptibility to the following agents was tested: cephalothin (KF, Oxoid, 30 μg), nalidixic acid (NA, Oxoid, 30 μg), flumequine (UB, Oxoid, 30 μg), ciprofloxacin (CIP, Oxoid, 5 μg), enrofloxacin (ENRO, Bayer Diagnostics, München, Germany, 5 μg), ampicillin (AMP, Oxoid, 25 μg), tetracycline (TE, Oxoid, 30 μg), and erythromycin (E, Oxoid, 15 μg). Isolates were considered resistant when the inhibition zone (including disk diameter of 6 mm) was $\leq$ 15 mm. For enrofloxacin this criterion was $\leq$ 17 mm, according to Bayer's directions. The significance of differences between the frequency of resistance was determined by using the two-tailed chi-square test. Differences between groups were considered to be significant at $P \leq 0.05$.

Serotyping

Campylobacter isolates were serotyped by using the method described by Penner and Hennessy (1980) and Jacobs-Reitsma *et al.* (1995). For this serotyping method, which is based on heat stable antigens, 65 sera were available.

Growth experiment with two *Campylobacter* strains with different serotypes

Strain 54 (WAU080193, O11, isolated from efflux of sewage purification plant) and strain 2648 (Spelderholt080193, O29, isolated from poultry caecum) were grown in brain heart infusion broth (0037-01-6, Difco Laboratories, Detroit, USA) at 37°C under micro-aerobic conditions (anaerobic jar, Don Whitley Scientific Limited, West Yorkshire, UK, flushed with 5% O_2, 10% CO_2 and 85% N_2). After 2 days incubation, the cultures were counted on Columbia agar base plates (CM331, Oxoid), containing 5% (v/v) defibrinated, lysed horse blood, cefoperazone, and amphotericin-B (SR155E, Oxoid, 32 and 10 mg/l medium, respectively) (CAB^+), mixed in 1:100 ratio. Of this mixed suspension 0.1 ml 10^{-6} dilution were inoculated into one single tube with 9 ml CCDB (in triplicate) and the CCDB tube was put through the normal MPN procedure, as described before for the water samples. After 2 days incubation, the CCDB suspension was counted on CAB^+ plates (colony forming units (CFU)/ml) and $\sqrt{n}$ colonies of the 10^{-6} plate were serotyped as described before.

RESULTS

***Campylobacter* in caecal and in water samples**

During the three sampling periods on Wednesday 16th September 1992, Thursday 12th November 1992, and Friday 8th January 1993, it appeared that the *Campylobacter* carrier rate in the broilers was mainly up to 80-100%, while some farmers delivered *Campylobacter*-free flocks or flocks with a fairly low carrier state (4-20%) (Tables 1, 2, and 3).

The numbers of *Campylobacter* in the poultry abattoir drain water and in the sewage and the efflux of the activated sludge system during the three sampling days,

which are represented as $\log_{10}$(MPN/100 ml) in Tables 1, 2, and 3 show similar trends. Already at the beginning of the slaughtering the poultry abattoir drain water was conside-

Table 1. Results from *Campylobacter* analyses of the processed broilers, the poultry abattoir drain water, and the sewage of the purification plant sampled on 16th September 1992.

Time of sampling	Percentage positive caecal samples (n)	Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml))	
		Poultry abattoir drain water	Sewage
16th September 1992			
6:20 h	—[a]	6.0	—
6:40 h	100% (25)[b]	>6.0	—
7:00 h	—	4.5	1.8
7:30 h	100% (25)	6.0	2.0
8:00 h	—	>6.0	2.0
8:30 h	100% (10)	—	—
9:00 h	100% (10)	4.7	1.9
9:25 h	100% (10)	—	—
10:00 h	100% (25)	5.7	—
10:30 h	60% (10)	—	—
11:00 h	—	—	1.9
11:20 h	100% (10)	—	—
12:00 h	100% (25)	3.5	—
12:45 h	100% (10)	—	—
13:30 h	80% (25)	—	—
13:45 h	—	6.0	—
14:00 h	—	—	1.5

[a] — = Not determined.

[b] (..) = Total number of analysed caecal samples in parentheses.

rably contaminated with *Campylobacter* (average > 5.8 $\log_{10}$(MPN/100 ml)). High numbers of *Campylobacter* were detectable in the poultry abattoir drain water during the whole day (average 5.1 $\log_{10}$(MPN/100 ml)). The lower carrier state of a few flocks did

Table 2. Results from *Campylobacter* analyses of the processed broilers, the poultry abattoir drain water, and the sewage of the purification plant sampled on 10th and 11th November 1992.

Time of sampling	Percentage positive caecal samples (n)	Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml))	
		Poultry abattoir drain water	Sewage
10th November 1992			
22:00 h	—[a]	6.0	—
11th November 1992			
6:00 h	—	>6.0	—
6:15 h	100% (25)[b]	—	—
6:45 h	100% (10)	—	—
7:15 h	90% (10)	—	—
8:00 h	100% (25)	4.5	1.8
9:30 h	100% (10)	—	—
10:00 h	100% (25)	6.0	2.0
10:50 h	100% (10)	—	—
12:00 h	—	>6.0	2.0
12:15 h	4% (25)	—	—
12:55 h	20% (10)	—	—
13:10 h	100% (25)	—	—
14:00 h	—	4.7	1.9
20:30 h	—	5.7	—

[a] — = Not determined.

[b] (..) = Total number of analysed caecal samples in parentheses.

not perceptibly influence the contamination level of the poultry abattoir drain water. Also the poultry abattoir drain water, which was drained during the evening and which consisted of cleaning water, was considerably contaminated (average 5.0 $\log_{10}$(MPN/100 ml). In a separate experiment on Sunday, 28th March 1993, the residue left behind in the fat floatation tank after emptying was sampled and analysed. This residue contained 5.7 $\log_{10}$(MPN/100 ml) of *Campylobacter*.

Table 3. Results from *Campylobacter* analyses of the processed broilers, the poultry abattoir drain water, and the sewage of the purification plant sampled on 7th and 8th January 1993.

Time of sampling	Percentage positive caecal samples (n)	Numbers of *Campylobacter* ($\log_{10}$(MPN/100 ml))	
		Poultry abattoir drain water	Sewage
7th January 1993			
22:00 h	—[a]	6.0	2
8th January 1993			
6:00 h	100% (25)[b]	5.3	2.8
7:00 h	100% (10)	—	—
8:00 h	90% (25)	4.3	1.0
9:00 h	80% (10)	—	—
10:00 h	—	4.6	2.0
10:30 h	0% (25)	—	—
12:00 h	0% (25)	4.2	<1.5
12:30 h	100% (25)	—	—
13:00 h	0% (25)	—	—
14:00 h	0% (25)	4.9	<1.5
22:00 h	—	2.1	—

[a] — = Not determined.

[b] (..) = Total number of analysed caecal samples in parentheses.

During the three sampling periods the *Campylobacter* numbers in the sewage and activated sludge efflux did not vary significantly during daytime and ranged within 1.5 - 2.8 $\log_{10}$(MPN/100 ml). The purification process resulted in a reduction of 1 $\log_{10}$ unit, however *Campylobacter* was still detectable in some efflux samples. The average pH, the oxygen pressure and the temperature in the activated sludge system and the temperature of the poultry abattoir drain water during the three sampling periods (Wednesday 16th September 1992, Thursday 12th November 1992, and Friday 8th January 1993) were similar (Table 4).

In order to determine the *Campylobacter* contamination level of the sewage when the abattoir was closed, the activated sludge sewage was sampled on several Sundays (6th December 1992 and 28th March 1993) and Monday mornings (22th December 1992 and 29th March 1993) and analysed for the numbers of *Campylobacter*. The average of the Sunday samples was 2 $\log_{10}$(MPN/100 ml) and of the Monday morning samples 1.5 $\log_{10}$(MPN/100 ml).

Antimicrobial susceptibility

Cephalothin resistance is a characteristic of *C. jejuni* subsp. *jejuni* and *C. coli* (Goossens and Butzler, 1992) and this antibiotic resistance was used as a control. Indeed, all the tested strains isolated from the caeca, the drain water of the poultry abattoir, and

Table 4. The pH, oxygen pressure, and temperature of the sewage and the poultry abattoir drain water.

Date	Sewage			Poultry abattoir drain water
	pH (-)	pO_2 (%)	t (°C)	t (°C)
16th Sept 1992	7.5	29.0	19	19
11th Nov 1992	7.4	37.8	13	20
8th Jan 1993	—[a]	22.6	10.1	17.3

[a] — = Not determined.

the sewage and activated sludge efflux, were resistant to cephalothin. The susceptibility results for the other selective agents tested on the isolates obtained on the three sampling days are summarized in Table 5. Ampicillin resistance was significantly higher among poultry abattoir drain water isolates than among caecal isolates, but the resistance was similar to those of the activated sludge isolates. The isolates obtained from the purification plant were more resistant to tetracycline and erythromycin than the isolates from the poultry abattoir drain water and the caeca ($P < 0.025$). Almost complete cross-resistance was found between the (fluoro)quinolones (Endtz *et al.*, 1991; Jacobs-Reitsma *et al.*, 1994b). Fifty-seven out of 227 (25%) isolates showed resistance to the four quinolones tested. Quinolone-resistant strains were significantly less frequently found in caecal samples than in poultry abattoir drain water ($P < 0.01$) and purification plant samples ($P < 0.001$).

Table 5. Antimicrobial susceptibility of *Campylobacter* isolates obtained on 16th September 1992, 11th November 1992, and 8th January 1993.

Antimicrobial agent	Resistant isolates from caeca (n=121)		Resistant isolates from poultry abattoir drain water (n=61)		Resistant isolates from purification plant[a] (n=45)	
	Number	%	Number	%	Number	%
Ampicillin	18	15	26	43	11	24
Tetracycline	22	19	8	13	15	33
Erythromycin	5	4	1	2	6	13
Quinolones[b]	18	15	18	30	21	47

[a] Including isolates from sewage and activated sludge efflux.

[b] The quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin were considered as one group.

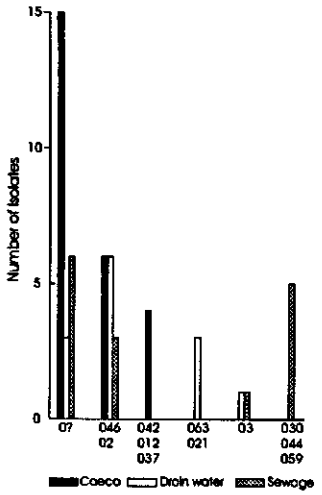

Fig. 1. Serotyping results of *Campylobacter* isolates obtained on 10th and 11th November 1992.

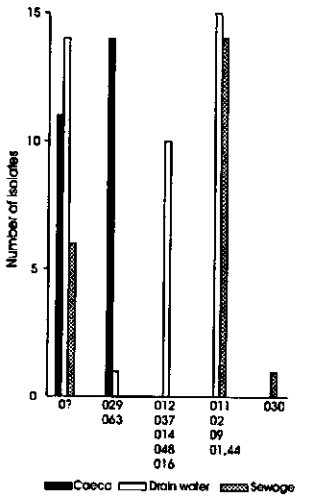

Fig. 2. Serotyping results of *Campylobacter* isolates obtained on 7th and 8th January 1993.

Serotypes

The isolates from the caeca, the abattoir drain water, and the sewage and the activated sludge efflux of 11th November 1992 and 8th January 1993 were serotyped and the results are summarized in Figs. 1 and 2, respectively. Nontypable strains were present in all types of samples. The broilers processed on 11th November 1992 mainly harboured the serotypes O46, O2, O42, O12, and O37 (Fig. 1). Only O46 and O2 were also observed in the poultry abattoir drain water and purification plant samples. Moreover, the poultry abattoir drain water contained also O63 and O21, the types O30, O1,44 and O59 could be only isolated from the purification plant. The broilers processed on 8th January 1993 harboured, besides many nontypable isolates, predominantly O29 and O63 (Fig. 2). From the poultry abattoir drain water, the serotypes O2, O11, O37, and O1,44 could be isolated frequently, while O29 was found only once and O63 was not observed. The serotypes O2, O1,44, and O11 were isolated from both sewage and the activated sludge efflux sampled on 8th January 1993.

Mixed growth experiment with two *Campylobacter* strains with different serotypes

The growth experiment with *Campylobacter* strains 54 (O11) isolated from sewage and 2648 (O29) isolated from poultry caecum in mixed cultures was performed in triplicate. The BHI cultures of 54 (O11) and 2648 (O29) contained 8.5 and 8.1 $\log_{10}$(CFU)/ml), respectively. These BHI cultures were mixed in a ratio of 1:100 volume units, respectively and subsequently 0.1 ml of this mixed suspension was inoculated to a CCDB tube. After performing the normal enrichment procedure the numbers of *Campylobacter* in these enrichment cultures were determined by plate counting. The CCDB culture contained after two days of incubation in average 8 $\log_{10}$(CFU/ml) *Campylobacter*. From each plate count, nine colonies were subcultured and serotyped. All 27 colonies from the three experiments were serotype O11.

DISCUSSION

This study was carried out to gain more insight into the *Campylobacter* contamination of sewage. For that purpose, in the period of September 1992 till February 1993,

the prevalence of *Campylobacter* was determined in a sewage purification plant as well as in the drain water of a connected poultry abattoir on three sampling days during 24 h periods. The numbers of *Campylobacter* in the sewage were expected to increase subsequent the starting of the slaughter process. In order to reveal possible transmission routes, the isolates were characterized by antimicrobial susceptibility testing and by serotyping.

On the three sampling days the *Campylobacter* carrier state of the processed broilers was mainly up to 80-100%, as also reported by Jacobs-Reitsma *et al.* (1994a). Already at the start of slaughtering, the poultry abattoir drain water was considerably contaminated with *Campylobacter* (average > 5.8 $\log_{10}$(MPN/100 ml)), which is in agreement with the observations of Höller (1988), Jones *et al.* (1990) and Koenraad *et al.* (1994; This thesis, Chapter 3). The high contamination degree so early in the morning can be explained by the observed survival of *Campylobacter* in the remainder still present in the fat floatation tank. Thus, the poultry abattoir releases a constant flow of *Campylobacter* on the sewer. An increase in *Campylobacter* contamination of the sewage due to the start of the slaughtering was not observed; the numbers of *Campylobacter* in the activated sludge plant did not vary during the sampling period. The purification process in the activated sludge system resulted in a reduction of the numbers of *Campylobacter* by 1 $\log_{10}$ unit, which agrees with the observed reduction in the Dutch monitoring programme (Koenraad *et al.*, 1994, This thesis, Chapter 3) and with the observations of Höller (1988).

Theoretically the experiment lasted long enough to detect *Campylobacter* in the sewage originating from the poultry abattoir drain water, because transport of the drain water to the purification plant takes approximately 1.5 h. Dilution can only partly explain the lower numbers of *Campylobacter* in the sewage compared to the numbers in the poultry abattoir drain water; the abattoir drained approximately 310 m^3 per day and the purification plant received in average 15,000 m^3 daily. If dilution was the only the factor, the numbers in the sewage would be in average 3.3 $\log_{10}$(MPN/100 ml). A negative effect of some bactericidal components in the sewage, entrapment of *Campylobacter* cells in sludge particles, ingestion by protozoa (King *et al.*, 1988) during transport to the activated sludge system may also be responsible for the reduction. Furthermore, these numbers indicate that possible other contamination sources may be of relatively less

importance in the purification plant investigated.

In agreement with the observations of Endtz *et al.* (1991) and Jacobs-Reitsma *et al.* (1994b) several isolates were found to be resistant to the quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin, especially the isolates obtained from the sewage purification plant samples. The activated sludge system isolates are a compilation of poultry and human isolates, and among both types of isolates quinolone resistance has emerged during the 1980s (Endtz *et al.*, 1991). The tetracycline- and erythromycin-resistant strains among the purification plant isolates could originate from human campylobacteriosis patients (Sjögren *et al.*, 1992; Wang *et al.*, 1984).

The patterns of antimicrobial susceptibility results and serotype results differed significantly between the various groups of samples and therefore, no transmission route could be deduced. This discrepancy can be caused by several factors. The residence time in the sewer and/or floatation tank determines which serotypes of *Campylobacter* will be found, so probably the isolates from the abattoir poultry abattoir drain water and the sewage purification plant reflect the pattern of *Campylobacter* harbouring in the intestines of the broilers processed during the last few days. Furthermore, from the performed mixed culture experiments it can be concluded that in enrichment medium the two serotypes did not multiply with an equal rate. This agrees with the observation that in the enrichment procedure no new dominant serotypes were isolated from the enrichment tubes with a higher dilution (data not shown). For these various factors, it is recommendable to avoid an enrichment procedure in an epidemiological investigation.

Summarizing, it may be concluded that the poultry abattoir drain water in this study provides a constant flow of *Campylobacter* into the sewer. The purification process in the activated sludge reduces the numbers, but *Campylobacter* is still detectable in the efflux. A transmission route from the poultry abattoir towards the purification plant could not be deduced from the serotyping and antimicrobial susceptibility results. This study also indicates that the numbers of *Campylobacter* in the sewage originating from other contamination sources may be negligible in the purification plant studied.

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CHAPTER 5

SPECIATION AND SUBTYPING OF *CAMPYLOBACTER* ISOLATES FROM SEWAGE PURIFICATION PLANTS AND DRAIN WATER FROM AN ASSOCIATED POULTRY ABATTOIR USING MOLECULAR TECHNIQUES

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In this study the distribution of phenotypes of *Campylobacter* strains in sewage and surface waters was investigated by subtyping and by speciation of isolates from various aquatic environments, including two municipal sewage purification plants (**spA** and **spB**) and drain water from a poultry abattoir (**dwA**). The isolates were speciated by the species-specific polymerase chain reaction technique and subtyped by polymerase chain reaction - restriction fragment length polymorphism technique based on the flagellin PCR products.

From all three reservoirs, no *C. lari* was isolated, and approximately 80% of the isolates could be identified as *C. jejuni* and the rest belonged to the *C. coli* species. The PCR-RFLP typing technique has a high discrimination level and is a suitable tool for comparing *Campylobacter* isolates from various sources and laboratories. The 182 isolates tested yielded 22 distinct *Dde* I profiles. No seasonality was observed in the profile distribution. So, for the Dutch situation it is unlikely that infections caused by contaminated surface waters contribute to the seasonality of human *Campylobacter* infections.

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INTRODUCTION

Campylobacter jejuni is a common cause of acute bacterial enteritis in man (Tauxe, 1992). In the Netherlands it has been estimated that the annual incidence of campylobacteriosis is 2,000 cases per 100,000 (Notermans and Hoogenboom-Verdegaal, 1992). The associated economic consequences in terms of lost working days and cost of treatment are considerable. Undercooked poultry meat is generally regarded as the predominant source of infection. However, *Campylobacter* is ubiquitous in the environment (Stelzer *et al.*, 1991) and exposure to untreated water is another recognised source of infection (Taylor *et al.*, 1983). *Campylobacter* can survive well in aquatic environments, especially when temperatures are low (Blaser *et al.*, 1980). Faeces from asymptomatic, wild and domestic animals, like cattle, swine, poultry, water-fowl, rodents, and dogs, all contribute to contamination of surface waters (Blaser *et al.*, 1984; Stelzer *et al.*, 1991). In particular poultry abattoir effluent may contain high numbers of *Campylobacter* (Höller, 1988; Jones *et al.*, 1990; Koenraad *et al.*, 1994).

The contribution of environmental *Campylobacter* strains as a source to human and animal *Campylobacter* infections is currently unknown. However, previous investigations indicate a seasonal variation (Jones *et al.*, 1990) exemplified by a decline in numbers of organisms observed in the sewage of an activated sludge system during the end of the summer (Koenraad *et al.*, 1994). Even though this seasonality contradicts that often observed in human infections of the developed world (Tauxe, 1992) the possibility cannot be excluded that *Campylobacter* cells with enhanced virulence properties are better able to survive aquatic environmental conditions during the summer months. Such anomalies need to be investigated further. Accurate methods for speciation and subtyping are particularly important in such studies.

Until recently *Campylobacter* speciation has been difficult. Discrimination between closely-related thermophilic species, *C. jejuni*, *C. coli*, and *C. lari*, on the basis of phenotypic charateristics is time-consuming and largely inaccurate (Jacobs-Reitsma *et al.*, 1994b; Penner, 1988; Totten *et al.*, 1987). Recently, a polymerase chain reaction (PCR) technique based on species-specific 23S rRNA fragments has been developed which may be suitable for rapid speciation of *Campylobacter* strains (Eyers *et al.*, 1993).

Techniques for *Campylobacter* subtyping have in the past caused even more difficulties. Conventional subtyping methods, such as biotyping (Lior, 1984; Skirrow and

Benjamin, 1980) and serotyping (Lauwers and Penner, 1984; Lior *et al.*, 1982) are not readily available and have poor levels of discrimination. Recently developed techniques for the restriction fragment length polymorphism (RFLP) analysis of the flagellin genes (Ayling *et al.*, 1995) appeared to provide a suitable and practical typing tool (Nachamkin *et al.*, 1993).

The aim of the current study was to utilize these newly developed epidemiological tools to speciate and subtype *Campylobacter* strains isolated from several aquatic sources. These sources have previously been described and include poultry abattoir drain water and an associated as well as an unrelated sewage purification plant sampled over a one-year period (Koenraad *et al.*, 1994).

MATERIALS AND METHODS

Campylobacter strains

Campylobacter isolates from the influx and efflux of two municipal plants were included in this study (Koenraad *et al.*, 1994; This thesis, Chapter 3); namely an activated sludge sewage purification plant (**spA**) and a trickling filter sewage purification plant (**spB**). The activated sludge system, with a capacity of 60,000 citizen equivalents, receives sewage from households and from various small industries, including a poultry abattoir. The trickling filter system, with a capacity of 130,000 citizen equivalents, receives domestic and industrial waste, but not from meat-processing industries. Furthermore, isolates obtained from the drain water of the poultry abattoir (**dwA**), connected with the activated sludge plant, were also characterized. The poultry abattoir is located at a distance of approximately 4 km from the activated sludge system. Samples were collected from all three reservoirs over a one-year period.

The samples were analysed by selective enrichment (42°C) in charcoal cefoperazone deoxycholate broth, after a pre-enrichment step (37°C) according to previously published methods (Koenraad *et al.*, 1994; This thesis, Chapter 3). Confirmation of suspected *Campylobacter* isolates was based on microscopic appearance, growth under micro-aerobic conditions in brain heart infusion broth (BHI, 0037-01-6 Difco Labora-

tories, Detroit, USA) at 42°C (+) and 25°C (–), oxidase activity (+) and catalase activity (+).

The isolates were stored at -80°C in BHI containing 20% (v/v) glycerol. For DNA preparations, isolates were cultured in BHI for 2 days at 42°C under micro-aerobic conditions.

DNA preparations

A BHI culture was swabbed onto Columbia agar base plates (CM 331, Oxoid, Basingstoke, UK) with 5% (v/v) defibrinated, lysed horse blood (CAB) and incubated for 2 days at 42°C under micro-aerobic conditions. Bacteria were harvested and washed in physiological salt solution (8.5 g/l NaCl) containing 1 g/l peptone (L34, Oxoid) and finally resuspended in 100 µl lysis buffer of the IsoQuick® Nucleic Acid kit (MXT-020-100, MicroProbe Corporation, Washington, USA). The nucleic acid extraction and purification procedure was carried out according to the manufacturer's instructions. The extraction is based on the chaotropic properties of guanidine isothiocyanate, which both disrupts cellular integrity and inhibits nuclease (DNAase and RNAase) activities, thereby providing both cell lysis and DNA stabilization. The pure nucleic acids were dissolved in 100 µl RNAase free water and stored at -20°C.

Speciation

The species were discriminated by PCR, based on the 23S rRNA gene (Eyers *et al.*, 1993). Briefly, a standard PCR reaction mixture (50 µl) contained 5 µl of the DNA solution and 45 µl of buffer to give final concentrations of 20 mM Tris-HCl (pH 8.8), 50 mM KCl, 3 mM $MgCl_2$, 0.01% (w/v) gelatin, 200 µM of each deoxyribonucleotide triphosphate (u1240, Promoga Corporation, Madison, USA), 1 U of AmpliTaq DNA polymerase (N808-1012, Perkin Elmer Cetus, Norwalk, USA), and 50 pmol of each primer. The reaction mixture was overlayed with 50 µl mineral oil (M3516, Sigma, St. Louis, USA) to prevent evaporation. Amplification was carried out using a thermocycler (Perkin-Elmer 480) with the following profile for 27 cycles: 1 min at 94°C; 1 min at 54°C, and 1 min at 74°C. Amplified samples were separated by agarose gel electrophoresis (1.5% [w/v], 1444964 Agarose MP, Boehringer Mannheim Biochemica, Mannheim, Germany) in a TAE buffer (40 mM Tris [pH 7.2] - 20 mM acetic acid - 50

mM EDTA) and visualised by ethidium bromide staining (0.5 μg/ml).

Typing by PCR-RFLP

PCR

Samples of the extracted DNA were assayed in a PCR-RFLP system, as described by Ayling *et al.* (1995). A standard PCR reaction mixture (50 μl) was composed of Saiki buffer (end concentration 3.0 mM $MgCl_2$, 1% [w/v] gelatin, 10 mM Tris-HCl [pH 8.5], 50 mM KCl), 5% (v/v) glycerol, 500 μM of each deoxyribonucleotide triphosphate (Promega), 2.5 Units of AmpliTaq DNA polymerase (Perkin Elmer Cetus), and 50 pmol of each primer and contained 2 μl extracted DNA solution. This solution was overlayed with 50 μl mineral oil to prevent evaporation. The amplification profile started with a denaturation step (94°C; 60 s) followed by 45 cycles of amplification in a thermocycler (Perkin Elmer 480). Temperature profile comprised 45 s at 94°C; 45 s at 55°C; and 2 min at 72°C. The programme was terminated by an extension step (58°C; 90 s) and an incubation step (72°C; 5 min). Primers, based on the flagellin genes, used were: Cj431: 5′-AAAGGATCCGCGTATTAACACAAATGTTGCAGC-3′, Cj432: 5′-AAAGGATCC-GAGGATAAACACCAACATCGGT-3′, and Cj433: 5′-GATTTGTTATAGCAGTTTCT-GCTATATCC-3′ (Ayling *et al.*, 1995).

Digestion

The PCR product was digested by incubating 12 μl PCR product with 10 units *Dde* I restriction enzyme (15238-025, Gibco BRL, Life Technologies Ltd, Gaithersburg, USA), 2 μl 10 × buffer containing 500 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$, 500 mM NaCl (Life Technologies Ltd), and 5 μl Millipore water for 2 h at 37°C. Two microliters of loading buffer (40% [w/v] sucrose in water containing 0.25% [w/v] bromophenol blue) were added to the digestion mixture to stop the digestion reaction. The digestion product was separated on a 2.5% (w/v) agarose gel (2.0% [w/v] NuSieve® GTG® Agarose [50082, FMC, Rockland, UK], 0.5% MP agarose [Boehringer Mannheim Biochemica]) in TAE buffer. The fragments were visualized by staining with ethidium bromide (0.5 μg/ml) (Ayling *et al.*, 1995). Profiles were compared by using the computerized Logitech Foto Touch scanning system and analysed by the Singularity

RFLP analyzer 1.21.

RESULTS

Campylobacter isolates (n = 182) from the three different reservoirs (**dwA**, **spA**, and **spB**) and collected over a one-year period were identified at the species level using the PCR technique with primers specific for *C. jejuni*, *C. coli*, and *C. lari*. Approximately 10% of the strains investigated could not be speciated by this technique. All the isolates identified as *C. jejuni* gave PCR products of 710 bp or 810 bp. The size of this PCR product was independent of the reservoir and of the season. The species distribution was similar for the three reservoirs investigated (Table 1). No *C. lari* strains were identified in isolates from any of the reservoirs.

For PCR-RFLP analyses, the bacterial culture, DNA extractions, PCR's, and digestions were performed in duplicate for each strain. Analysing standard strains and comparing the results between two independent laboratories (Central Veterinary Laboratory [United Kingdom] and Wageningen Agricultural University [The Netherlands] de-

Table 1. The distribution of the species among the isolates from reservoirs **dwA**, **spA**, and **spB**.

Reservoir	Number tested	Number of isolates speciated	
		C. jejuni (%)[a]	*C. coli* (%)
dwA[b]	78	56 (72)	16 (21)
spA	60	44 (73)	11 (18)
spB	44	30 (68)	8 (18)

[a] Percentage of total isolates (n = 165), which could be identified as specific species.

[b] **dwA** = Poultry abattoir drain water, which is drained on the activated sludge system (**spA**).
spA = Municipal sewage purification plant; an activated sludge system.
spB = Municipal sewage purification plant; a trickling filter system.

monstrated the consistency of this typing method. The 1.49 kb PCR products from the flagellin genes of each of 182 isolates were digested with *Dde* I to yield a diversity of profiles each comprising two to five fragments sized between 124 bp and 1,075 bp

Table 2. Details of *Campylobacter* strains used for PCR-RFLP analyses.

Lane no. in Fig. 1	Isolate	Reservoir	Date of isolation	PCR-RFLP profile	Lane no. in Fig. 1	Isolate	Reservoir	Date of isolation	PCR-RFLP profile
2	WAU705[a]	spA[b]	05 08 92	14[c]	22	WAU1119	dwA	10 03 93	17
3	WAU1000	spB	27 01 93	31	23	WAU1280	dwA	21 04 93	16
4	WAU905	spA	09 12 92	15	24	WAU1120	dwA	10 03 93	27
5	WAU1053	spA	12 02 93	15	25	WAU1159	dwA	26 03 93	27
6	WAU721	spA	05 08 92	34	26	WAU1160	dwA	26 03 93	28
7	WAU719	spA	05 08 92	16	27	WAU1162	dwA	26 03 93	25
8	WAU761	spA	26 08 92	17	28	WAU1182	dwA	28 03 93	28
9	WAU724	spA	05 08 92	18	29	WAU1184	dwA	28 03 93	29
10	WAU731	dwA	05 08 92	19	30	WAU1269	spA	21 04 93	30
11	WAU1229	dwA	31 03 93	20	31	WAU631	spB	24 06 92	30
12	WAU738	dwA	05 08 92	21	32	WAU671	spB	15 07 92	31
13	WAU754	dwA	26 08 92	22	33	WAU527	spB	01 04 92	32
14	WAU763	dwA	26 08 92	23	34	WAU526	spB	01 04 92	33
15	WAU1155	spA	26 03 93	23	35	WAU522	spB	01 04 92	19
16	WAU810	spA	21 10 92	24	36	WAU929	spB	09 12 92	15
17	WAU824	dwA	21 10 92	25	37	WAU1067	dwA	22 02 93	25
18	WAU1161	dwA	10 03 93	26	38	WAU1216	spB	31 03 93	31
19	WAU1212	spB	31 03 93	35	39	WAU1116	dwA	10 03 93	24

[a] WAU = Wageningen Agricultural University, The Netherlands.

[b] For abbreviations see Table 1.

[c] For PCR-RFLP profiles see Fig. 1.

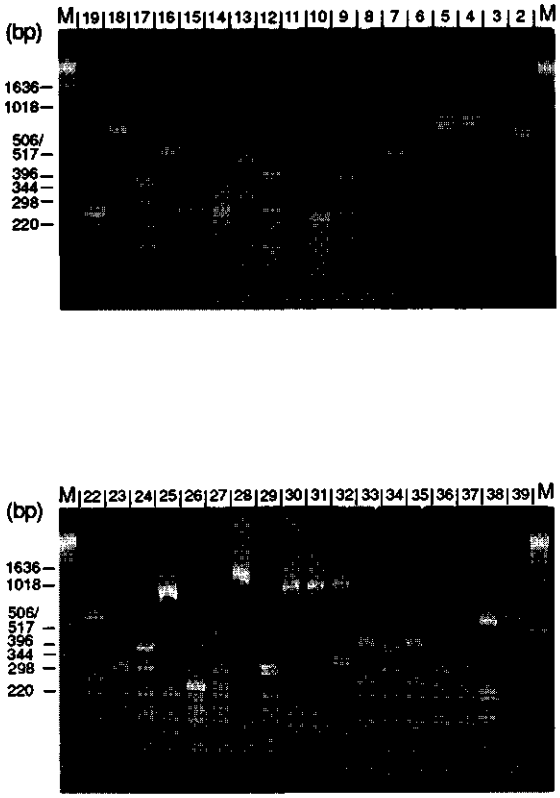

Fig. 1. Review gel electrophoresis of distinct PCR-RFLP profiles from *Campylobacter* isolates originating from poultry abattoir drain water (**dwA**) and sewage purification plants (**spA** and **spB**).

Lanes:

1, 20, 21, 40: Molecular weight marker. Fragment sizes are shown besides the lanes.

2 to **19** and **22** to **39:** PCR-RFLP profiles; all isolates are shown as indicated in Table 2.

(Fig. 1). Among the isolates tested 22 distinct *Dde* I profiles were distinguished. None of the 22 profiles did match with previously described 13 profiles from the United Kingdom (Ayling *et al.*, 1995) and therefore the 22 profiles observed in this investigation were designated profile numbers extending the previous scheme (Table 2). Approximately 52% (95 out of 182) of the strains fell into only 4 profiles (profiles **14, 15, 22,** and **23**).

Hunter (1990) suggested that discriminatory power can be defined mathematically as the probability that two strains chosen at random from a population of unrelated strains will be distinguished by a particular typing method and he defined a numerical index of discriminatory power, which could be calculated as 0.92 for this typing method.

The distribution of the profiles among the isolates from the three reservoirs is illustrated in Fig. 2. The ranked order of prevalence of the various profiles was dependent on the source. Only 6 profiles (profiles **14, 22, 15, 23, 32,** and **19**) were re-

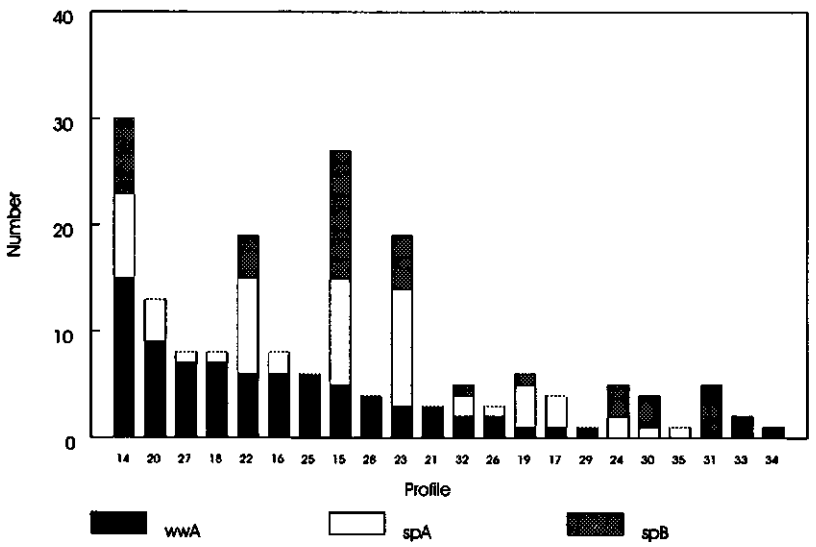

Fig. 2. Distribution of PCR-RFLP profiles among 182 *Campylobacter* isolates obtained from poultry abattoir drain water (**dwA**) and sewage purification plants (**spA** and **spB**), with the profiles on the horizontal axis according to decreasing order of prevalence among the poultry abattoir drain water isolates.

presented in isolates from each of the three reservoirs. Some profiles were unique to isolates from **dwA** (profiles **25**, **28**, **21**, and **29**), **spB** (profiles **31**, **33**, and **34**) or **spA** (profile **35**). It is notable that no profiles were confined to **dwA** and **spB** only.

Samples were collected from **spA**, **spB**, and **dwA** over a one year-period. From each sample, various strains expressing multiple profiles were isolated. There was no evident seasonality in any of the profiles isolated from any of these reservoirs.

DISCUSSION

In this study the distribution and diversity of *Campylobacter* in the influx and efflux from two municipal sewage purification plants in the Netherlands was investigated. The sewage purification plants differed in their purification mechanisms; one being an activated sludge plant (**spA**), the other a trickling filter system (**spB**). In addition isolates were obtained from the drain water (**dwA**) of a poultry abattoir which drained into **spA**. Both sewage purification plants received waste from households and various small industries. However, only **spA** received waste from a meat-processing plant, namely the poultry abttoir. The isolates were charaterized both at the species level, by species-specific PCR (Eyers *et al.*, 1993) and at the subtype level, by PCR-RFLP profile based on the variation of the flagellin genes (Ayling *et al.*, 1995).

The molecular technique used for speciation (Eyers *et al.*, 1993) was very easy to perform. Unfortunately, 10% of the *Campylobacter* strains did not result in a PCR product with the available species-specific primers (Eyers *et al.*, 1993). The proportion of unspeciated strains was equally distributed between the three sources. No explanation is currently available for the inability to speciate these anomalous strains, using these primers. However, these strains had phenotypic charateristics of *C. jejuni/coli*. An additional multiprimer PCR, including primer sets of several species (Van Der Plas *et al.*, 1993), was found to be unsuitable because of nonspecific amplification (data not shown). The species distribution was independent of the reservoir investigated; about 80% of isolated strains were *C. jejuni*, 20% were *C. coli*, while *C. lari* was not found. These results confirm previous studies, using phenotypic techniques (Jacob *et al.*, 1991). However, in other investigations (Stelzer *et al.*, 1991) the proportion of *C. coli* isolates was

significantly higher (66%) and *C. lari* was also recoverable. The reasons for these differences are unknown but may reflect differences between species in sensitivity to selective antibiotics (Bolton *et al.*, 1983; Ng *et al.*, 1985) and survival in aquatic environments (Korhonen and Martikainen, 1991).

The PCR-RFLP technique has only recently been developed (Alm *et al.*, 1993; Ayling *et al.*, 1995; Owen *et al.*, 1993) and its value in the epidemiological investigation of *Campylobacter* is still being established (Ayling *et al.*, 1995). Nevertheless, the evidence to date suggest that there are a number of advantages over alternative typing methods. In particular the technique is readily available, has a high level of discrimination and provides similar results between laboratories. Moreover, nontypable isolates were not obtained.

From the 182 isolates investigated 22 PCR-RFLP profiles were distinguishable. More than half (52%) of the isolates were confined to four profiles (profiles **14**, **15**, **22**, and **23**) suggesting that certain strains either have enhanced abilities to colonise host sources or have enhanced survival possibilities in the environment. However, the absence of profile variation with season would suggest the environmental susceptibility not to be a factor in this distribution.

There were marked differences in the prevalence of profiles among the isolates from the various reservoirs. Strains of six profiles were common to all three sources indicating that those strains which were present in poultry can also occur in waste from human sources. Strains of a further 10 profiles were either confined to both **dwA** and **spA** or unique to **dwA** only. This is consistent with the likelyhood that strains from poultry abattoir, **dwA**, would be represented in the receiving sewage purification plant, **spA**. However, as these profiles are not observed in strains from **spB**, this may suggest that at least some strains may be confined to poultry only and may not occur in humans. Previous experimental evidence using *in vitro* and *in vivo* models has indicated the presence of some *Campylobacter* strains in the environment with reduced virulence (Newell *et al.*, 1985) and which may not, therefore, be pathogenic to man. In contrast, strains of the remaining six profiles were from **spA** and/or **spB** only. Thus no profiles were observed which were confined to strains from **spB** and **dwA**. It, therefore, is possible that the source of some *Campylobacter* strains infecting humans, and hence present in human sewage effluent, is not poultry.

Interestingly all of the isolates from the drain water of the poultry abattoir had different profiles from the 13 profiles previously observed in poultry in the United Kingdom. Whether this discrepancy is a true reflection of the geographical variation in *Campylobacter* strains present in the Netherlands and the United Kingdom has yet to be determined by the investigation of larger numbers of samples and comparable isolates from similar sources.

The absence of seasonality in the profile distribution is consistent with previous observations relative to the prevalence in sewage and broilers (Koenraad *et al.*, 1994; Jacobs-Reitsma *et al.*, 1994a). Moreover, total *Campylobacter* numbers were previously shown to decline in the summer months (Jones *et al.*, 1990). It, therefore, seems unlikely, at least in the Netherlands, that infections caused by contaminated surface waters contribute to seasonality of human infections.

Despite these, and other investigations the sources of the majority of sporadic cases of human campylobacteriosis remain unproven. In the past such studies have been limited by the availability of adequate subtyping of strains. This study has utilised a recently developed molecular typing scheme to investigate the potential health risk of surface waters contaminated with *Campylobacter*. However, not all environmental *Campylobacter* strains may be pathogenic for man. In addition the results suggest that although poultry may have an important role in human infections, other sources cannot be ignored. The introduction of such molecular epidemiomological tools will enable routes of transmission of *Campylobacter* infections to be definitively established in the future.

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CHAPTER 6

ANTIBIOTIC SUSCEPTIBILITY OF *CAMPYLOBACTER* ISOLATES FROM SEWAGE AND POULTRY ABATTOIR DRAIN WATER

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In this study, the *in vitro* susceptibility of 209 *Campylobacter* strains to the quinolones nalidixic acid, flumequine, ciprofloxacin and, enrofloxacin, and to ampicillin, tetracycline, and erythromycin was tested by the disk diffusion method. The strains were isolated from poultry abattoir drain water (**dwA**) and two sewage purification plants (**spA** and **spB**). Sewage purification plant **spA** received mixed sewage, including that from a poultry abattoir, whereas **spB** did not receive sewage from any meat-processing industry. The quinolone resistance of the **dwA** isolates ranged from 28% for enrofloxacin to 50% for nalidixic acid. The strains isolated from the sewage purification plants were more susceptible to the quinolones with a range of 11 to 18% quinolone resistance for **spB** isolates to 17-33% quinolone resistance for **spA** isolates. The **dwA** isolates showed highest ampicillin (28%) and tetracycline (34%) resistance.

This study shows clearly that the quinolone resistance of *Campylobacter* bacteria is highest in the plant receiving sewage from a poultry abattoir. The occurrence of *Campylobacter* in the aquatic environment and the resistance to various antimicrobial agents are important to be monitored, as surface waters are recognized as possible sources of infection.

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INTRODUCTION

Campylobacter jejuni is recognised as one of the most important causes of acute diarrhoeal disease in humans throughout the world (Taylor and Blaser, 1991). Four sources appear to account for nearly all cases of campylobacteriosis: poultry meat products, raw milk, untreated surface water, and pets. Most cases of campylobacteriosis are single cases and related to the consumption of (undercooked) poultry meat. However, outbreaks are mostly related to consumption of raw milk and untreated surface water (Aho *et al.*, 1989; Hudson *et al.*, 1991; Riordan *et al.*, 1993).

Campylobacter organisms found in surface water most probably originate from wild and domestic animals (Park *et al.*, 1991), water run-off from farmland following heavy rainfall, industrial waste water and efflux from sewage purification plants (Jones *et al.*, 1990; Koenraad *et al.*, 1994). In particular, poultry abattoir efflux may contain high numbers of *Campylobacter* and as these organisms can easily pass sewage purification plants, it constitutes an important contamination source for surface water (Brennhovd *et al.*, 1992; Höller, 1988; Jones *et al.*, 1990; Koenraad *et al.*, 1994). An epidemiological study on the distribution and diversity of *Campylobacter* strains in a sewage purification plant and poultry abattoir drain water drained on this plant, showed several common phenotypes (Koenraad *et al.*, 1995).

Most of the *Campylobacter* infections are self limiting. However, if diarrhoea is frequent and/or bloody, or high fever is present, treatment with antimicrobial agents is indicated. Erythromycin is still the drug of choice (Skirrow and Blaser, 1992) but nowdays fluoroquinolones are considered to be a good, safe but expensive alternative for the treatment of human *Campylobacter* enteritis (Mattila *et al.*, 1993). However, Endtz *et al.* (1991) reported an increase of quinolone resistance of *Campylobacter* strains isolated from human stools and poultry products in the Netherlands between 1982 and 1989. In this country the quinolones enrofloxacin and ciprofloxacin were introduced into veterinary and human medicine in 1987 and 1988, respectively. The prevalence of quinolone-resistant strains isolated from poultry products increased during the period 1982-1989 from 0 to 14% and that of human isolates increased from 0 to 11% (Endtz *et al.*, 1991). Most recently, Jacobs-Reitsma *et al.* (1994b) observed that 29% of the *Campylobacter* isolates from poultry were quinolone-resistant. This rapid increase in quinolone resistance may have implications for the treatment of human diarrhoeal diseases.

Until recently, *Campylobacter* speciation was difficult. Discrimination between closely-related species, *C. jejuni*, *C. coli* and *C. lari*, on the basis of phenotypic characteristics was time-consuming and not always correct (Penner, 1988; Totten *et al.*, 1987). A polymerase chain reaction (PCR) technique based on species-specific 23S rRNA fragments has been developed which may be suitable for rapid speciation of *Campylobacter* strains (Eyers *et al.*, 1993).

In the present investigation, the prevalence of antibiotic resistance in *Campylobacter* isolates obtained from aquatic environments was determined. *Campylobacter* strains isolated from surface water, sewage, and poultry abattoir drain water, were speciated by the PCR technique (Eyers *et al.*, 1993) and their *in vitro* susceptibility to ampicillin, tetracycline, and erythromycin and the quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin, was tested by the disk diffusion method (Bauer *et al.*, 1970). The quinolones have similar chemical structures and therefore, resistance to the four agents tested can be considered as resistance to one component. Ampicillin and oxytetracycline are frequently used in Dutch broiler production and therefore included in this study. The performance standards for antimicrobial disk susceptibility tests, as recommended by the National Committee Clinical Laboratory Standards (1990), which is a communication forum in the USA providing international standards, were evaluated. The evaluation was performed by calculating and plotting the population distributions of the measured inhibition diameters for the various antimicrobial agents.

MATERIALS AND METHODS

Campylobacter strains

Isolates from influx and efflux of two municipal sewage purification plants; namely an activated sludge plant (**spA**) and a trickling filter system (**spB**) were tested. The activated sludge plant, with a capacity of 60,000 citizen equivalents, receives sewage from households and from various small industries, including a poultry abattoir. The trickling filter system, with a capacity of 130,000 citizen equivalents, receives both domestic and industrial drain water, but not from any meat-processing industry. Furthermore, *Campylobacter* strains isolated from the drain water of the poultry abattoir (**dwA**),

connected with the activated sludge plant, were included in the present study. This poultry abattoir is located at approximately 4 km from the activated sludge plant.

The samples were analysed under micro-aerobic conditions by selective enrichment (42°C) in charcoal cefoperazone deoxycholate (CCD) broth, after a pre-enrichment step (37°C). *Campylobacter* strains were isolated during micro-aerobic incubation on selective CCD plates (Koenraad *et al.*, 1994). Confirmation of suspected *Campylobacter* isolates was based on microscopic appearance (spiral-shaped cells with rapid motility), resistance to cephalothin (KF, Oxoid, Basingstoke, UK, 30 μg), growth under micro-aerobic conditions in brain heart infusion broth (BHI, 0037-01-6 Difco Laboratories, Detroit, USA) at 42°C (+) and 25°C (—), presence of oxidase and catalase activity (+). The isolates were stored at -80°C in BHI containing 20% (v/v) glycerol. For DNA preparations and antibiotic susceptibility testing, isolates were precultured in BHI for 2 days at 42°C under micro-aerobic conditions.

Table 1. Susceptibility criteria for inhibition zone diameters for the antimicrobial agents nalidixic acid, flumequine, ciprofloxacin, enrofloxacin, ampicillin, tetracycline, and erythromycin.

Antimicrobial agent	Abbreviation	Disk content (μg)	Criteria[a] (mm)
Nalidixic acid	NA	30	≥ 19
Flumequine	UB	30	≥ 18[b]
Ciprofloxacin	CIP	5	≥ 21
Enrofloxacin	ENRO	5	≥ 18[b]
Ampicillin	AMP	25	≥ 17
Tetracycline	TE	30	≥ 19
Erythromycin	E	15	≥ 23

[a] Criteria for nalidixic acid, ciprofloxacin, ampicillin, tetracycline, and erythromycin according to National Committee for Clinical Laboratory Standards (1990).

[b] The standards for flumequine and enrofloxacin are deduced from the observations of Endtz *et al.* (1991).

Antibiotic susceptibility testing by disk diffusion

Antibiotic susceptibility was determined by the disk diffusion method described by Bauer *et al.* (1970). The method was adjusted to the characteristics of *Campylobacter* by Jacobs-Reitsma *et al.* (1994b). The BHI suspension was swabbed onto Mueller-Hinton (CM337, Oxoid) plates supplemented with 5% (v/v) defibrinated, lysed horse blood (MH). Susceptibility to the following agents was tested: nalidixic acid (NA, Oxoid, 30

Table 2. Antibiotic resistance of 209 *Campylobacter* aquatic isolates and 617 *Campylobacter* poultry isolates.

Antimicrobial agent	Resistant isolates				p[c]
	poultry[a] (n=617)	dwA[b] (n=99)	spA[b] (n=66)	spB[b] (n=44)	
Nalidixic acid	236 (38%)	49 (50%)	22 (33%)	8 (18%)	<0.01
Flumequine	204 (33%)	30 (30%)	13 (20%)	6 (14%)	<0.01
Ciprofloxacin	203 (33%)	29 (29%)	12 (18%)	5 (11%)	<0.01
Enrofloxacin	181 (29%)	28 (28%)	11 (17%)	6 (14%)	<0.05
Ampicillin	263 (43%)	33 (33%)	15 (23%)	7 (16%)	<0.001
Tetracycline	110 (18%)	34 (34%)	16 (24%)	11 (25%)	<0.01
Erythromycin	27 (4%)	15 (15%)	5 (8%)	8 (18%)	<0.001
All Quinolones[d]	181 (29%)	28 (28%)	11 (17%)	5 (11%)	<0.02
Multi[e]	159 (26%)	29 (29%)	23 (35%)	10 (23%)	NS[f]

[a] Poultry isolates as mentioned in Jacobs-Reitsma *et al.* (1994b).

[b] **dwA** = Poultry abattoir drain water, which is drained on sewage purification plant **spA**.
spA = Sewage purification plant A.
spB = Sewage purification plant B, without meat-processing industries in its drainage area.

[c] Two-tailed chi-square test.

[d] Resistant to the quinolones tested.

[e] Resistant to more than one antimicrobial agent, considering the quinolones as one component.

[f] NS = Not Significant.

μg), flumequine (UB, Oxoid, 30 μg), ciprofloxacin (CIP, Oxoid, 5 μg), enrofloxacin (ENRO, Bayer Diagnostics, München, Germany, 5 μg), ampicillin (AMP, Oxoid, 25 μg), tetracycline (TE, Oxoid, 30 μg), and erythromycin (E, Oxoid, 15 μg). Per MH plate, 4 disks were placed and after incubation for 2 days (37°C, micro-aerobic conditions) the diameter of the inhibition zone (including disk diameter = 6 mm) was measured with a callipers. As recommended by the National Committee Clinical Laboratory Standards (NCCLS), Subcommittee on Antimicrobial Susceptibility Testing, *Escherichia coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used as control micro-organisms (National Committee Clinical Laboratory Standards, 1990). The applied susceptibility criteria as recommended by the NCCLS are given in Table 1.

DNA preparation and speciation

Cultures grown in BHI were swabbed onto Columbia agar base plates (CM 331, Oxoid) with 5% (v/v) defibrinated, lysed horse blood (CAB) and incubated for 2 days at 42°C under micro-aerobic conditions. Bacteria were washed in physiological salt solution (8.5 g/l NaCl) containing 1 g/l peptone (L34, Oxoid) and harvested by centrifugation. The nucleic acids were extracted by the IsoQuick® Nucleic Acid kit (MXT-020-100, MicroProbe Corporation, Washington, USA) according to the manufacturer's instructions. The species were identified by PCR, based on the 23S rRNA gene (Eyers *et al.*, 1993). The PCR was performed as described in Koenraad *et al.* (1995; This thesis, Chapter 5).

Statistical analyses

Hypotheses about the frequency of antibiotic resistance in the various categories of *Campylobacter* isolates were tested by the two-tailed chi-square test, considering $P \leq 0.05$ to be significant.

RESULTS

The antibiotic susceptibility of 209 *Campylobacter* strains isolated from sewage purification plants (**spA**, **spB**) and poultry abattoir drain water (**dwA**) was determined.

For 60 isolates selected at random, duplicate culturing and determination of susceptibility gave reproducible inhibition diameters (data not shown).

The susceptibility results of 209 *Campylobacter* isolates obtained from aquatic environments are compared with the data of 617 *Campylobacter* poultry isolates (Jacobs-Reitsma, 1994b) (Tables 1 and 2). Comparing the susceptibility from the four sources, the resistance to nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin is significantly lower among **spB** isolates. The **dwA** isolates have a similar resistance to flumequine, ciprofloxacin, and enrofloxacin as the poultry isolates. The resistance to all quinolones was lower among the aquatic isolates from **spA** and **spB** than among the poultry and **dwA** isolates. The isolates obtained from the sewage purification plant **spB** were more susceptible to ampicillin than the isolates from the other three sources ($P<0.001$). However, the **spB** isolates were less susceptible to erythromycin ($P<0.01$). The susceptibility to tetracycline did not differ between the isolates of the various sources.

Speciation by PCR was performed on 153 isolates; 121 (79%) were identified as *C. jejuni* and 32 (21%) belonged to the *C. coli* species (Table 3). *C. coli* isolates were more resistant to tetracycline ($P<0.001$) and erythromycin ($P<0.02$). Resistance to the quinolones and to ampicillin did not differ between the species. The *C. jejuni* and *C. coli*

Table 3. Antibiotic resistance of 121 *C. jejuni* isolates and 32 *C. coli* isolates.

Antimicrobial agent	Resistant *C. jejuni* isolates (n=121)	Resistant *C. coli* isolates (n=32)	P[a]
All Quinolones[b]	26 (21%)	5 (16%)	NS[c]
Ampicillin	28 (23%)	3 (9%)	NS
Tetracycline	23 (19%)	16 (50%)	< 0.001
Erythromycin	11 (9%)	8 (25%)	<0.02

[a] Two-tailed chi-square test.

[b] Cross-resistant to nalidixic acid, flumequine, ciprofloxacin and enrofloxacin.

[c] NS = Not Significant.

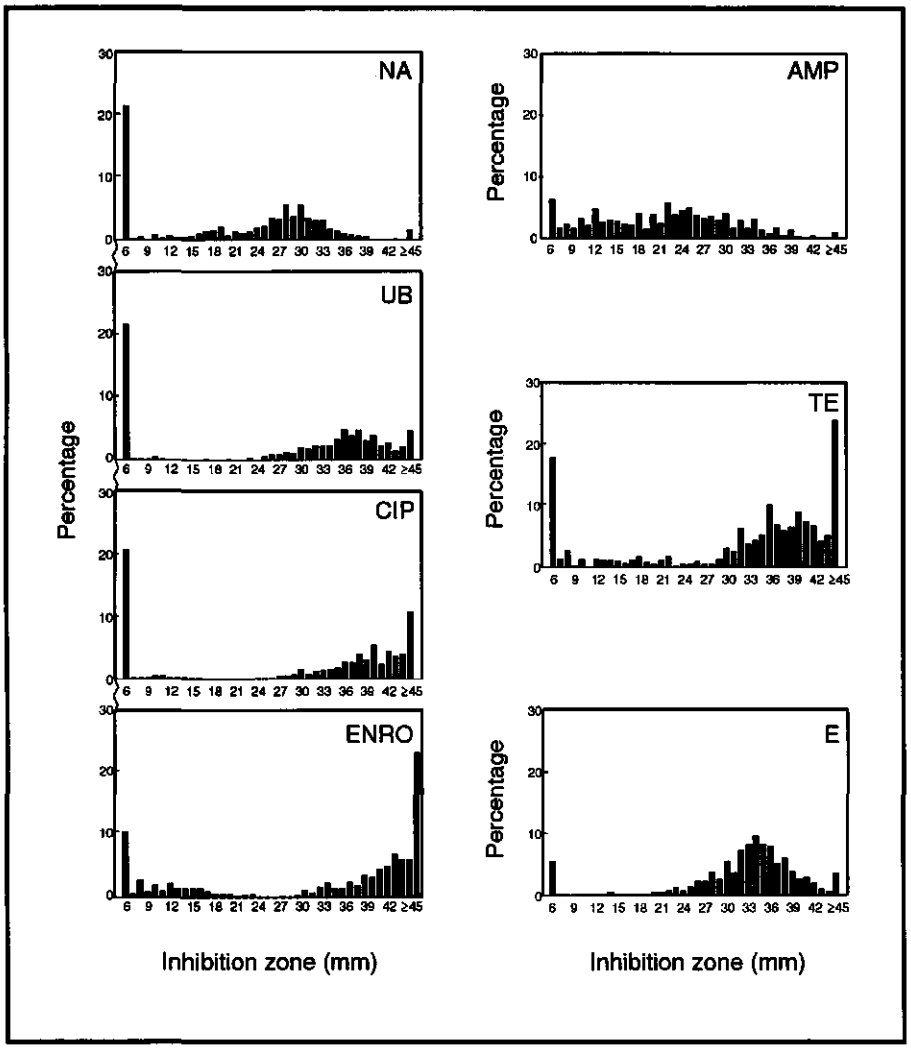

Fig. 1. Population distributions of antibiotic susceptibility (as measured in inhibition zone diameters) of 617 poultry *Campylobacter* isolates and 209 aquatic *Campylobacter* isolates to nalidixic acid (NA), flumequine (UB), ciprofloxacin (CIP), enrofloxacin (ENRO), ampicillin (AMP), tetracycline (TE), and erythromycin (E).

isolates showed a similar extent of multi-resistance, which was defined as resistance to more than one agent considering the quinolones as one component.

For the various antimicrobial agents, the population distribution of the inhibition diameters of these *Campylobacter* strains isolated from aquatic environments and poultry (Jacobs-Reitsma, 1994b) together were calculated and are shown in Fig. 1. The population distributions were similar for both strain collections. All population distributions showed a variation in frequency at different inhibition diameters, with exception of the rather flat distribution for the ampicillin inhibition diameters.

DISCUSSION

In the present study, the prevalence of antibiotic resistance among aquatic *Campylobacter* isolates was studied with respect to the treatment of water-borne cases of campylobacteriosis. Strains isolated from sewage (**spA**, **spB**) and poultry abattoir drain water (**dwA**) were tested on their susceptibility to the quinolones nalidixic acid, flumequine, ciprofloxacin, and enrofloxacin, and to ampicillin, tetracycline, and erythromycin by the disk diffusion method.

Among the *Campylobacter* isolates from the poultry abattoir drain water (**dwA**) percentages of strains resistant to the quinolones, ampicillin, and tetracycline were similar to those of the poultry isolates obtained in the investigation of Jacobs-Reitsma *et al.* (1994b). The isolates derived from sewage purification plant **spA** showed a lower percentage of strains resistant to the quinolones, ampicillin and tetracycline than the isolates from the poultry abattoir drain water (**dwA**). The lower percentage of resistant strains is probably due to dilution with household and other drain water, in which *Campylobacter* isolates will have a lower degree of resistance. Quinolones, ampicillin, and tetracycline are frequently applied in veterinary medicine. The resistance to these veterinarily applied agents is lowest among the isolates from sewage purification plant **spB**, which is in agreement with the fact that this municipal plant does not receive drain water from meat-processing industries. However, the erythromycin resistance is highest among these **spB** isolates. These erythromycin-resistant *Campylobacter* isolates might originate from human campylobacteriosis patients (Sjögren *et al.*, 1992).

The resistance to quinolones and ampicillin did not differ among the aquatic *C. jejuni* and *C. coli* isolates, which is at variance with the observations of several investigators (Endtz *et al.*, 1991; Jacobs-Reitsma *et al.*, 1994b). The aquatic *C. coli* isolates were more resistant to erythromycin and tetracycline. This agrees with the observations of Navarro *et al.* (1993), Rautelin *et al.* (1991), and Reina *et al.* (1992).

The disk diffusion method described by Bauer *et al.* (1970) is easy to perform and the reproducibility is high (data not shown). For the various antimicrobial agents, the population distributions of the inhibition diameters were not dependent on the source of the isolates (results not shown). The cut-off values (susceptibility criteria) recommended by the National Committee Clinical Laboratory Standards (1990) for the size of the inhibition diameters to discriminate between susceptible and resistant strains (Table 1) are mainly based on results obtained with *Staphylococci* and *Pseudomonas*. However, as shown in Fig. 1 they cannot readily be employed for *Campylobacter*, for which separate cut-off values should be established.

This investigation showed clearly that the resistance of the *Campylobacter* isolates from the two sewage purification plants reflects the extent of veterinary activities in the drainage area of the plants. Resistance to quinolones can readily be induced by enrofloxacin treatment of *Campylobacter* colonized broiler chicks (Jacobs-Reitsma *et al.*, 1994a). *Campylobacter* can survive the purification process in the sewage plants (Koenraad *et al.*, 1994; This thesis, Chapters 3 and 4), so the efflux will be a vehicle for the spread of resistant *Campylobacter* organisms in the environment. As surface waters are recognised as sources of human *Campylobacter* infections, it is important to continue surveillance of the resistance displayed by *Campylobacter* strains isolated from surface waters and other aquatic environments.

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CHAPTER 7

TEMPERATURE-DEPENDENT MEMBRANE FATTY ACID AND CELL PHYSIOLOGY CHANGES IN COCCOID FORMS OF *CAMPYLOBACTER JEJUNI*

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The effect of temperature and the availability of nutrients on the transition of spiral *Campylobacter jejuni* cells to coccoid forms was investigated. Cells incubated at 4°C in nutrient-deficient media remained culturable the longest. To study the phenomenon, ATP levels, protein profiles and fatty acid compositions were monitored. During storage the levels of intracellular ATP were highest in cells incubated at low temperatures (4°C and 12°C) and remained constant after a small initial decrease. During the transformation no alteration in protein profiles could be detected. Inhibition of protein synthesis by chloramphenicol did not influence the transition, and gamma-radiation had no effect on the process. Membrane fatty acid composition of *Campylobacter* cocci formed at low temperature was found to be almost identical to that of spiral cells, whereas that of cocci formed at 25°C was clearly different. Combining these results, it is concluded that the formation of cocci is not an active process. Cocci formed at 4°C show characteristics similar to spirals and these cocci may well play a role in the contamination cycle of *C. jejuni*. However, spiral *Campylobacter* cells can also play an important role in this cycle since these cells remain culturable for a long period of time especially under nutrient-poor and low temperature conditions.

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INTRODUCTION

Campylobacter jejuni is an important cause of food-borne infections and is associated mostly with chicken products. Although much research has been carried out, the environmental contamination cycle of this pathogen is not completely elucidated (Humphrey *et al.*, 1993; Van Der Giessen *et al.*, 1992). When *Campylobacter* is excreted in the environment, this micro-organism usually does not grow, since the minimum growth temperature of this micro-organism is 32°C (Doyle and Roman, 1981) and therefore survival is more important rather than growth under environmental conditions. It is generally known that *Campylobacter* cells are transformed from the spiral form into a coccoid form under unfavourable conditions. Although this coccoid form, which is evident at both growth and environmental temperatures (Rollins and Colwell, 1986), is not culturable using available techniques, it may nevertheless play a role in survival of *Campylobacter* during the contamination cycle.

The transformation to a viable but nonculturable form under unfavourable environmental conditions has been described for a variety of pathogenic micro-organisms such as *Legionella*, *Vibrio*, *Salmonella* and *Shigella*, and appears to be reversible when the conditions improve (McKay, 1992). Resuscitation is influenced by various specific conditions for different bacteria. For *Campylobacter*, Rollins and Colwell (1986) have provided preliminary evidence which suggests that reversion from coccoid to spiral forms occurred following animal passage. These results agree with observations by Colwell *et al.* (1985) where a nonculturable *Vibrio cholerae* was found to be pathogenic in animal passage experiments. Infectivity of *Campylobacter* cocci has also been reported by Jones *et al.* (1991), Saha *et al.* (1991), and Stern *et al.* (1994) who observed the appearance of *Campylobacter* spiral forms after administration of a cell suspension which apparently consisted only of nonculturable coccoid forms to mice, rats, and chicks, respectively. Further evidence for a role of nonculturable forms in the contamination cycle of *Campylobacter* was given by Pearson *et al.* (1993), who demonstrated that nonculturable forms of the bacterium, observed in water supplies by direct immuno-fluorescence microscopy, gave rise to *Campylobacter* colonization of chickens following consumption of this water. It is notable however, that Medema *et al.* (1992) could not isolate *Campylobacter* from the caeca of chicks, seven days after admission of nonculturable forms. Also, in previous experiments in our laboratory, when cocci were given to mice, rabbits,

and humans, no *Campylobacter*-specific antibodies could be detected and furthermore, the bacterium could not be isolated from stool samples in a 30-days period after administration (Beumer *et al.*, 1992).

Another approach to investigate the viability of coccoid forms, is to determine the physiological state of the cells. Indeed, viability of coccoid *Campylobacter* forms could be deduced from work of Rollins and Colwell (1986), who used the slightly modified method of Kogure *et al.* (1979) to determine the direct viable count in order to observe cell elongation. Moran and Upton (1986, 1987a, 1987b), however, have concluded that the coccoid form is a degenerative state, following from the observation of decreased levels of peptides, nucleic acids and decreased activity of superoxide dismutase in cocci. These authors also described a lack of cell integrity by electron microscopy.

Such contradictory results may well be caused by the different conditions under which cocci were formed and also by different conditions under which reversion experiments or viability assays were performed. This study therefore, analyses the process of ageing of spiral *C. jejuni* cells under different environmental conditions such as decreased nutrient availability and low temperature. Samples were taken at regular time intervals and culturability and cell morphology were examined. To monitor metabolic changes, intracellular ATP concentrations were determined and membrane fatty acid analyses were performed. Furthermore, the effect of chloramphenicol-induced inhibition of protein synthesis and DNA-damaging gamma radiation on the process of coccus formation was investigated.

MATERIALS AND METHODS

Campylobacter strains and culture conditions

The *C. jejuni* strain, part of our laboratory collection, WAU104 was isolated from sewage (Koenraad *et al.*, 1994). Furthermore, we isolated strain LU101090 from chicken liver. Strain 85Y500, isolated from human faeces, and B258, isolated from chicken faeces, were obtained from the National Institute of Public Health and Environmental Protection (Bilthoven, The Netherlands). *C. jejuni* strain ATCC 33560 was originally

isolated from bovine faeces. All strains were maintained in 15-20% (v/v) glycerol in brain heart infusion broth (BHI, 0037-01-6, Difco Laboratories, Detroit, USA) with glass beads at -80°C. For culturing, one glass bead was removed and inoculated in 10 ml BHI. After 30-48 h the strains were subcultured in 50-500 ml BHI for 20-28 h. Plate counts were performed by spread plating decimal dilutions of cell suspensions on Columbia agar base (CM331, Oxoid, Basingstoke, UK) with 5% (v/v) defibrinated, lysed horse blood (CAB). All cultures were incubated micro-aerobically at 37°C (unless otherwise stated) in jars or incubators flushed with a gas mixture of 5% O_2, 10% CO_2 and 85% N_2.

Survival curves of *C. jejuni*

Cells grown at 37°C in BHI, were harvested by centrifugation at 3,000 × *g* and resuspended in either BHI (pH 7.4), 50 mM potassium phosphate buffer (PPB, pH 7), or 50 mM potassium phosphate buffer containing 0.85% (w/v) NaCl (PPBS, pH 7). Cell suspensions were incubated stationary in the dark at 4°C, 12°C and 25°C. Samples were taken regularly to determine plate counts on CAB, presented as $\log_{10}$(colony forming units(CFU)/ml) and for examination of morphology using phase contrast microscopy (total magnification 1,600 ×, Zeiss standard 20, Oberkochen, Germany).

ATP measurements

For determination of ATP concentration, 1 ml of cell suspension was centrifuged (20,000 × *g*). The supernatant was frozen (for determination of extracellular ATP concentration) and the pellet was resuspended in 1 ml fresh BHI or buffer (for determination of intracellular ATP concentration) and subsequently frozen. For ATP analyses, frozen samples of cells and supernatants of cells were thawed at 4°C and the ATP concentration of 100 µl of sample was determined in a Biocounter M2010 (Lumac, Landgraaf, The Netherlands) according to the manufacturer's instructions with the NRB/LUMIT-PM kit (LUMAC 9268-7, Lumac). Standard curves were constructed with ATP (A 5394, Sigma, Chemical Co., St. Louis, USA) diluted in BHI, PPBS and PPB. Cell volumes were estimated to be approximately the same for spiral and coccoid cells, although minor differences might arise in cell length of the spirals (Griffiths, 1993) and in diameter of the cocci (data not shown). From the observed dimensions, the internal volume of one (spiral or coccoid) cell was estimated to be 6×10^{-16} l.

Two-dimensional gel electrophoresis

Cell suspensions of spirals and cocci formed in BHI following incubation at 4°C and 25°C (strains WAU104, B258, and ATCC 33560) were harvested and frozen as a pellet. For protein analyses, pellets were thawed at 4°C and incubated for 30 min at 37°C in lysis buffer (9 M urea, 2% [v/v] Triton X-100, 2% [v/v] 2-mercaptoethanol, 2% [v/v] Pharmalyte 3-10 and 8 mM phenylmethylsulfonyl fluoride). Two-dimensional gel electrophoresis was performed with Immobiline DryStrip, pH 3.0-10.5 (no. 18-1016-61, Pharmacia Biotech AB, Uppsala Sweden) and ExcelGel SDS, gradient 8-18 (no. 80-1255-53, Pharmacia Biotech AB) according to the manufacturer's instructions. After two-dimensional electrophoresis, gels were silver stained, recorded with a Sony XC 77CE camera and image analyses were performed with the Gemini program (Applied Imaging Int. Ltd., Tyne & Wear, UK).

Chloramphenicol treatment

Addition of chloramphenicol at 10 μg/ml resulted in growth inhibition of *C. jejuni* indicating that protein synthesis was indeed inhibited under these conditions. An overnight culture was used to inoculate (0.1% v/v) six 150 ml volumes of BHI which were incubated for 20 h micro-aerobically at 37°C. Chloramphenicol (10 μg/ml) was added to three cultures and all were incubated for another 5 h at 37°C to allow the antibiotic to enter the cells. Cell suspensions with and without chloramphenicol were subsequently incubated stationary in the dark at 4°C, 25°C, and 37°C. Samples were removed regularly (every two days during the first week, twice during the second week and once a week subsequently) and assessed microscopically to determine cell morphology, and for ability to grow on agar surfaces.

Radiation of cells

Overnight *C. jejuni* cultures were exposed to ionizing gamma-radiation (2 kGy) at the pilot radiation plant (PROVO, Wageningen, The Netherlands). Cell suspensions (100 ml) were subsequently incubated stationary at 4°C or 37°C in the dark. Samples were taken at regular intervals to determine plate counts and morphology (see above).

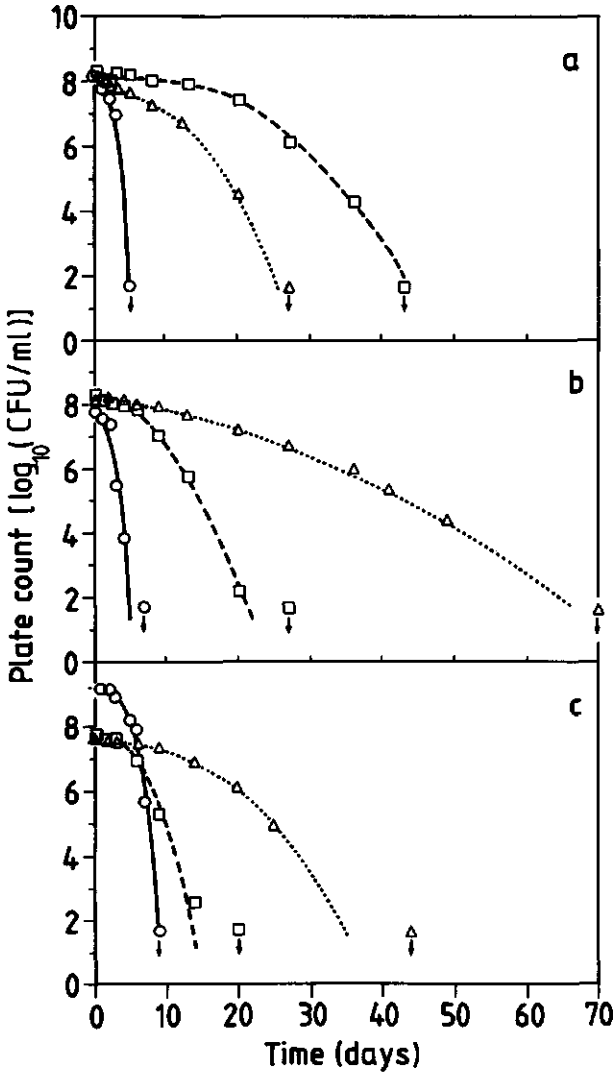

Fig. 1. Survival curves of *C. jejuni* strain WAU104 in PPB (a), PPBS (b) and BHI (c). Cells were incubated at 4°C (△), 12°C (□) and 25°C (○). Points with downward arrows indicate values below detection limit ($\log_{10}$(CFU/ml) ≤ 1.7).

Fatty acid analyses

To determine the fatty acid composition, 500 ml of various strains (*Campylobacter* spirals or cocci formed at different temperatures) were harvested by centrifugation at 3,000 × *g* as already described. Fatty acids from these cell suspensions were methylated and extracted as described by Miller and Berger (1985). For separation, detection, and identification, The Midi Microbial Identification System (MIS, Microbial ID, Inc., Newark, USA) was used. The MIS consists of a Hewlett Packard HP5890A gas-liquid chromatograph with a 25 m x 0.2 mm 5% methylphenyl silicone fused silica capillary column, H_2 as carrier gas and a flame-ionization detector, an automatic sampler, an integrator and a program which identifies the fatty acids, using data of a fatty acid library and a calibration mix of known fatty acids (Microbial ID, Inc.). Principal component analysis was performed on the data and fatty acid compositions of the same cell types were averaged from spiral *C. jejuni* suspensions (n = 11) and from cocci suspensions formed at 4°C, 12°C or 25°C (n = 10, n = 10 and n = 7, respectively). Statistical analyses of the data was performed using the Student's *t* distribution (two-tailed) considering $P \leq 0.01$ to be significant.

RESULTS

Effect of temperature on survival of *C. jejuni* in different media

Survival curves of *C. jejuni* strain WAU104 cells resuspended in PPBS and in BHI show the same characteristics; a decrease in culturability below the detection limit ($\log_{10}$(CFU)/ml) ≤ 1.7) in 40-70 days, 15-20 days and 6-8 days at 4°C, 12°C, and 25°C, respectively (Fig. 1). Cells in PPBS remained culturable for a longer period of time than cells incubated in BHI. In contrast, following resuspension in PPB, culturability dropped below the detection limit in 30, 45, and 5 days at 4°, 12°, and 25°C, respectively. Cells maintained in this buffer at 12°C remained culturable significantly longer than cells at 4°C. Similar results were obtained with the other *C. jejuni* strains (data not shown).

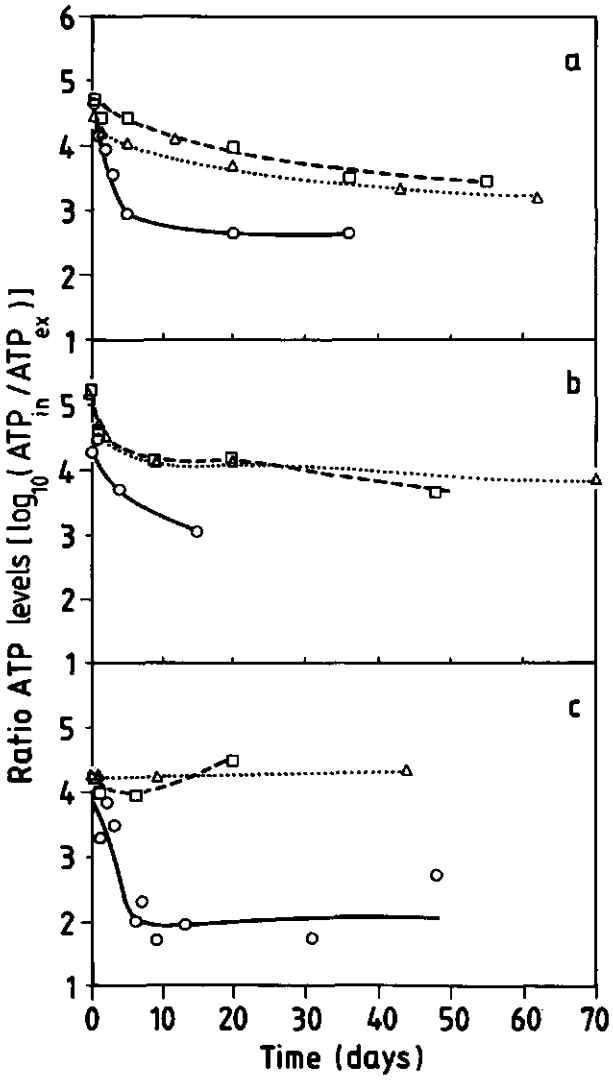

Fig. 2. Ratio of intracellular and extracellular ATP level during ageing of *C. jejuni* strain WAU104 cells in PPB (a), PPBS (b) and BHI (c). Cells were incubated at 4°C (△), 12°C (□), and 25°C (○). Ratio ATP_{in} : ATP_{ex} was determined as described in Materials and Methods.

ATP measurements

During the first week of incubation, a decrease in intracellular ATP level (ATP_{in}), an indication of the cells' energetic state, and an increase in extracellular ATP level (ATP_{ex}), an indication of ATP leakage, were observed in all experiments. After this first week, the levels remained quite constant for several weeks. To facilitate comparison, the ratios of ATP_{in}:ATP_{ex} were calculated and these data are shown in Fig. 2. At 25°C, the ratio is lower in all media when compared to the ratios measured in cells stored at 4° and 12°C.

Cell protein composition analyses

To explore the possible syntheses of new (stress) proteins as means of adaptation, two-dimensional protein profiles of *Campylobacter* spirals and cocci formed at 4°C and 25°C were compared. No major differences in protein patterns of spirals and the various cocci could be detected (Fig. 3). Image analyses indicated similar amounts of spots for spirals (315 spots) and cocci formed at 4°C (306 spots) and 25°C (310 spots). Minor differences observed might be due to limited proteolysis and protein turnover. This might also explain the bulk increase of low molecular weight compounds in cocci formed at 25°C. Similar results were obtained with the other *Campylobacter* strains.

Effect of chloramphenicol on formation of coccoid cells

Addition of chloramphenicol (10 μg/ml) to a growing culture inhibited growth immediately indicating that protein synthesis was blocked (data not shown). The ability of chloramphenicol-treated cells to form colonies on agar plates results from dilution of the antibiotic-containing medium during plating to subinhibitory levels. No irreversible damage due to chloramphenicol treatment was apparent. Cocci appeared in both treated and untreated cell suspensions after 14 and 4 days of incubation at 4°C and 25°C, respectively, whereas the culturability dropped below the detection limit in 43 and 10 days, respectively (Table 1). At 37°C cell counts from suspensions without chloramphenicol remained high for several weeks. Addition of chloramphenicol caused non-culturability in 6 days at 37°C. Microscopic analyses indicated that coccoid forms arose as frequently following chloramphenicol treatment as without the antibiotic at all temperatures tested, indicating that protein synthesis is not essential for the formation of

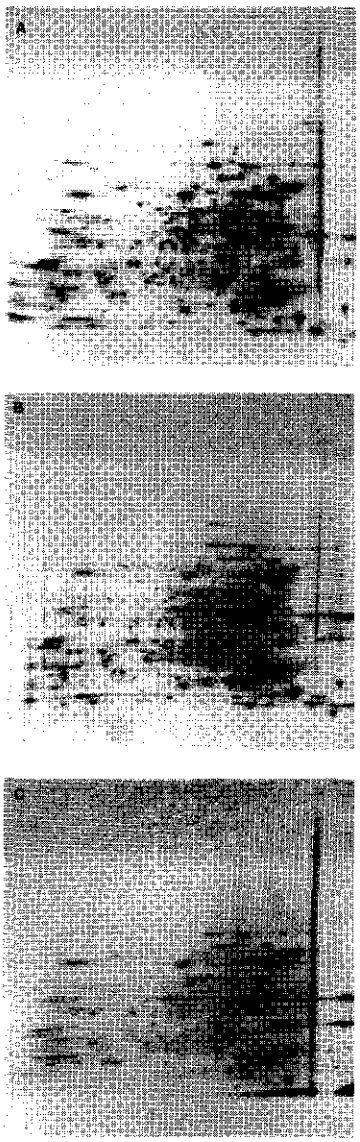

Fig. 3. Two-dimensional protein profiles of *C. jejuni* strain ATCC 33560. Spirals (A) and cocci formed at 4°C (B) and 25°C (C). First dimension: isoelectric focusing pH 10.5 (left) - 3 (right), second dimension: SDS PAGE (8-18%) high molecular weight (top) - low molecular weight (bottom). Proteins were visualized with silver stain.

cocci. Similar results were obtained with the four other strains tested.

Effect of gamma-radiation

The effect of gamma-radiation resulting in DNA damage was examined to investigate whether intact DNA is needed for the formation of cocci. Before radiation, plate counts on CAB of all five strains were approximately $\log_{10}$(CFU/ml)= 8.6 to 8.9. Immediately after radiation (2 kGy) all five, which were observed to be still in the spiral form yielded no colonies as a result of DNA damage, most likely. Microscopic analyses, however, showed that cells were still as motile as nonradiated cells, indicating that cellular energetics had not been affected. Strikingly, in both the radiated and the non-radiated cell suspensions, cocci were observed after 3-5 days at 37°C and after 2-3 weeks at 4°C. Furthermore, no differences were observed in one-dimensional protein analyses of cocci resulting from radiated and untreated cultures (data not shown).

Table 1. Culturability and transformation to coccoid forms of *Campylobacter jejuni* strain WAU104 during ageing of cultures in the absence and presence of chloramphenicol (10 μg/ml).

Storage temperature (°C)	Chloramphenicol	Observation of cocci (days)	Nonculturability[a] (days)
4	+[b]	14	43
4	−	14	43
25	+	4	10
25	−	4	10
37	+	1	6
37	−	1	> 40

[a] Period of time after which plate counts were below detection limit (cells > 99% in coccoid form).

[b] + = Chloramphenicol present.
− = Chloramphenicol absent.

Fatty acid analyses

Although *C. jejuni* is unable to grow at temperatures below 32°C, it was of interest whether adaptation to lower temperatures could be observed to result in changes in the cytoplasmic membrane. Principal component analyses were used to compare the fatty acid compositions of the various samples and a two-dimensional reproduction of a multidimensional analysis was obtained. Analyses of fatty acid profiles of the spiral and various coccoid cell suspensions showed that those of cocci formed at 25°C grouped in a distinct cluster (Fig. 4). Fatty acids profiles of cocci formed at 4°C and 12°C could not easily be discriminated from those of spirals. Only minor differences in fatty acid patterns between strains occurred and fatty acid compositions of the same cell types were therefore averaged to facilitate comparison. Significant differences in amounts of individual fatty acids (contributing more than one per cent of the total amount of phospholipids) could be observed (Fig. 5). Cocci formed at 25°C have a different fatty acid composition when compared to spiral cells: a significant increase in the amount of 16:0 and 18:0 fatty acids and a decreased concentration of 14:0, 16:1 and 19:0 fatty acids. Cocci

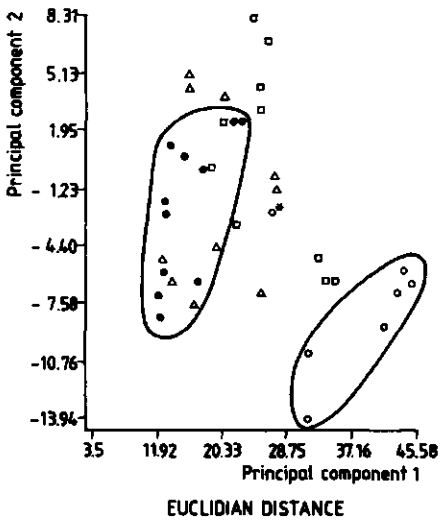

Fig. 4. Two-dimensional principal component analysis of fatty acids of spirals and coccoid cells of *C. jejuni*. Symbols: Fatty acids of spirals (●) and cocci formed at 4°C (△), 12°C (□), and 25°C (○). The spot marked by an asterisk is closer to the 25°C cluster than to the spiral cluster when compared in three-dimensional space.

formed at 12°C show significant increase in 16:0, 18:1 and 18:0 and a decrease in 14:0 and 16:1 fatty acids. The fatty acid composition of cocci formed at 4°C is quite similar to that of spirals, only the amount of 14:0 fatty acids is decreased and 18:1 fatty acids are increased. These results indicate that the fatty acid composition of cocci is strongly influenced by the temperatures at which they are formed.

DISCUSSION

To study the process of ageing of *Campylobacter jejuni* cells, the effect of temperature and resuspension/storage media on the culturability was analyzed. BHI was used as an environment rich in nutrients, PPBS was used as a poor environment but with addition of saline to prevent osmotic shock. PPB was used as an environment comparable with water, containing just a low concentration of phosphate buffer to keep the pH at 7.

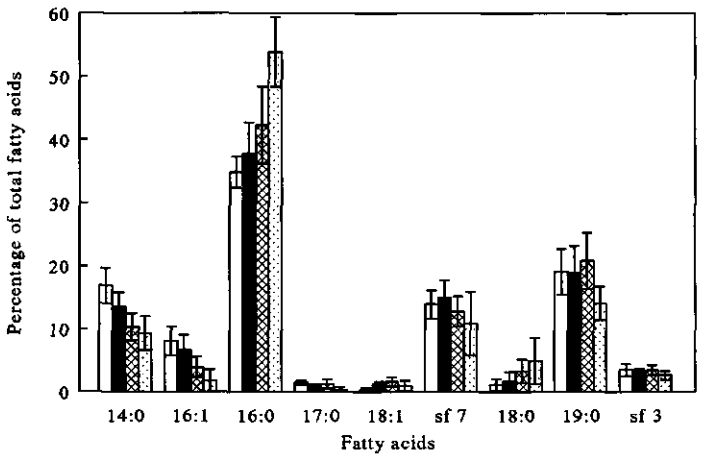

Fig. 5. Fatty acid composition of *C. jejuni* spirals and various coccoid forms. Due to closely eluting fatty acids, no discrimination could be made between 12:0 aldeh., 16:1 iso or 14:0 3OH (represented as sf3) and 18:1 ω7c/ω9t/ω12t, 18:1 ω9c/ω12t/ω7c or 18:1 ω12t/ω9t/ω7c (represented as sf7). Spirals (▭), cocci formed at 4°C (■), 12°C (▩), and 25°C (▨); 16:1 = 16:1 ω7c; 18:1 = 18:1 ω9c; 17:0 = 17:0 cyclo; 19:0 = 19:0 cyclo ω8c.

In all media the culturability decreased faster at higher storage temperatures (Fig. 1), which can be explained by higher metabolic activity of the cells at these temperatures. Cells remain culturable for a longer period of time in PPB and PPBS when compared to BHI. This is likely due to low metabolic activity resulting from decreased nutrient availability leading to a slower rate of transformation to a nonculturable state. The remarkable fact that cells in PPB become nonculturable at a slower rate at 12°C as compared to 4°C, is difficult to explain, but this phenomenon occurred with all the strains tested. Perhaps the combination of osmotic shock (transfer from BHI to PPB) and the greater temperature shock (from the growth temperature of 37°C to the storage temperature of 4°C) is responsible for the observed results. Apparently, temperature is a major factor determining the rate at which cells become nonculturable whereas the presence of nutrients seems to stimulate rather than to retard the process of coccus formation. This is in agreement with results from experiments performed by Rollins and Colwell (1986) who observed, with *C. jejuni* cells suspended in a streamwater microcosm at low temperature, that the culturability was extended by comparison to that observed at higher temperatures.

To determine the energetic state of the cells during ageing, ATP concentrations were determined at regular time intervals. Intracellular ATP concentration decreased while extracellular ATP increased during the first week of incubation. Subsequently, levels remained, for the most part, constant, indicating that the cells are able to maintain a certain level of ATP while their culturability is below the detection limit. Apparently, there is no strict correlation between the ratio ATP_{in} : ATP_{ex} and culturability. Cells incubated at 4°and 12°C show similar ATP_{in} : ATP_{ex} ratios in all media tested. At both temperatures, the ratios decreased during the first week and then remained quite constant at a log-ratio of 3.5-4. In contrast, cells kept at 25°C showed a lower log-ratio of 2 and relatively more ATP present outside the cells due to increased ATP leakage and/or cell lysis. These observations suggest that cocci formed at 25°C are more seriously damaged than cocci formed at low temperatures. While ATP levels, and in some instances adenylate energy charge, have been related to cell viability during starvation of *Escherichia*, *Zymomonas* and *Peptococcus* (Kjelleberg *et al.*, 1987), apparently in *C. jejuni* no such relationship exists between ATP levels and viability.

Whole-cell protein profiles of spirals and cocci formed at different temperatures

were compared to explore the possibility of new proteins being formed during the process of coccus development. The induction of stress proteins in starvation situations has been described for several micro-organisms, such as *Escherichia* (Reeve *et al.*, 1984) and *Vibrio* (Holmquist *et al.*, 1993). However, in *C. jejuni* the appearance of newly synthesized proteins in coccoid forms could not be detected. This was confirmed in experiments in which protein synthesis was blocked by chloramphenicol (Sjögren *et al.*, 1992; data not shown) where formation of coccoid *Campylobacter* cells proceeded at a rate similar to untreated cells (Table 1). Exposure of cells to gamma-radiation induces single and/or double strand breaks in the DNA thereby preventing DNA transcription (Ward, 1975). *C. jejuni* was not able to form colonies after gamma-radiation (2 kGy) which is consistent with a D_{10} value of 0.2 (Clavero *et al.* 1994). However, radiation of the cells did not influence the process of coccus formation (data not shown). Furthermore, the protein composition of these coccoid cells was essentially the same as that of coccoid cells derived from untreated cultures (see above). These results indicate that *Campylobacter* cocci formation can occur in the presence of protein synthesis inhibitors and gamma-radiation, therefore *de novo* synthesis of proteins may not be required for the transition of spirals to coccoid forms suggesting that this is a passive process in *C. jejuni*.

Fatty acid analysis is currently employed for identification of a wide variety of bacteria, since it involves a relatively simple sample preparation and has a high reproducibility (Welch, 1991). This method has also been used successfully for the identification of *Campylobacter* species (Leaper and Owen, 1981; Wait and Hudson, 1985) and differentiation to subspecies level (Coloe *et al.*, 1986). The fatty acid compositions of spirals described in this paper were comparable to those previously published for *Campylobacter* (Coloe *et al.*, 1986; Moore *et al.*, 1994), the predominant acids in spirals being 14:0, 16:1, 16:0, 18:1 and 19:0. Linder and Oliver (1989) described an increase of short chain fatty acids in nonculturable cells of *Vibrio vulnificus* formed at 5°C, which was explained by the need of the micro-organism to maintain fluidity of the membrane at this low temperature. In *Listeria monocytogenes* a similar phenomenon has been described in that cells grown at low temperature exhibit increased amounts of short chain length fatty acids (Püttmann *et al.*, 1993). Significantly however, *L. monocytogenes* and *V. vulnificus* are able to grow at these low temperatures whereas *C. jejuni* only grows at temperatures

above 32°C. Therefore, we investigated whether an adaptation of the fatty acid composition could be observed in coccoid cells formed at temperatures below 32°C, i.e. those to which *C. jejuni* will be exposed in the environment. Strikingly, the fatty acid composition of cocci formed at 4°C and to a lesser extent at 12°C, showed a remarkable similarity to the fatty acid composition of spirals. Apparently, *C. jejuni* does not adapt the composition of fatty acids in the membrane during transition to the coccoid form at low temperatures. The fatty acid composition of cocci formed at 25°C does differ significantly, however, from that of spirals (Figs. 4 and 5). The observed increase in 16:0 and 18:0 fatty acids and the concomitant decrease in the amounts of 14:0, 16:1, and 19:0 fatty acids at 25°C is not in line with an active adaptation of the fatty acid composition to low temperature (see above) and might be caused by nonspecific reactions. The induced changes in the fatty acid composition in these cocci apparently result in leaky membranes such that ATP is lost from the cells (Fig. 2). We have previously shown by using a PCR technique that cocci formed at high temperatures (25°C and 37°C) had a significantly reduced DNA content in contrast to cocci formed at low temperatures where DNA levels were similar to that of spiral cells (Hazeleger *et al.*, 1994). The transformation from spirals to coccoid cells has previously been studied extensively at higher temperatures, ranging from 30°C to 42°C, and in these studies a significant degeneration of these coccoid cells was observed (Moran and Upton, 1986, 1987a, 1987b). Notably, the conditions applied in these studies do not mimic the environmental conditions to which *C. jejuni* cells are exposed upon excretion by the human or animal host into the environment i.e. low nutrient concentrations and low temperature.

In conclusion, there are many different coccoid forms of *C. jejuni* and their distinct characteristics are determined by the conditions under which they are formed. Furthermore, *de novo* protein synthesis is not required for the transition of *Campylobacter* spirals to coccoid forms indicating that this a passive process. Cocci formed at high temperatures show significant degeneration, whereas cocci formed at 4°C show characteristics similar to that of spirals. The latter cocci may well play a role in the contamination cycle of *C. jejuni*. An important role of spiral *Campylobacter* cells in this cycle cannot be excluded since these forms remain culturable for more than two months under environmental conditions.

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CHAPTER 8

AN ESTIMATION OF WATER-BORNE *CAMPYLOBACTER* INFECTIONS: RECREATION WATER AS POTENTIAL SOURCE OF HUMAN CAMPYLOBACTERIOSIS[1]

Sentinel studies showed that in the Netherlands 1,800-2,300 per 100,000 individuals suffer annually from campylobacteriosis. Several case-control studies identified eating undercooked chicken, drinking undisinfected water, drinking raw milk, and to a lesser degree living in a household with a cat or dog as main risk factors. For the Dutch situation, no information about the contribution of water in human *Campylobacter* infections is known. In the present study, the contribution of contaminated recreational water in human *Campylobacter* infections is estimated. The estimation is based on the observed contamination level of recreational waters, the reported dose-response relationship, and the known infection experiments in human volunteers. The incidence of campylobacteriosis due to exposure to contaminated recreational waters is estimated to range between 1.2 and 170 per 100,000 individuals. This exercise indicates that the attribution of water-borne campylobacteriosis cases is low but may be higher as assumed till now.

[1] **Authors:** P.M.F.J. Koenraad and S.H.W. Notermans.

INTRODUCTION

In the early 1980s *Campylobacter* species were recognized as a major cause of diarrhoeal disease. From sentinel and population studies in the United States of America and the Netherlands, the annual incidence of campylobacteriosis could be estimated to be 1,000/100,000 (Sacks *et al.*, 1986) and 1,800-2,300 per 100,000 individuals (Notermans and Van De Giessen, 1993). Main risk factors in human *Campylobacter* infections, identified by case-control studies, are consumption of undercooked chicken meat (Kapperud *et al.*, 1992), drinking untreated water (Rautelin *et al.*, 1990), drinking raw milk (Harris *et al.*, 1987), and to a lesser degree living in a household with a cat or dog (Deming *et al.*, 1987). A case-control study conducted in the United States of America showed that eating poultry accounted for the majority of all human campylobacteriosis cases, but still 8% could be attributed to drinking contaminated water (Seattle-King County Department of Public Health, 1984). Especially large outbreaks are associated with drinking undisinfected recreational water (Aho *et al.*, 1989; Alary and Nadeau, 1990; Millson *et al.*, 1991). Indeed, many studies report a high level of contaminated surface waters with *Campylobacter* (Stelzer and Jacob, 1991; Koenraad *et al.*, 1994; Stampi *et al.*, 1992).

In the Netherlands, sentinel studies were carried out to estimate the annual incidence of human campylobacteriosis (Notermans and Van De Giessen, 1993). However, no information about the contribution of water in the infections is available. In the present study, the contribution of contaminated water in human *Campylobacter* infections is estimated. This estimation is based on the observed contamination of recreational waters (Koenraad *et al.*, 1994), the dose-response relationship deduced by Rose and Gerba (1991), and infection experiments in human volunteers as reported by Black *et al.* (1992).

STARTING-POINTS

The estimation of human *Campylobacter* infections caused by ingestion of *Campylobacter* cells present in recreational waters is composed of the following aspects;

* Number of individuals that may be exposed to *Campylobacter*, which can be deduced

from the number of day trips with water recreation as the main activity (Netherlands Central Bureau of Statistics, 1992).

* Number of *Campylobacter* cells, to which humans staying near/in recreational waters, may be exposed (Koenraad *et al.*, 1994; This thesis, Chapter 3).
* Number of infected persons, which can be deduced from the dose-response relation as observed by Rose and Gerba (1991).
* The assumption that illness, defined as the manifestion of clinical symptoms of *Campylobacter* enteritis, is preceeded by infection.

Assumptions concerning infection caused by *Campylobacter*.

The dose-response model of Rose and Gerba (1991; Table 1) is based on the hypothesis that only a single organism needs to survive and multiply in the gastro-intestinal tract of the host to cause an infection and provoke a response. With this model the probability for one exposed person to become infected can be estimated. Infected individuals are defined as persons who gave a *Campylobacter*-positive stool sample but not necessarily with campylobacteriosis symptoms.

Assumptions concerning illness caused by *Campylobacter*

Only infected individuals can become ill and develop symptoms specific for campylobacteriosis, namely diarrhoea and/or fever. Black *et al.* (1992) initiated volunteer

Table 1. Dose-response model for probability for a person to contract a *Campylobacter* infection (Rose and Gerba, 1991).

Dose-response model of Rose and Gerba (1991):

$$P_i = 1 - (1+N/\beta)^{-\alpha}$$

P_i = Probability for one person to become infected.
N = Average exposure to *Campylobacter* cells.
α, β = Parameters that describe the infectivity of the organism. For *Campylobacter*, α and β have the values of 0.039 and 55, respectively.

studies to establish a model of *C. jejuni* infection, including establishing the number of organisms needed to cause illness. A shortcoming of these experiments was the lack of challenges with low numbers of *Campylobacter* cells. An actual dose-response relation between exposure and illness could not be deduced in this study. However, it could be deduced that 33% of the infected individuals really develop enteritis (Black *et al.*, 1992).

Number of individuals exposed to *Campylobacter* during recreation

During 1990-1991, the Netherlands Central Bureau of Statistics investigated several features of one-day outings, such as frequencies and main activities (Netherlands Central Bureau of Statistics, 1992). A day trip was defined as a recreative excursion during at least two hours (ending with home-coming in own house), excluding visits to family or acquintances. Every day trip represented one individual. The number of day trips in 1990-1991 with swimming, sunbathing and picnicking near a pond or lake as main activities are summarised in Table 2 as function of the season. In 1990-1991, 8.6×10^6 day trips with swimming or picnicking as main activities were made.

Occurrence of *Campylobacter* in recreational waters

In a survey on the occurrence of *Campylobacter* in sewage purification plants (Koenraad *et al.*, 1994; This thesis, Chapter 3), recreational waters in the neighbourhood were also sampled and analysed on the numbers of *Campylobacter* by the most probable number technique. The detection limit of the applied enrichment procedure is 1.5 $\log_{10}$ (MPN/100 ml). Out of 25 samples, 16 analyses confirmed the presence of *Campylobacter*. The numbers of *Campylobacter* in the positive samples ranged from 1.6 to 3.6 $\log_{10}$ (MPN/100 ml).

CALCULATIONS

Only the summer day trippers are included in the estimation, because it is likely that only this category actually is exposed to water. The number of day trippers going out during spring, autumn and winter is only 5% of the total number. Furthermore, it is assumed that a variable number of the summer day trippers are exposed to *Campylobac-*

ter cells (5%, 10% or 25%). The number of *Campylobacter* cells to which an individual is exposed, is dependent on the ingested volume of recreational water, so the exposure is calculated for various volumes (2ml, 5ml, and 10ml).

The probability for one person of contracting a *Campylobacter* infection is estimated from the observed numbers of *Campylobacter* in recreational waters (Koenraad *et al.* 1994), and by using the model of Rose and Gerba (1991). Swimming in water with an average concentration of 1×10^{3} *Campylobacter* cells per 100 ml and ingesting 10 ml water, would result in an average exposure of 100 cells and subsequently in a probability of 4×10^{-2} of contracting a *Campylobacter* infection. The number of exposed individuals ranged from 4×10^{5} to 2×10^{6}, depending on the percentage of swimming persons during their day trip. So in this calculation example, the numbers of infected persons in the Dutch population at large would range from 1.6×10^{4} to 8×10^{4}. In Table 3, the estimated number of infected persons per 100,000 are presented as function of the percentage of swimming day trippers and of the ingested volume. The numbers of infected persons ranged from 3.6 to 506.0 per 100,000 exposed persons.

From Black *et al.* (1992), it could be estimated that 33% of the infected persons

Table 2. Day trips in the Netherlands, involving water recreation near ponds or lakes, as function of the season (1990-1991) (Netherlands Central Bureau of Statistics, 1992).

Season (1990-1991)	Number of day trips (×1,000)
	Recreation near pond and/or lake[a]
Autumn	129
Winter	48
Spring	276
Summer	8,179
Total (1990-1991)	8,632

[a] Day trips with swimming, sunbathing or picnicking as main activities.

really develop symptoms of campylobacteriosis. From the estimated number of infected

persons (Table 3), it can be calculated that the number of human *Campylobacter* enteritis cases due to exposure to contaminated surface water would range between 1.2 and 170 per 100,000 individuals.

DISCUSSION

Based on the dose-response model of Rose and Gerba (1991) it can be estimated

Table 3. Estimation of the number of *Campylobacter* infections due to exposure to contaminated surface water, as function of digested volume and percentage of exposed day trippers.

Average concentration of *Campylobacter* cells[a] (Number/100 ml)	Digested volume (ml)	Estimated number of *Campylobacter* infections per 100,000 exposed persons[b] as function of percentage exposed day trippers[c]		
		5%	10%	25%
1×10^2	2	3.6	7.1	17.8
	5	8.7	17.3	43.3
	10	16.6	33.2	83.0
2.5×10^2	2	8.7	17.3	43.3
	5	20.3	40.7	102.0
	10	37.1	74.2	185.0
5×10^2	2	16.7	33.2	83.0
	5	37.1	74.2	185.0
	10	63.7	127.0	318.0
1×10^3	2	30.7	61.5	154.0
	5	63.7	127.0	318.0
	10	101.0	202.0	506.0

[a] Derived from Koenraad *et al.* (1994).

[b] Estimated from the dose-response model of Rose and Gerba (1991).

[c] It is assumed that 8.2×10^6 persons are exposed to *Campylobacter* during recreation near a lake (Netherlands Central Bureau of Statistics, 1992).

that in the Netherlands annually between 3.6 and 506 per 100,000 persons become infected with *Campylobacter*, due to exposure of contaminated recreational water. This estimated risk of human *Campylobacter* infections caused by exposure to contaminated recreational water has however its limitations:

* Only day trips with main activities swimming, sunbathing or picnicking near a lake or pond are included in this study. Holiday-makers, persons active in water-sports or persons sojourning near the sea are not included.
* In this study the ratio of infected persons and ill persons was derived from the volunteer study reported by Black *et al.* (1992). These authors challenged with *Campylobacter* strains isolated from patients. The environmental *Campylobacter* strains in these aquatic environments could have different virulence and infectivity features (Newell *et al.*, 1985). Furthermore, the effect of the survival of *Campylobacter* strains in aquatic environments on their infectivity is still unknown.
* The effect of acquired immunity after repeated exposure is not accounted for in the present estimation. In this context, it should be considered that 67% of the infected persons do not develop symptoms (Black *et al.*, 1992).

In this study it is estimated that in the Netherlands during summer between 1.2 and 170 per 100,000 exposed individuals could suffer from campylobacteriosis caused by exposure to contaminated recreational water. For the total Dutch population, this annual incidence would range between 180 and 27,000 campylobacteriosis cases. It is obvious that this exercise has its shortcomings. However, combining the estimated annual Dutch incidence of 1,800-2,300 campylobacteriosis cases per 100,000 individuals (Notermans and Van De Giessen, 1993) with the reported 8% attribution of *Campylobacter* human infections caused by exposure to contaminated water observed in a case-control study in the United States of America, results in a comparable incidence of 26,000 individuals per year. This exercise indicates that the incidence of human *Campylobacter* infections attributed to exposure to contaminated water may not be negligible. A national case-control study on human *Campylobacter* infections has to be recommended in order to determine precisely the annual incidence of campylobacteriosis and the attribution of water-borne cases.

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CHAPTER 9

GENERAL DISCUSSION[1]

INTRODUCTION

During the last decade clear evidence has been obtained that in developed countries campylobacteriosis is a commonly occurring enteritis, with considerable economic and social consequences. *Campylobacter* infections are likely to be more common than *Salmonella* infections (Notermans and Van De Giessen, 1993) and the lower reported incidence rates merely reflect the limitations of surveillance as currently carried out (Blaser *et al.*, 1983).

It has been established that the intestinal tract of many warm-blooded animals is the natural habitat of *Campylobacter*. As a consequence of the faecal origin of this organism, warm-blooded animals are the main sources of contaminated food. However, transmission routes have not yet been completely elucidated. In the elucidation of the transmission routes and contamination cycles of *Campylobacter*, it has to be taken into account the phenomenon of the so-called viable but nonculturable stage of *Campylobacter* (Rollins and Colwell, 1986). Furthermore, the significance of water in the transmission of the *Campylobacter* is not yet clear.

Poultry products, raw milk, untreated surface water, and pets are considered as the dominant sources of infection. Knowledge about the significance of these vectors in transmitting *Campylobacter* organisms has been gained during the past two decades (Griffiths and Park, 1990; Park *et al.*, 1991; The National Advisory Committee on Microbiological Criteria for Foods, 1994). Outbreak-associated cases are related to consumption of raw milk and untreated surface water, and single cases of campylobacteriosis may be associated with the consumption of undercooked poultry meat and to a lesser degree with contact with pets (Tauxe, 1992).

In summary, the significance of sewage and surface waters in the epidemiology of *Campylobacter* as well as the role of viable but nonculturable cells has to be established.

[1] Part of this chapter is submitted as a review. Authors: P.M.F.J. Koenraad, F.M. Rombouts, and S.H.W. Notermans.

Therefore, in this thesis the occurrence, the survival possibilities and the origins of *Campylobacter* in sewage and surface waters were investigated in an attempt to assess the public health risk. Additionally, the contribution of nonculturable coccoid *Campylobacter* cells was evaluated.

DETECTION OF *CAMPYLOBACTER* IN SEWAGE

The procedures for the detection of *Campylobacter* from food and environmental samples consists generally of an enrichment step, which includes when necessary a non-selective pre-enrichment, a selective enrichment, and an isolation step (Fricker, 1987). A great variety of enrichment broths and isolation media has been proposed for the isolation of *Campylobacter* from clinical, veterinary, and food samples (Goossens *et al.*, 1986). However, it is advisory to compare the efficacy of these various media for each specific application, which in this thesis was the isolation of *Campylobacter* from sewage samples. Eight combinations of two enrichment broths ([a] Preston broth {PB} and [b] charcoal cefoperazone deoxycholate broth {CCDB}) with four isolation media ([1] Columbia agar base {CAB^+}; [2] *Campylobacter* blood-free selective medium-modified CCDA-Preston {CCDA}; [3] *Campylobacter* blood-free selective medium-modified CCDA improved {$CCDA^+$}, and [4] *Campylobacter* agar base Karmali {KAR}) were evaluated by applying the most probable number technique (Table 1, Chapter 2). The isolation efficacy of the various combinations did not differ significantly; this agrees with the observations of Höller (1991) and Dousse *et al.* (1993), but is at variance with the results of Korhonen and Martikainen (1990). Only based on practical aspects, such as blood availability, costs, and convenience of preparation, the charcoal-based media can be recommended for isolation and were used in this thesis for detecting *Campylobacter* in sewage.

Conventional isolation and the subsequent identification *Campylobacter* by biochemical tests lasts three days. In Chapter 3 the applicibility of an immunological agglutination assay and the culture PCR assay as described by Giesendorf *et al.* (1992) were evaluated for rapid identification and detection of *Campylobacter* in sewage enrichments. For the individual enrichments, the PCR culture assay had a significantly

higher detection ability than the conventional isolation on CAB^+ only, or on at least CAB^+ or CCDA. Isolation on at least one of four isolation media tested had also a lower detection ability than PCR. For qualitative analyses concerning the presence or absence of *Campylobacter* in sewage samples, PCR and conventional culturing could detect similar numbers of *Campylobacter*-positive samples.

Results presented in Chapter 2 indicate an effect of the amount of enriched sewage. For qualitative detection by conventional isolation, enrichment of 1 ml of a 10^{-1} or 10^{-2} dilution of sewage in 9 ml enrichment broth is recommendable. However, PCR can detect *Campylobacter* quite well in enrichments of 1 ml undiluted sewage or in enrichments of 1 ml of the 10^{-1} dilution. *Campylobacter* cannot easily compete with the accompanying flora (Rosef *et al.*, 1987) and therefore, it is likely that a dilution step prior to enrichment reduces the influence of competing flora.

The applicability of the latex agglutination assay (LAA) was also evaluated by comparing the results obtained by the conventional isolation procedure. Overall, the LAA could detect only 39% of *Campylobacter*-containing enrichments. Especially, for PB enrichments LAA had a low overall predictive value for positive enrichments (4%). The low overall predictive value of LAA can be explained by the observed high detection limit of the LAA (6 $\log_{10}$(CFU/ml) (Hazeleger *et al.*, 1992; This thesis, Chapter 2). Such high numbers are not always observed in *Campylobacter*-positive enrichments. Other investigators have also shown a low predictive value as well as a high rate of false positive reactions for immunological assays (Reina and Muñoz, 1993).

For qualitative analyses, both the conventional culturing procedure and the PCR assay are satisfactory. However, within the conventional isolation procedure diluted sewage should be enriched but for PCR analyses undiluted enriched sewage may be applied. From the results presented in this thesis, a quantitative, unambiguous method with a high recovery rate, high selectivity and general applicability for the detection of *Campylobacter* in sewage could not be recommended.

CAMPYLOBACTER IN SEWAGE

A two-year survey was carried out on the occurrence of *Campylobacter* in three

sewage purification plants; two trickling filter systems and one activated sludge system (Table 1, Chapter 3). This survey clearly showed that sewage may be considerably contaminated with this pathogen in numbers ranging from 2.6 to 3 $\log_{10}$(MPN/100 ml). Similar high levels of sewage contamination are reported by Höller (1988), Stampi *et al.* (1992), and Stelzer and Jacob (1991). It became clear that the number of *Campylobacter* organisms present in sewage is strongly related to the type of industries in the drainage area of the purification plant, as was also observed by Höller (1988) and Jacob *et al.* (1991). Especially, poultry abattoir drain water was found to be highly contaminated with *Campylobacter* (more than 7 $\log_{10}$(MPN/100 ml)). When meat-processing industrial activities are absent, the numbers of *Campylobacter* found in sewage were lower: 2.3 $\log_{10}$(MPN/100 ml). In these cases the numbers of *Campylobacter* organisms may originate from infected humans. Sentinel studies carried out in the Netherlands, have revealed that 2-3% of the Dutch population suffers annually from *Campylobacter* enteritis (Notermans and Hoogenboom-Verdegaal, 1992).

For the activated sludge system, dilution by a factor of 50 during transport in the sewerage could only partly explain the observed lower numbers in sewage (3 $\log_{10}$ (MPN/100 ml)) as compared to those in poultry abattoir drain water (5 to 7 $\log_{10}$ (MPN/100 ml)). Therefore, for the activated sludge system it is likely that other contamination sources than the drain water of the poultry abattoir, are negligible in numbers.

Many investigators observed a seasonal variation in the numbers of *Campylobacter* present in sewage. Both the counts and frequency of isolation usually peak around the summer months May to September (Höller, 1988; Jones *et al.*, 1990; Stampi *et al.*, 1992). In our studies, only for the activated sludge system there appeared to be a seasonal variation in the occurrence of *Campylobacter* in sewage. Furthermore, the trend of the variation in our studies was reversed as compared to the former studies; during August the numbers were in average 1 $\log_{10}$ unit lower than during the rest of the year. This decrease in *Campylobacter* numbers concurred with the onset of the increase in infections of chickens and humans (Jacobs-Reitsma *et al.*, 1994; Hoogenboom-Verdegaal *et al.*, 1990). Therefore, it is likely that the decline of the sewage contamination should be explained by an environmental factor, such as water temperature, affecting the survival of this pathogen in water.

For the activated sludge system several environmental parameters (temperature,

pH, pO_2, intensity of sunlight and rainfall) were available and a possible correlation with the prevalence of *Campylobacter* was studied (Table 4, Chapter 3). Water temperature ranged between 10.7 and 20.1, pH was rather constantly around 7 and the average pO_2 was 42%. Rainfall determined as weekly average varied between 0 and 8.6 mm. Also the intensity of sunlight varied weekly; range 137-2607 J/cm^2. However, the occurrence and reduction of *Campylobacter* in the activated sludge system was not correlated with any of these parameters, which agrees with the observations of Höller (1988). On the contrary, Gondrosen (1986) observed that the survival of this pathogen in aquatic environments was influenced by temperature. Furthermore, Jones and Telford (1991) presumed a lethal effect of sunlight. However, in our case a lethal effect of sunlight is questionable, because of the rather high turbidity of sewage and the low degree of exposure to sunlight.

In all three purification plants, the numbers of *Campylobacter* were reduced, in the activated sludge system with 1 $\log_{10}$ unit (=90%) and in the trickling filter by 0.6 $\log_{10}$ unit (=75%). The decline in the activated sludge system was similar to the values reported by Betaieb and Jones (1990), Höller (1988) and Jacob *et al.* (1991), but was lower than the decrease reported by Stampi *et al.* (1992). The reduction observed in the trickling filter was much lower than the 99.9% abatement which was observed by Arimi *et al.* (1988) in a similar system.

During aerobic purification as much oxygen as possible is dissolved in the sewage, resulting in an average pO_2 of 40% in the investigated activated sludge system. Beforehand, *Campylobacter* had already been exposed to oxygen in the sewers. Therefore, the total effect of the exposure to oxygen is difficult to quantify. Considering the oxygen sensitivity of *Campylobacter*, it is remarkable that *Campylobacter* is still detectable in the effluxes of these systems.

Since *Campylobacter* can easily be isolated from surplus sludge, the reduction can partly be explained by attachment of cells to particles, as reported by Arimi *et al.* (1988) and Höller and Schomakers (1994) or by ingestion by protozoa (King *et al.*, 1988).

In summary, the prevalence and reduction of *Campylobacter* in sewage purification plants is probably influenced by several factors such as pO_2 and attachment to particles. However, the reduction is not complete and numbers of *Campylobacter* in the efflux varying from 1.6 to 2 $\log_{10}$(MPN/100 ml) are common.

CAMPYLOBACTER IN SLUDGE

A considerable number of the analysed samples of both fresh surplus sludge and sedimented sludge were contaminated with *Campylobacter* organisms and the numbers estimated by the most probable number technique averaged 2.4 $\log_{10}$(MPN/100 ml). In these studies (Chapter 3), aerobic digestion of sludge eradicated *Campylobacter* completely. These findings agree with the observations of Betaieb and Jones (1990), Jones *et al.* (1990), and Stelzer and Jacob (1991). Therefore, the spread of *Campylobacter* in the environment due to agricultural application of aerobically digested sewage sludge may be assumed to be a low risk.

CAMPYLOBACTER IN SURFACE WATER

As already indicated, *Campylobacter* can pass sewage purification plants and thus effluxes of these plants may contribute to *Campylobacter* contamination of surface waters. The investigation described in this thesis (Chapter 3) clearly showed that surface waters are indeed regularly contaminated with this pathogen. The efflux of a purification plant may contain 1.6 $\log_{10}$(MPN/100 ml), or even 2 $\log_{10}$(MPN/100 ml) when a meat-processing plant is present in the drainage area. The observed numbers of *Campylobacter* in surface waters varied between 1.5 and 4.2 $\log_{10}$(MPN/100 ml) when a meat-processing industry was present in the drainage area. When meat-processing industries are absent, the numbers of *Campylobacter* present in surface waters are lower; in average 1.6 $\log_{10}$(MPN/100 ml). The results of Stelzer and Jacob (1991) confirm our findings. The daily release of *Campylobacter* from a purification plant, which receives drain water from meat-processing industries, with average numbers of *Campylobacter* of 2 $\log_{10}$ (MPN/100 ml) in the efflux and with a flow rate of 40,000 m^3 per day, amount about 4×10^{10} *Campylobacter* cells. Consequently surface waters, which receive these effluxes, are continuously contaminated with this pathogen. Therefore, a larger reduction of numbers of *Campylobacter* during purification has to be recommended. However, the contribution of wild-living birds in the contamination of surface waters has to be taken into account as well.

SOURCES OF *CAMPYLOBACTER* PRESENT IN SEWAGE AND SURFACE WATERS

The significance of poultry abattoir drain water for providing a constant flow of *Campylobacter* onto sewage purification plants and indirectly on surface waters became obvious from both the two-year survey (Chapter 3) and the short-term investigation in the activated sludge system (Chapter 4). In order to prove that poultry abattoirs contribute in the *Campylobacter* contamination of sewage, the isolates from these two investigations were characterized by serotyping, by polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) typing and by antimicrobial susceptibility testing.

In the short-term investigation (Chapter 4), the serotyping results showed some common types among poultry abattoir drain water and sewage, but a transmission route from *Campylobacter* types present in the processed broilers via drainage of waste water towards the sewage plant could not be deduced. Considering differences in survival possibilities of various *Campylobacter* types, it is likely that the transport in the sewerage affects the types observed in sewage. Therefore, in this short-term investigation, the sewage and the poultry abattoir drain water probably reflect the pattern of *Campylobacter* harboured in the intestines of broilers processed during the last few days. In this short-term investigation 40% of the isolates were nontypable, which hampers the research on the transmission route.

The diversity and distribution of *Campylobacter* types in two purification plants were studied by determining the PCR-RFLP profile of the isolates gathered in the two-year survey. Both sewage purification plants, an activated sludge system and a trickling filter system, received waste waters from households and various small industries. The activated sludge system received additionally waste from a poultry processing plant. Isolates obtained from the drain water of this poultry abattoir were also subtyped (Chapter 5).

The PCR-RFLP technique, recently developed by Ayling *et al.* (1995) and based on the variety in the flagellin genes, appeared to be a consistent *Campylobacter* typing scheme with a high typability and discrimination level. In the study 22 profiles were distinguished and the profiles observed were all different from the previously described profiles in poultry in the United Kingdom (Ayling *et al.*, 1995). The epidemiological consequence of this discrepancy should be determined by the investigation of larger

numbers of samples from aquatic reservoirs and poultry. No specific PCR-RFLP type dominated during the various seasons of the sampling period.

Approximately 50% of the isolates were confined to only four profiles, suggesting that certain strains have either enhanced abilities to colonise host sources or have enhanced survival possibilities in the environment (Chapter 5). Six profiles were common in all three sources indicating that the types of strains which were present in poultry also occur in waste water from human sources. Strains of further four profiles were confined to the poultry abattoir drain water and the sewage of the activated sludge system, which received this drain water. These profiles were not observed in strains from the trickling filter, which suggests that at least some poultry strains may not occur in humans. It is likely that the source of some *Campylobacter* strains infecting humans, and hence present in human sewage, is not poultry. Thus, the results obtained indicate that although poultry may have an important role in human infections, other sources cannot be ignored.

Results from antimicrobial susceptibility testing (Chapter 6) showed that aquatic *Campylobacter* isolates from the activated sludge system were more resistant against quinolones (17% resistant strains) and against ampicillin (23% resistant strains) than the *Campylobacter* isolates from the trickling filter system (11% and 16% resistant strains, respectively). This difference reflects again the fact that the activated sludge does receive waste from a meat-processing industry. These observed quinolone-resistant aquatic *Campylobacter* isolates may originate from poultry (Jacobs-Reitsma *et al.*, 1994) and humans (Endtz *et al.*, 1991). The observation of rising resistance is alarming as quinolones are mentioned as alternative to erythromycin treatment for some campylobacteriosis patients. It is recommended to survey the resistance level among aquatic *Campylobacter* isolates. The disk diffusion assay (Bauer *et al.*, 1970) used in this thesis, would be suitable for such a survey. However, the sensitivity criteria recommended by the National Committee Clinical Laboratory Standards (1990) should be evaluated on their applicability for *Campylobacter* bacteria.

In summary, from the typing results and the antimicrobial susceptibility data, it can be concluded that poultry abattoir drain water contribute to the *Campylobacter* contamination of sewage, and thus indirectly to the numbers of *Campylobacter* in surface waters.

VIABLE BUT NONCULTURABLE STAGE OF *CAMPYLOBACTER*

The role of the viable but nonculturable stage of *Campylobacter* in the transmission route is a matter of debate. Therefore, in this thesis several physiological parameters of *Campylobacter* cocci formed under various conditions were compared with spiral *Campylobacter* cells (Chapter 7). The transformation into cocci was carried out as a function of the nutrient level of the storage medium and the temperature. A higher temperature increased the rate of coc formation and the presence of nutrients seemed to stimulate the transformation, which agrees with observations of Rollins and Colwell (1986).

The energetic status of the *Campylobacter* cocci was studied by determination of the intracellular and extracellular ATP levels. The intracellular ATP concentration of cocci incubated at 4 and 12 °C was much higher than the extracellular concentration. This indicates that cocci formed at 4°C and 12°C can maintain a pool of energy-rich components.

Production of new proteins during ageing was studied by comparing whole-cell protein patterns. However, no newly synthesized proteins could be observed in the *Campylobacter* cocci, which agrees with the observations of Jacob *et al.* (1993). Furthermore, the transformation from spiral cells to cocci, was not influenced by the addition of chloramphenicol in the storage medium or by a lethal dose of gamma-radiation. These observations concerning the metabolism of proteins indicate that the ageing of spirals with the resulting changes in morphology is a passive process.

Many micro-organisms can survive and grow at temperatures far below optimum growth temperatures by adjusting the fatty acid composition of the cell wall in order to maintain the fluidity of the membrane (Lindner and Oliver, 1989). In the experiments described in Chapter 7, *Campylobacter* cocci formed at various temperatures were investigated on this adaptation ability. It appeared that cocci formed at 4°C and 12°C had a similar fatty acid composition as spiral cells.

These physiological experiments on the nonculturable coccoid forms of *Campylobacter* produced at different temperatures in liquid stationary media indicate that these are not survival forms, but merely degenerative cells. However, certain viability properties appear to be best preserved in cocci formed and stored at 4°C. These results are in agreement with reports on failure to provoke an infection in warm-blooded models

after challenging with coccoid *Campylobacter* cells (Beumer *et al.*, 1992; Fearnley *et al.*, 1994; Medema *et al.*, 1992) but opposite results have been reported also. In this respect it should be noted that spiral *Campylobacter* cells have high colonization properties (Jacobs-Reitsma, 1994) and the presence of a few culturable cells in preparations of coccoid cells cannot readily be ruled out. In summary, it can be concluded that the role of these viable but nonculturable *Campylobacter* cells in the transmission routes is probably negligible. This underlines the significance of culturable *Campylobacter* cells in surface waters.

CONCLUSIONS

The surveys described in this thesis on the occurrence of *Campylobacter* in sewage purification plants, show that sewage and surface waters are generally contaminated with this pathogen. Sewage, which included domestic and industrial waste which did not originate from meat-processing industries, contained in average 2.3 $\log_{10}$ (MPN/100 ml) *Campylobacter* cells. When a poultry abattoir drained its processing water on the sewerage, the contamination level was much higher and ranged from 2.6 to 3 $\log_{10}$(MPN/100 ml). The cause was probably the high contamination rate of the drain water of poultry abttoirs; up to 7 $\log_{10}$(MPN/100 ml).

Purification in aerobic plants, like an activated sludge system or a trickling filter reduced the numbers of *Campylobacter* at most with one $\log_{10}$ unit. Consequently, *Campylobacter* could be isolated from the effluxes of these purification plants and these effluxes may contribute to the contamination of surface waters. *Campylobacter* was isolated from various surface waters, with numbers up to 2 $\log_{10}$(MPN/100 ml). Considering the low infectious dose of *Campylobacter* (Black *et al.*, 1992), precautions should be considered because some of these surface waters are used for recreational activities. Furthermore, from the dose-response relation deduced by Rose and Gerba (1991), it could be estimated that the incidence of human *Campylobacter* infections attributed to exposure to surface water may not be negligible. However, these estimations (as carried out in Chapter 8) should be confirmed by a directed case-control study.

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SUMMARY

Campylobacter bacteria are one of the most important causes of human diarrhoeal diseases. Sentinel and population studies carried out in the Netherlands, indicated that about 2,000 per 100,000 people suffer from campylobacteriosis annually. A case-control study in the United States of America showed that 50% of all cases was caused by consumption of undercooked poultry meat, and 8% could be attributed to drinking of contaminated surface water. The natural habitat of *Campylobacter* is the intestinal tract of warm-blooded animals. However, at low temperatures this pathogen may survive fairly well in the external environment, especially in aquatic niches. As untreated waters are recognised as sources of infection, knowledge on the prevalence of *Campylobacter* in sewage and surface waters is important. Therefore, the aim of the investigations described in this thesis was to obtain a better understanding of *Campylobacter* epidemiology in sewage and surface waters. Furthermore, the role of the nonculturable, coccoid *Campylobacter* cells in the transmission routes was evaluated by determining several physiological parameters as an indication of their viability.

In Chapter 2, combinations of blood- and charcoal-based media (Table 1, Chapter 2) were evaluated on their efficacy of enrichment and isolation. The polymerase chain reaction (PCR) technique and the latex agglutination assay (LAA) were tested on their applicability as tools for rapid detection of *Campylobacter* in enrichments. No significant difference in efficacy was observed for the various enrichment broths and isolation media tested. The LAA had a low positive predictive value of 39% and therefore, this assay is not recommended to be used as a rapid detection method. The PCR culture assay is suitable for the detection of *Campylobacter* in sewage enrichments; the method has a higher detection ability than the conventional culturing procedure. For qualitative analysis, both the conventional culturing procedure and PCR assay are satisfactory. However, within the conventional isolation procedure diluted sewage should be enriched and for a qualitative PCR analysis enriched undiluted sewage may be applied.

A two-year survey was carried out on the occurrence of *Campylobacter* in three sewage purification plants; two trickling filter systems and one activated sludge system (Table 1, Chapter 3). This survey clearly showed that sewage is considerably contaminated with this pathogen: up to 2.6-3 $\log_{10}$(MPN/100 ml). The numbers of *Campylobacter*

isolated from the different plants could be related to the presence of poultry abattoirs in the drainage areas. When meat-processing industrial activities were absent, the numbers of *Campylobacter* present in sewage were lower: 2.3 $\log_{10}$(MPN/100 ml). Drain water of poultry abattoir was shown to be highly contaminated with *Campylobacter*; up to 7 $\log_{10}$(MPN/100 ml) (Chapters 3 and 4).

Purification in an activated sludge tank reduced the *Campylobacter* counts by 1 $\log_{10}$ unit and in a trickling filter system the decline was 0.6 $\log_{10}$ unit (Chapter 3). The occurrence and reduction was not correlated to water temperature, oxygen pressure, pH, intensity of rainfall or of sunlight. Despite the purification, *Campylobacter* was still present in the efflux. Furthermore, the surface waters near discharge points of the plants were regularly contaminated with this pathogen. The observed numbers varied between 1.5 and 4.2 $\log_{10}$(MPN/100 ml). As the efflux of sewage purification plants is a vehicle of this pathogen in the environment, a larger reduction of numbers of *Campylobacter* during purification has to be recommended.

A considerable number of the analysed samples of both fresh surplus sludge and sedimented sludge were *Campylobacter* positive (average 2.4 $\log_{10}$(MPN/100 ml; Chapter 3). In these studies, aerobic digestion eradicated *Campylobacter* completely from sludge. Therefore, the spread of *Campylobacter* in the environment due to agricultural application of aerobic digested sewage sludge may be assumed to be a low risk.

In particular, the activated sludge system was investigated in more detail, in order to reveal sources of the *Campylobacter* sewage contamination. For this plant, it is likely that other contamination sources than drain water of the poultry abattoir, are negligible in numbers. *Campylobacter* isolates obtained from the activated sludge system and the drain water of the poultry abattoir were characterized by serotyping. The serotyping results (Chapter 4) showed some common types among poultry abattoir drain water and sewage, but a transmission route from *Campylobacter* present in the processed broilers towards the sewage could not be deduced. However, 40% of the isolates were nontypable, which hampers the research on transmission routes.

The diversity and distribution of *Campylobacter* types in two purification plants were studied by determining the polymerase chain reaction - restriction fragment length polymorphism (PCR-RFLP) profile of the isolates gathered in the two-year survey (Chapter 5). Apart from the purification system, also the composition of waste differs

between these two plants. Only the activated sludge system receives drain water from a meat-processing industry, namely a poultry abattoir. Isolates obtained from the drain water of this poultry abattoir were also subtyped. In this study 22 PCR-RFLP profiles were distinguished and no specific PCR-RFLP type was predominant in the various seasons of the sampling period. From the profile distribution among the isolates of the three sources, it is suggested that some poultry strains may not occur in humans. Furthermore, it is likely that the source of some *Campylobacter* strains infecting humans, and hence present in human sewage, is not poultry.

The difference in composition of sewage of the activated sludge system and the trickling filter was also obvious from the antimicrobial susceptibility patterns of the *Campylobacter* isolates (Chapter 6). Results from antimicrobial susceptibility testing showed that aquatic *Campylobacter* isolates from the activated sludge system were more resistant against quinolones (17% resistant strains) and against ampicillin (23% resistant strains) than the isolates from the trickling filter system (11% and 16% resistant strains, respectively). It is recommended to survey the resistance level among *Campylobacter* isolates.

To study the phenomenon of transformation from spiral to coccoid *Campylobacter* cells, ATP levels, protein profiles, and fatty acid compositions were monitored in coccoid preparations at different temperatures and in different media (Chapter 7). Especially, the temperature had an effect on the rate of transformation and the decline of culturability. Cells incubated at low temperatures (4°C and 12°C) could maintain very well an ATP gradient, however a survival strategy which is based on protein and fatty acid metabolism was not observed. These physiological experiments on the nonculturable coccoid forms of *Campylobacter* indicate that these are merely degenerative cells. However, certain viability properties appear to be best preserved in cocci formed and stored at 4°C. Considering the many reports on failure to provoke infection in a warm-blooded model with these coccoid cells, it is concluded that the role of these viable but nonculturable *Campylobacter* cells in the transmission routes is probably negligible.

This thesis is a contribution to the knowledge on the epidemiology of *Campylobacter* in aquatic environments. Application of the results on the occurrence of *Campylobacter* in these aquatic reservoirs to the reported dose-response relationship for this pathogen (Chapter 8), indicates that the number of water-borne *Campylobacter* infections may not be negligible.

SAMENVATTING

Campylobacter-bacteriën zijn een van de belangrijkste veroorzakers van acute diarree bij de mens. Een bevolkingsonderzoek heeft aangetoond dat zich in Nederland jaarlijks ongeveer 315.000 gevallen van campylobacteriosis voordoen. Uit een onderzoek in de Verenigde Staten, met als doel de oorzaak van humane *Campylobacter* infecties te bepalen, bleek dat 50% van de infecties wordt veroorzaakt door het eten van onvoldoende verhit vlees van pluimvee. Verder liet dit onderzoek zien dat consumptie van ongezuiverd water verantwoordelijk is voor 8% van de campylobacteriosis-gevallen.

Campylobacter maakt deel uit van de darmflora van warmbloedige dieren maar kan ook in het externe milieu overleven, vooral in waterige reservoirs. Daar ongezuiverd water in sommige landen een belangrijke besmettingsbron is, is kennis van het gedrag van *Campylobacter* in riool- en oppervlaktewater van belang voor de volksgezondheid. Het doel van de onderzoeken die in dit proefschrift zijn beschreven, was dan ook om meer inzicht te krijgen in de overlevingskansen van *Campylobacter* in riool- en oppervlaktewater. Daarnaast werd de rol van de niet-kweekbare, coccoide *Campylobacter*-cellen in de transmissieroute geëvalueerd. Als indicatie voor de levensvatbaarheid van de coccen werden verschillende fysiologische parameters onderzocht.

In hoofdstuk 2 is ingegaan op de methodiek van de bepaling van *Campylobacter* in rioolwater. Ten behoeve van de conventionele ophopingsmethode werden verschillende ophopings- en isolatiemedia vergeleken. Deze media hadden bloed of koolstof als basis. Ook werden de polymerase kettingreactie (PCR) en de latex agglutinatietest geëvalueerd als mogelijke snelle detectie- en identificatiemethoden van *Campylobacter* in de ophopingscultures. Voor rioolwatermonsters hadden de geteste ophopings- en isolatiemedia een vergelijkbaar detectievermogen. De latex agglutinatietest bleek echter niet geschikt te zijn om *Campylobacter* in ophopingen van rioolwater aan te tonen. De geteste PCR bleek geschikt te zijn. Per individuele ophoping lag het detectievermogen van de PCR beduidend hoger dan dat van de conventionele kweektechniek. Echter, voor kwalitatieve analyses om de aan- of afwezigheid van *Campylobacter* in rioolwatermonsters te bepalen, bleken beide methoden geschikt te zijn.

Om inzicht te krijgen in het voorkomen van *Campylobacter* in rioolwater werd

gedurende twee jaar een monitoringsprogramma uitgevoerd in drie verschillende rioolwaterzuiveringsinstallaties. De zuiveringsstappen in deze installaties waren gebaseerd op twee systemen: het actief slibsysteem en het oxydatiebed (tabel 1, hoofdstuk 3). Om mogelijke bronnen van de *Campylobacter*-besmetting van het rioolwater op te sporen, werden ook de afvalstromen van pluimveeslachterijen bemonsterd en geanalyseerd. Tevens werden omgevingsfactoren als temperatuur, regenval en lichtintensiteit bepaald om een eventueel patroon in de besmetting te kunnen verklaren. Uit het onderzoek bleek dat rioolwater in hoge mate besmet is met *Campylobacter* (2.3 $\log_{10}$(MPN/100 ml) en dat de aantallen significant hoger liggen (2.6-3 $\log_{10}$(MPN/100 ml) als in het drainagegebied van de zuiveringsinstallatie pluimveeslachterijen aanwezig zijn. Het afvalwater van deze slachterijen bleek inderdaad sterk verontreinigd te zijn met *Campylobacter* (7 $\log_{10}$ (MPN/100 ml), hoofdstukken 3 en 4).

Zuivering in een actief slibtank reduceerde het aantal *Campylobacter*-bacteriën met gemiddeld 1 $\log_{10}$ eenheid en behandeling in een oxydatiebed gaf een verlaging van aantallen met 0.6 $\log_{10}$ eenheid. *Campylobacter* was dan ook nog steeds aanwezig in het gezuiverde water. Daarmee vormen de effluenten van rioolwaterzuiveringssystemen een besmettingsbron van oppervlaktewater die aantoonbaar besmet waren (1.5-4.2 $\log_{10}$ (MPN/100 ml)). Via deze effluenten kan *Campylobacter* zich dus in het milieu verspreiden. Een hogere reductie tijdens de zuivering zou gewenst zijn.

Ook het verse, overtollige actieve slib en het gesedimenteerde slib bleken veelvuldig *Campylobacter* positief te zijn (gemiddeld 2.4 $\log_{10}$(MPN/100 ml)). In dit onderzoek bleek dat na aerobe gisting *Campylobacter* niet meer aantoonbaar was. Redenerend vanuit de preventie van de verspreiding van *Campylobacter* mag de toepassing van aeroob verteerd rioolslib als veilig worden beschouwd.

Als vervolg op dit onderzoek werd de actief slibzuivering nader bekeken met als doel bronnen van de *Campylobacter*-besmetting op te sporen. Voor deze zuivering is het aandeel van andere bronnen dan het afvalwater van pluimveeslachterijen in de besmetting waarschijnlijk te verwaarlozen. Om informatie te verkrijgen over transmissie zijn de verkregen isolaten gekarakteriseerd met serotypering (hoofdstuk 4). Een aantal serotypes was zowel in het afvalwater van de pluimveeslachterij aanwezig als in het rioolwater van de actief slibzuivering. Hieruit kon echter geen verder bewijs worden afgeleid voor de transmissieroute van vleeskuikens verwerkt in de slachterij naar de zuiveringsinstallatie.

In dit deelonderzoek was 40% van de isolaten evenwel niet te typeren, hetgeen de studie naar de transmissieroute sterk bemoeilijkte.

In het onderzoek beschreven in hoofdstuk 5 is getracht een transmissieroute af te leiden door de isolaten verkregen uit het monitoringsprogramma, dat twee jaar in beslag nam, te karakteriseren met behulp van de "polymerase chain reaction-restriction fragment length polymorphism" (PCR-RFLP) techniek. Deze typeringsmethode is gebaseerd op de variatie in de flagelline genen van *Campylobacter*. De isolaten waren afkomstig van de eerder genoemde lokaties: het afvalwater van een pluimveeslachterij, het rioolwater van een actief slibinstallatie en het rioolwater van een oxydatiebed. De samenstelling van het rioolwater dat bij deze zuiveringen werd gebruikt, liep sterk uiteen, aangezien alleen de actief slibinstallatie afvalwater van een vleesverwerkend bedrijf kreeg. Met behulp van de PCR-RFLP methode konden 22 types worden onderscheiden. Onder de types van de waterisolaten was geen seizoensinvloed waar te nemen. Een aantal types kwam in alle drie lokaties voor en sommige alleen in het oxydatiebed. Dit is een indicatie dat enkele *Campylobacter*-stammen van pluimvee niet bij de mens voorkomen. Verder is het mogelijk dat sommige types die de mens infecteren via het rioolwater, niet van pluimvee afkomstig zijn.

Van de *Campylobacter*-isolaten uit het monitoringsprogramma werd naast het PCR-RFLP-type ook de gevoeligheid voor quinolonen nalidixinezuur, flumequine, ciprofloxacin, enrofloxacin en verder voor ampicilline, tetracycline en erythromycine bepaald (hoofdstuk 6). Bij de isolaten van de actief slibzuivering is de gevoeligheid voor de quinolonen en ampicilline lager dan bij de isolaten van het oxydatiebed. Dit verschil weerspiegelt het feit dat de actief slibinstallatie afvalwater ontvangt van een vleesverwerkend bedrijf en dat in het rioolwater van het oxydatiebed geen industrieel afval zit.

Als onderdeel van het *Campylobacter*-onderzoek, zoals beschreven in dit proefschrift, is getracht de levensvatbaarheid van de niet-kweekbare kiemen van *Campylobacter* te bepalen (hoofdstuk 7). De kweekbaarheid bleek sneller te dalen bij een hogere incubatietempera-tuur. Fysiologische experimenten met coccen- en spiralen-cultures lieten zien dat cellen geïncubeerd bij lage temperaturen in staat zijn om een ATP-gradiënt in stand te houden. Een actief eiwit- en vetzuurmetabolisme gericht op overleving kon echter niet worden waargenomen. De fysiologische eigenschappen van coccen ontstaan bij incubatie bij 4°C, vertonen de meeste gelijkenis met spiralen. Desalniettemin

verkeren de niet-kweekbare, coccoide *Campylobacter*-cellen waarschijnlijk in een degeneratief stadium. Dat is zeker het geval als daarbij de publikaties over de infectieproeven, waarin tevergeefs getracht werd warmbloedige (dier)modellen te infecteren met niet-kweekbare *Campylobacter*-cellen, in ogenschouw worden genomen. De conclusie is dat de niet-kweekbare coccoïde cellen uit een oogpunt van infectie en het in stand houden van kringlopen van weinig of geen betekenis zijn.

Dit proefschrift vormt een bijdrage aan de kennis van het gedrag van *Campylobacter* in waterige milieus. De *Campylobacter*-incidentie door blootstelling aan besmet recreatiewater kan worden geschat als het *Campylobacter*-besmettingsniveau in het door Rose and Gerba gepubliceerde dosis-responsmodel wordt toegepast. Deze schatting, beschreven in hoofdstuk 8, laat zien dat de aantallen humane *Campylobacter*-infecties veroorzaakt door blootstelling aan besmet recreatiewater, tot nu toe werden onderschat.

With agreement from Mr. H. De Kort (June, 28th, 1995).

NAWOORD

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Petra

CURRICULUM VITAE

Op 14 juni 1966 werd Petronella Magdalena Francisca Josephina Koenraad in Sittard geboren. Zij behaalde in 1984 het Atheneum-B diploma aan het plaatselijke Bisschoppelijk College. In datzelfde jaar begon zij de studie Levensmiddelentechnologie aan de toenmalige Landbouwhogeschool in Wageningen. In de doctoraalfase werden de hoofdvakken Industriële Microbiologie (Prof. dr. ir. J.A.M. de Bont) en Proceskunde (Prof. dr. ir. J. Tramper) gevolgd. De eerste stageperiode heeft zij bij TNO in Zeist (ir. J.P.J. de Jong, in samenwerking met ir. M.E. Vermuë, sectie Proceskunde, Landbouwuniversiteit Wageningen) doorgebracht. Daarna is zij vier maanden bij de University of Lund in Zweden (dr. O. Holst) op stage geweest om vervolgens in augustus 1990 af te studeren aan de Landbouwuniversiteit.

Van oktober 1990 tot november 1994 was zij assistent in opleiding (AIO) werkzaam bij de vakgroep Levensmiddelentechnologie, sectie Levensmiddelenchemie en -microbiologie aan de Landbouwuniversiteit. Het wetenschappelijke onderzoek, beschreven in dit proefschrift, werd onder leiding van Prof. dr. ir. F.M. Rombouts en R.R. Beumer uitgevoerd. Het toenmalige Ministerie van Welzijn, Volksgezondheid en Cultuur was opdrachtgever van het project. Aansluitend op haar promotieonderzoek werkte zij als onderzoeker een half jaar bij Friki-Plukon in Wezep.

Vanaf 24 juli 1995 is zij als Hoofd Microbiologisch Laboratorium werkzaam bij de Inspectie Gezondheidsbescherming (de Keuringsdienst van Waren) in 's-Hertogenbosch.

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