Water Research 187 (2020) 116421

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Tracing the animal sources of surface water contamination with *Campylobacter jejuni* and *Campylobacter coli*

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ARTICLE INFO

Article history:
Received 30 June 2020
Revised 9 September 2020
Accepted 10 September 2020
Available online 20 September 2020

Keywords:
Campylobacter coli
Campylobacter jejuni
source-attribution
surface water type
livestock, season

ABSTRACT

Campylobacter jejuni and *C. coli*, the primary agents of human bacterial gastroenteritis worldwide, are widespread in surface water. Several animal sources contribute to surface water contamination with *Campylobacter*, but their relative contributions thus far remained unclear. Here, the prevalence, genotype diversity, and potential animal sources of *C. jejuni* and *C. coli* strains in surface water in the Netherlands were investigated. It was also assessed whether the contribution of the different animal sources varied according to surface water type (i.e. agricultural water, surface water at discharge points of wastewater treatment plants [WWTPs], and official recreational water), season, and local livestock (poultry, pig, ruminant) density. For each surface water type, 30 locations spread over six areas with either high or low density of poultry, ruminants, or pigs, were sampled once every season in 2018-2019. *Campylobacter* prevalence was highest in agricultural waters (77%), and in autumn and winter (74%), and lowest in recreational waters (46%) and in summer (54%). In total, 76 *C. jejuni* and 177 *C. coli* water isolates were whole-genome sequenced. Most *C. coli* water isolates (78.5%) belonged to hitherto unidentified clones when using the seven-locus sequence type (ST) scheme, while only 11.8% of the *C. jejuni* isolates had unidentified STs. The origin of these isolates, as defined by core-genome multi-locus sequence typing (cgMLST), was inferred by comparison with *Campylobacter* strain collections from meat-producing poultry, laying hens, adult cattle, veal calves, small ruminants, pigs, and wild birds. Water isolates were mainly attributed to wild birds (*C. jejuni*: 60.0%; *C. coli*: 93.7%) and meat-producing poultry (*C. jejuni*: 18.9%; *C. coli*: 5.6%). Wild bird contribution was high among isolates from recreational waters and WWTP discharge points, and in areas with low poultry (*C. coli*) or high ruminant (*C. jejuni*) densities. The contribution of meat-producing poultry was high in areas with high density of poultry, springtime, agricultural waters and WWTP discharge points. While wild birds and poultry were the main contributors to *Campylobacter* contamination in surface water, their contribution differed significantly by water type, season, and local poultry and ruminant densities.

1. Introduction

Campylobacteriosis is the most frequently reported zoonosis in Europe, with an estimated 70 thousand cases annually in the

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https://doi.org/10.1016/j.watres.2020.116421

Netherlands alone (~17 million inhabitants) (Pijnacker et al., 2019). *Campylobacter jejuni* and *Campylobacter coli* are the two species of the *Campylobacter* genus that account together for over 90% of human campylobacteriosis cases in Europe (Centre of Disease Control and Prevention, 2017; European Food Safety and European Centre of Disease Prevention and Control, 2018). Besides gastroenteritis, a *Campylobacter* infection can result in more severe diseases, such as Guillain-Barré syndrome, reactive arthritis, and irritable bowel disease, which strongly contribute to the disease burden of campylobacteriosis (Halvorson et al., 2006; Nachamkin et al., 1998; Ternhag et al., 2008). Although up to 80% of all human campylobacteriosis cases can be attributed to the poultry reservoir, several epidemiological studies have shown that only 40% of these poultry-associated cases can be explained by the consumption of chicken meat (Friesema et al., 2012; Mughini Gras et al., 2012; Veilinga and van loock, 2002; Wagenaar et al., 2013; Wagenaar et al., 2015; Wilson et al., 2008). Most interventions to control *Campylobacter* infections have focussed on spread through the food production chain, particularly poultry meat, with limited effects. Accordingly, there has been no appreciable decrease in the incidence of human campylobacteriosis so far (European Food Safety and European Centre of Disease Prevention and Control, 2018). This emphasizes the need to study transmission routes other than food (Sears et al., 2011; Stern et al., 2003), such as those involving the aquatic environment, as *Campylobacter* is commonly found in surface water contaminated with animal faeces, sewage effluent, and agricultural runoff (Jones, 2001).

Even though *Campylobacter* is believed to survive poorly outside the host, some specialist strains have been found to be successfully adapted to survival outside an animal host in certain sylvatic (Hepworth et al., 2011), farmland (French et al., 2005) and environmental niches (Colles et al., 2011; French et al., 2005; Sopwith et al., 2008). These strains are generally more resistant to physical stress than other strains (Sopwith et al., 2008). *Campylobacter* also has the ability to convert into a viable but non-culturable state, to advert conditions while being outside the host (Collins and Colwell, 1986; Murphy et al., 2006). These characteristics indicate that surface water serves more as a vehicle of transmission for *Campylobacter* among animals, from animals to humans and vice versa, rather than as an amplifying reservoir per se.

A previous source attribution study has shown that poultry and wild birds are the most important contributors to surface water contamination with *C. jejuni* and *C. coli* in the Netherlands and Luxembourg, followed by ruminants and pigs (Mughini-Gras et al., 2016). The relative contributions of wild birds and poultry seemed to vary with season, water type, and the magnitude of the local poultry production, suggesting substantial dissemination of *Campylobacter* into the environment from poultry farms in poultry-rich regions. Although the aforementioned study quantified the contributions of different animal sources to *C. jejuni* and *C. coli* contamination in surface water, the authors acknowledged that the interpretation of their findings was limited by the extensive use of non-local and non-recent source data, retail food data, and coarse spatial resolution of the analyses (Mughini-Gras et al., 2016). Therefore, further testing of the previously formulated hypotheses by using more representative data in additional smaller-scale analyses was necessary.

Due to the paucity of epidemiological research on non-foodborne transmission routes of *Campylobacter*, innovative control measures to limit *Campylobacter* spread into the environment have not yet been developed. Although the aquatic environment seems to contribute to the transmission of *Campylobacter* to humans, the extent to which this is determined by fecal pollution from different types of livestock and wildlife remains unclear. This study aimed to quantify *Campylobacter* prevalence and genotype diversity in surface water, as well as the relative contributions of several putative animal sources to surface water contamination with *C. jejuni* and *C. coli* in the Netherlands using high-throughput genomic data derived from whole-genome sequencing (WGS). Additionally, potential effects of local livestock density, type of surface water, and season were assessed.

2. Materials and methods

2.1. Water samples

2.1.1. Study areas

Water samples were collected in areas which largely varied in densities of specific livestock groups in the Netherlands. Specifically, six areas were selected, with either a high or a low density of poultry (i.e. broiler chickens, laying hens and turkeys combined), pigs, or ruminants (i.e. cattle, sheep and goats combined). To this end, the livestock density per municipality (number of animals/km2) was calculated per livestock group based on official agricultural census data and land surface per municipality available at the time of this study set-up (December 2018) from Statistics Netherlands (CBS, 2018a,b). Then, the first and last quintiles of the frequency distributions of the density of each livestock group were calculated, which thereby defined the high and low densities of each livestock group in question (high poultry density area: 19,677 poultry/km2; low poultry density area: <1 poultry/km2; high pig density area: 2,729 pigs/km2; low pig density area: 4 pigs/km2; high ruminant density area: 451 ruminants/km2; low ruminant density area: 26 ruminants/km2). The corresponding areas were geographically identified for each livestock group separately (Fig. 1) using ArcMap 10.5 (ESRI). However, those areas could contain multiple livestock types, as the different livestock types are widely spread and mixed throughout the Netherlands (Smit and Heederik, 2017). The selection was based on the following criteria: i) a municipality could only be included in one livestock density area; ii) the areas needed to contain enough surface water sampling sites to allow for data collection (see also Section 2.1.2); iii) the areas needed to be of comparable size.

2.1.2. Sampling sites

Three different types of surface freshwater were selected: i) recreational water at official bathing sites that have to comply with European bathing water legislation (EUR-Lex., 2006) and therefore generally have relatively low levels of faecal contamination; ii) surface water (e.g. drainage ditches, irrigation canals, etc.) in farmlands and pastures that are mainly faecally contaminated by agricultural activities (i.e. run-off from farms, grazing fields and crops fertilized with manure or accessed by free-ranging animals, etc.); and surface water at the discharge sites of effluents of wastewater treatment plants (WWTP), which are a source of contamination with faecal material mainly of human origin. Within each of the six study areas (Fig. 1), 15 surface water sampling sites were selected, i.e. five sites for each of the three types of surface water. Each of the selected 90 sampling sites was sampled four times, once per season (summer: June to August; autumn: September to November; winter: December to February; spring: March to May), resulting in a total of 360 planned water samples.

2.1.3. Water sample collection and analysis

Sampling was performed between April 2018 and February 2019 by an accredited contractor (OMEGAM-Water B.V.). Water samples were taken according to the ISO 19458:2006 procedure and immediately cooled and transported to the laboratory at the Dutch National Institute for Public Health and the Environment (RIVM), where they were stored at 4°C and analyzed within 24 hours from sampling. Samples in a total volume of 1000 ml were

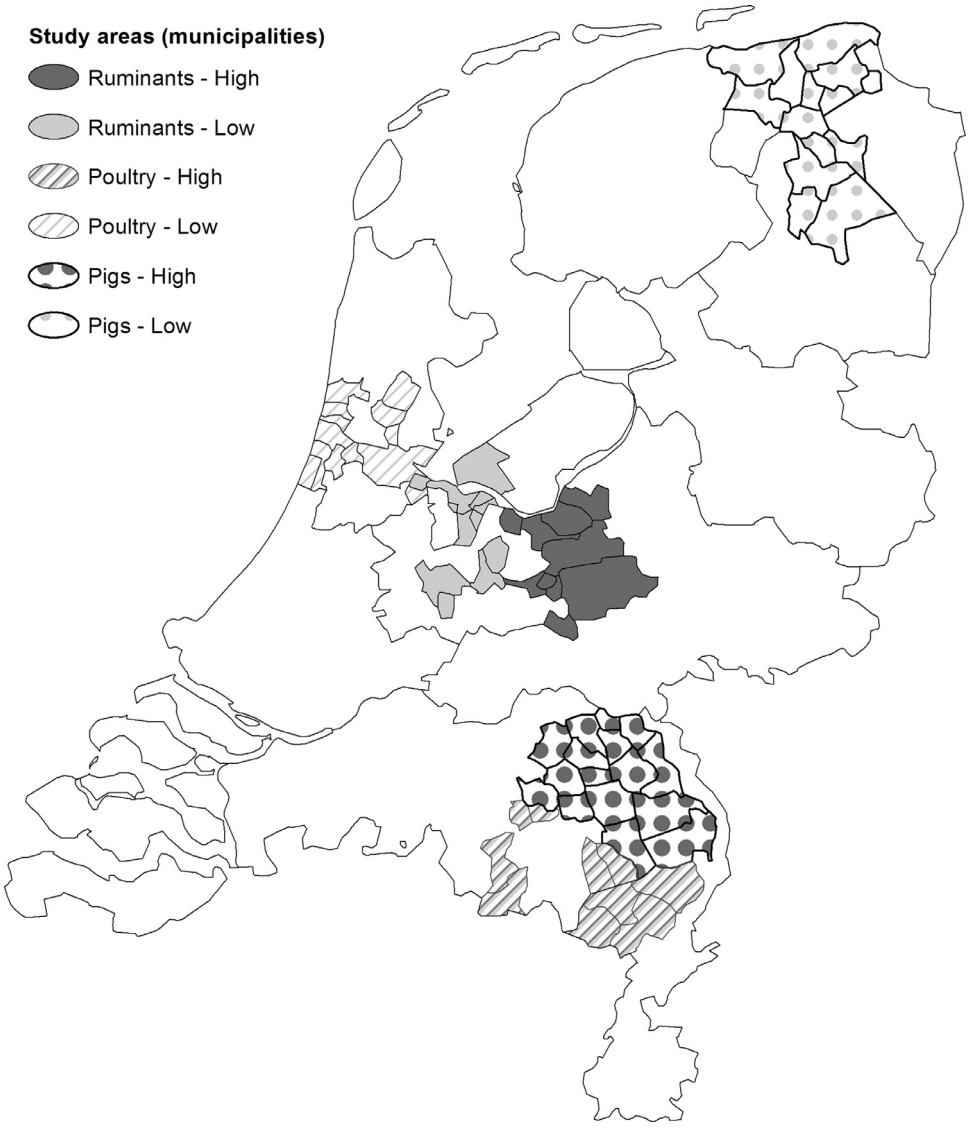

Fig. 1. Representation of the six study areas for surface water sampling with high and low densities of poultry, pigs, or ruminants.

filtered using 0.45 µm cellulose-based membrane filters (Millipore). The filters were placed in Preston broth and incubated under microaerobic conditions using CampyGen sachets (Oxoid) for 48 h at 37°C. Samples were then streaked (10 μl) on modified charcoal cefoperazone deoxycholate (mCCDA) agar and the plates were incubated under microaerobic conditions for 48 h at 41.5°C. From each sample, a maximum of five colonies was inspected by light microscopy for *Campylobacter* characteristics, and a maximum of five visually confirmed colonies per sample were analyzed using Matrix-Assisted Laser-Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS, Bruker Microflex LT, Germany) for species identification. Per individual sample, one *C. jejuni* isolate and one *C. coli* isolate was selected at random for whole-genome sequencing (WGS).

2.2. Animal data collection

2.2.1. Livestock

Livestock isolates of *C. jejuni* and *C. coli* from faecal samples and carcasses were collected at farms and slaughterhouses by Wageningen Bioveterinary Research (WBVR) and Wageningen Food Safety Research (WFSR), in collaboration with the RIVM and the Netherlands Food and Consumer Product Safety Authority (NVWA). This was done within the framework of established and nationally representative surveillance programs for zoonotic agents (Opsteegh et al., 2018) and antimicrobial resistance (de Greeff et al., 2019) in food-producing animals, as well as routine inspection and testing activities by veterinary services, in the Netherlands during 2014-2019. Additional isolates from small ruminants (sheep and goats) were obtained through a small-scale internal project including *ad-hoc* sampling events at farms in the Netherlands conducted by engaging field veterinarians collaborating with the Veterinary Microbiological Diagnostic Centre (VMDC) of Utrecht University.

Isolates were obtained from faecal samples analysed without enrichment by direct streaking onto mCCDA (Oxoid) plates following the ISO 10272-1:2017 procedure, whereas carcass samples were analysed with an enrichment step in accordance with the same procedure. Species identification was performed using MALDI-TOF MS.

2.2.2. Wild birds

Fresh droppings or cloacal swabs of some of the most widespread species of waterfowl, pigeons and gulls in the Nether-

lands were collected by Wageningen Ecological Research (WER) in June and December 2018 to cover both the summer and winter seasons. Sampling was performed by convenience at different locations across the country selected based on previous studies (Lange et al., 2013), which did not include the water sampling sites. Both herbivorous (i.e. geese, mute swan, ducks, common wood pigeon) and omnivorous/piscivorous (gulls, great cormorant) bird species were sampled, as the latter species have higher *Campylobacter* concentrations in their feces, whereas herbivorous birds produce more feces per day (de Lange, 2013).

2.3. Whole-genome sequencing (WGS)

All gathered *Campylobacter* isolates from both surface water and animals were subject to WGS. DNA isolation was done using the UltraClean® Microbial DNA Isolation Kit (Qiagen, USA). WGS was performed on Illumina Hiseq and NextSeq platforms (Illumina, USA) using 2 × 150-bp reads. Genomes were assembled with SPAdes v3.10.1 (Bankevich, 2012) and checked for completeness and contamination using CheckM (Parks et al., 2015); genomes with >5% contamination or <95% completeness were excluded. The sequences were deposited in ENA Sequence Read Archive project PRJEB38253.

A standard core genome multilocus sequence typing (cgMLST) scheme for *Campylobacter* was applied (Cody et al., 2017) using Seemanns' MLST tool to scan contig files against traditional PubMLST typing schemes (https://github.com/tseemann/mlst) modified for cgMLST schemes (https://github.com/aldertzomer/cgmlst). The cgMLST profile was assessed using the sequence definitions in BIGSdb (accessed at November 9th, 2019). Additional searches for missing genes were performed using the Basic Local Alignment Search Tool (BLAST) v2.5.0 (Altschul et al., 1990) on the assembled genomes. For the alleles not yet present in BIGSdb, multiple alignments of each locus were performed using MAFFT v7.407 (Katoh et al., 2002) and these were assigned unique identification numbers. All the loci for which none of these approaches provided unambiguous results were considered as missing. Loci with missing allele numbers in >5% of the isolates were excluded from the analysis. For description purposes, the sequence types (STs) based on the conventional 7-locus MLST scheme (Dingle, 2001) were also derived from the WGS data.

2.4. Data analysis

2.4.1. Prevalence and ST diversity in surface water

The prevalence of *C. jejuni* and *C. coli* (and their different STs) in surface water was calculated for the different types of surface water, seasons and livestock density areas. Simpson's diversity index was calculated to quantify the diversity of STs (Anandan et al., 2014). The structure of the *Campylobacter* population was visualized using both conventional MLST- and cgMLST-based minimum spanning trees (MST) to appreciate interrelationships and clusters among the *Campylobacter* isolates.

2.4.2. Analysis of Molecular Variance (AMOVA)

To test for genetic differentiation in STs between the sources, Φ-statistics were estimated using analysis of molecular variance (AMOVA) (Excoffier, 1992), an extension of the analysis of variance (ANOVA) that focusses on the (genetic) heterogeneity between groups. If the mutual heterogeneity between two sources was not significant, they were pooled into a new group. AMOVA was conducted using the R packages "poppr" (version 2.8.5) and "hierfstat" (version 0.04-22) in R (version 3.6.0) (RCT, 2015).

2.4.3. Source attribution analysis

The *C. jejuni* and *C. coli* isolates from surface water were attributed to the putative animal sources as defined by the AMOVA. cgMLST-based source attribution analysis using an established population genetics model, i.e. STRUCTURE (version 2.3.4), was performed to estimate relative probabilities for each *Campylobacter* strain found in surface water to originate from each of the animal sources (Hubisz et al., 2009; Pritchard et al., 2000). A model with no admixture and with the "USEPOPINFO" flag was used to determine the ancestry of the isolates to be attributed, i.e. the surface water isolates. Therefore, each animal population was considered as discrete and the origin of each water isolate i was estimated under the assumption that the isolate comes directly from one of the K animal sources, with a prior probability for each source of $1/K$ (Porras-Hurtado et al., 2013; Pritchard et al., 2009). The USEPOPINFO flag was used to pre-specify the population of origin of the animal isolates as to assist inference of the origin for the water isolates, whose (animal) populations of origin were set as unknown (Pritchard et al., 2009). By pre-setting the populations of origin of the animal isolates based on the AMOVA results, the cluster structure corresponded to the pre-defined populations, which were in agreement with the genetic information and made the output more interpretable. The very few missing alleles (0.3% of the total), minimized by performing additional searches and blasting, as well as by excluding loci with considerable and systematic missingness over the isolates, were then handled with the default software function, which ignores missing data when updating parameters. The length of the burning period was set at 1,000, followed by 10,000 iterations, which were able to provide adequate convergence of parameter estimation. The overall proportion of surface water isolates attributed to a given source was then calculated as the sum of the relative probabilities for that source of the surface water isolates divided by the total number of surface water isolates. The 95% confidence intervals (CIs) for the attribution were derived in R with the "boot" (version 1.3-24) package to provide bootstrapped values of the average attributions per source with 1,000 replications.

2.4.4. Effects of livestock density, water type and season

Significance testing of the differences in attribution estimates (i.e. the source probabilities) for the surface water isolates between the livestock density areas, types of water, and seasons, was performed using multiple linear regression analysis in R. A logarithmic transformation of the outcome variable (i.e. source probabilities) was applied. For this analysis, the attribution estimates for meat-producing poultry and laying hens were combined into 'poultry', and those for adult cattle, veal calves and small ruminants into 'ruminants' in order to reflect the livestock groups used in the definition of the livestock density areas. Finally, the multivariate shared relationships of the variables type of water, season and livestock density, with the attributions of surface water isolates were explored using canonical correlation analysis (CCA).

3. Results

3.1. Isolate collection

In total, 360 water samples were planned to be taken during this study. However, due to a few sampling locations being temporarily inaccessible or being without water due to drought at a given sampling event, a total of 348 samples were eventually collected. In total, 411 isolates (304 *C. coli* and 107 *C. jejuni*) were obtained from those surface water samples. From each individual sample, only one *C. jejuni* isolate and one *C. coli* isolate was selected at random for WGS. This resulted in a selection of 253 water isolates (177 *C. coli* and 76 *C. jejuni*). In total, 570 *C. jejuni* and 152 *C. coli* isolates were obtained from different livestock species (Table 1) and 47 *C. jejuni* and 15 *C. coli* isolates were obtained from wild birds (Supplementary Material, Table S1). This resulted in a

Table 1
Total number of *Campylobacter* isolates obtained from each livestock group.

Livestock	Total number of isolates (N)	*C. jejuni* isolates (N)	*C. coli* isolates (N)
Broiler chickens	200	186	14
Laying hens	56	55	1
Turkeys	38	37	1
Beef cattle	96	96	0
Dairy cattle	62	61	1
Veal calves	49	39	10
Small ruminants	111	86	25
Pigs	110	10	100

Table 2
Total number of surface water samples tested (N), number and percentage of *C. jejuni* and *C. coli* positive samples (Pos and %) per surface water type and season.

	Spring			Summer			Autumn			Winter			Total		
	N	Pos	%	N	Pos	%	N	Pos	%	N	Pos	%	N	Pos	%
Agricultural waters	30	24	80	26	17	65	26	20	77	29	24	83	111	85	77
WWTP discharge points	30	22	73	30	21	70	30	23	77	30	25	83	120	91	76
Recreational waters	30	10	33	29	8	28	29	20	69	29	16	55	117	54	46
Total	90	56	62	85	46	54	85	63	74	88	65	74	348	230	66

Table 3
Total number of surface water samples tested (N) and percentage (%) of *C. jejuni* and *C. coli* positive samples per livestock density areas.

	Low poultry density N (%)	High poultry density N (%)	Low pig density N (%)	High pig density N (%)	Low ruminant density N (%)	High ruminant density N (%)
Samples	59 (100)	52 (100)	60 (100)	58 (100)	58 (100)	60 (100)
Positive	42 (71)	31 (60)	45 (75)	35 (60)	44 (76)	33 (55)
C. coli [a]	37 (63)	21 (40)	39 (65)	27 (47)	36 (62)	20 (33)
C. jejuni [a]	7 (12)	17 (33)	11 (18)	10 (17)	13 (22)	20 (33)

[a] The sum of the number of positive samples of *C. jejuni* and *C. coli* is not equal to the number of positive samples, because each surface water sample can contain multiple isolates of *C. jejuni* and *C. coli*.

total of 1037 *Campylobacter* isolates (253 from surface water and 784 from animals) which were subjected to WGS. As was described in Section 2.3, 88 loci with missing allele numbers in >5% of the isolates were excluded, resulting in 1,255 loci with 99.7% complete allele numbers in the whole dataset.

3.2. Prevalence and STs in surface water

3.2.1. Prevalence

The overall *Campylobacter* prevalence in surface water samples was 66% (Table 2). Prevalence was highest in agricultural waters (77%) and at WWTP discharge points (76%), and lowest in official recreational waters (46%). Prevalence was highest in autumn (74%) and winter (74%) and lowest in spring (62%) and summer (54%).

Prevalence was generally higher in the low livestock density areas (poultry 71%, pigs 75%, and ruminants 76%) as compared to the high livestock density areas (poultry 60%, pigs 60%, and ruminants 55%) (Table 3). However, in areas with high poultry and ruminant densities, *C. jejuni* prevalence was higher, but *C. coli* prevalence was lower, as compared to areas with low poultry and ruminant densities. In general, *C. coli* was more often isolated than *C. jejuni*, except for the high ruminant density area where the prevalence was the same for both *Campylobacter* species (33%). Also in the high poultry density area, the difference in prevalence between *C. coli* (40%) and *C. jejuni* (33%) was small compared to that in the other areas.

3.2.2. Sequence types

Overall, 105 (41.5%) sequenced *Campylobacter* isolates had known STs, whereas about 60% of the isolates (58.5%) had thus far unidentified STs. Most *C. coli* water isolates (78.5%) belonged to those unidentified clones, while only 11.8% of the *C. jejuni* isolates had an unknown ST. The four most prevalent STs in surface water were ST45 (n=10, 4.0%), ST1766 (n=5, 2.0%), ST137(n=4, 1.6%) and ST2654 (n=4, 1.6%). The prevalence of STs differed considerably between the three different types of surface water. While only 15 of 54 isolates from recreational waters had known STs, which were each detected once, isolates from WWTP discharge points and agricultural waters had higher proportions of known STs: 41/91 and 49/85 isolates (i.e. 45,1% and 57,7%), respectively. At WWTP discharge points, the two most common STs were ST2654 (n=4, 4.4%) and ST137 (n=3, 3.3%), whereas the two most common STs in agricultural waters were ST45 (n=9, 10.6%) and ST1766 (n=4, 4.7%). An overview of all STs found in surface water and STs grouped by season, water type and livestock density area are reported in Figure S1, Supplementary Material.

3.2.3. Source heterogeneity

The AMOVA showed that there was significant genetic heterogeneity between most of the sources. Non-significant heterogeneity was observed between broilers and turkeys, and between dairy cattle and beef cattle. Therefore, these sources were combined into 'meat-producing poultry' (i.e. broilers and turkeys) and 'adult cattle' (i.e. dairy and beef cattle) for further analyses. The Φ-values and corresponding p-values for each pair of sources are reported in Table S2, Supplementary Material.

3.2.4. ST diversity among Campylobacter isolates from surface water

Simpson's diversity index based on the STs found in surface water was 0.96 for *C. jejuni* and 0.94 for *C. coli* (overall 0.97), indicating the probability that two isolates randomly selected from surface water belong to different STs. Diversity was lowest in areas with low pig density, at WWTP discharge sites, and in spring, and highest in areas with high ruminant density, recreational waters and in winter (Table 4).

Fig. 2. Core-genome MLST-based minimum spanning tree showing the population structure of the *C. jejuni* isolates from surface water and from the different animal sources.

Table 4
Simpson's index of diversity of *Campylobacter* STs from surface water.

Variable	Total	*C. jejuni*	*C. coli*
Overall	0.97	0.96	0.94
Livestock density area			
Low ruminant density	0.94	0.88	0.88
High ruminant density	0.95	0.95	0.00
Low poultry density	0.92	0.83	0.83
High poultry density	0.93	0.92	0.75
Low pig density	0.88	0.81	0.67
High pig density	0.91	0.74	0.88
Water type			
Agricultural waters	0.94	0.88	0.89
WWTP discharge points	0.93	0.93	0.91
Recreational waters	0.96	0.91	0.75
Season			
Spring	0.92	0.88	0.75
Summer	0.94	0.86	0.90
Autumn	0.94	0.91	0.86
Winter	0.96	0.94	0.91

3.2.5. Population structure

The cgMLST-based MSTs visualizing the population structure of the *C. jejuni* and *C. coli* isolates are shown in Fig. 2 and Fig. 3, respectively. For *C. jejuni* (Fig. 2), surface water and wild bird isolates generally clustered together, but surface water isolates were also found among isolates from other sources, specifically meat-producing poultry and laying hens. The *C. coli* tree showed significant clustering of the surface water and wild bird isolates, which were clearly separated from the isolates of the other sources (Fig. 3). A similar structure was appreciable in the MSTs based on conventional MLST (Figure S2 and Figure S3, Supplementary Material).

3.3. Attribution of animal sources to surface water

Most *C. jejuni* isolates in surface water could be attributed to wild birds (60.0%, 95%CI: 48.9-71.3%), followed by meat-producing poultry (18.9%, 95%CI: 10.5-26.8%), small ruminants (9.9%, 95%CI: 4.4-16.4%), adult cattle (6.9%, 95%CI: 2.3-12.4%), laying hens (4.1%, 95%CI: 0.4-8.8%), and veal calves (0.2%, 95%CI: 0.0-0.5%) (Fig. 4). The attribution of *C. jejuni* in surface water to pigs was too small to be sized. The vast majority of *C. coli* isolates in surface water could also be attributed to wild birds (93.7%, 95%CI: 90.4-96.4%), followed by meat-producing poultry (5.6%, 95%CI: 3.1-8.9%) and small ruminants (0.6%, 95%CI: 0.1-1.7%), while the other animal sources accounted altogether for <0.1% of *C. coli* isolates.

When the attribution estimates were split by livestock density area, wild birds were again the predominant contributor to *C. jejuni* contamination in surface water in all areas (Table 5). The second contributor was meat-producing poultry in most areas, except for the area with low pig density where small ruminants where the second most important contributor. The contribution of wild birds to *C. jejuni* contamination in surface water was higher in the low poultry density area (99.4%) than in the high poultry density area (59.2%). The opposite was observed for meat-producing poultry: in the high poultry density area, the contribution of meat-producing poultry to *C. jejuni* contamination in surface water (24.9%) was higher than in the low poultry density area (0.3%). In both the low and high ruminant density areas, small ruminants and laying hens ranked respectively as third and fourth most important contributors to *C. jejuni* contamination in surface water, without large differences in the contributions between the two areas. In the low ruminant density area, however, the contribution of adult cattle (6.8%) was higher than in the high ruminant density area where there was no detectable contribution of adult cattle at all. Also for *C. coli*, the contribution of wild birds was higher in the low poultry density area (93.2%) than in the high poultry density area (87.0%),

Fig. 3. Core-genome MLST-based minimum spanning tree showing the population structure of the *C. coli* isolates from surface water and from the different animal sources.

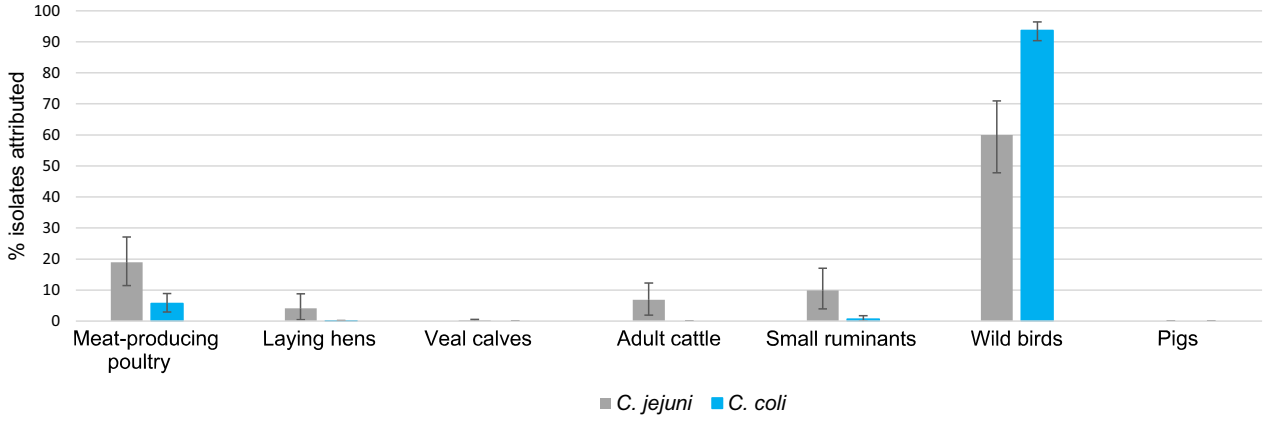

Fig. 4. Overall attributions of *C. jejuni* and *C. coli* isolates from surface water to the animal sources based on cgMLST. Error bars indicate 95% confidence intervals.

although the difference was smaller than for *C. jejuni*. The opposite was observed for meat-producing poultry (low poultry density area: 4.1%; high poultry density area: 12.8%). There were no sizeable contributions of other sources of *C. coli* water contamination.

Wild birds were the predominant contributor to *C. jejuni* and *C. coli* contamination in all three water types (*C. jejuni*: recreational waters 92.3%, agricultural waters 53.5%, and WWTP discharge points 51.8%, *C. coli*: recreational waters 98.9%; WWTP discharge points 95.0%; agricultural waters 88.4%). Meat-producing poultry was the second most important contributor in all three types of water for both *C. jejuni* and *C. coli*. Finally, wild birds were also the largest contributor to surface water contamination with both *C. jejuni* and *C. coli* in all seasons. For *C. jejuni*, the second most important contributor in spring was cattle, while meat-producing poultry was the second most important contributor in the other seasons. For *C. coli*, meat-producing poultry was the second most important contributor in all seasons.

3.4. Effects of livestock density, water type and season on the attribution estimates

The differences in attribution estimates for the *C. jejuni* and *C. coli* isolates from surface water (described for each source in Section 3.2) between livestock density areas, water types, and seasons were further tested for statistical significance using multiple linear regression. The significant differences are summarized in Table 6.

For *C. jejuni*, significantly higher attributions to wild birds were associated with high ruminant density (β=9.21, 95%CI 1.62;16.80), recreational waters (β=15.31, 95%CI 8.04;22.57), and agricultural waters (β=5.66, 95%CI 0.92;10.40). Furthermore, the attributions to ruminants were negatively associated with high ruminant density (β=-10.66, 95%CI -20.42;-0.79), and positively associated with agricultural waters (β=11.19, 95%CI 1.79;20.59) and WWTP discharge points (β=0.10, 95%CI 0.77;20.05), mostly during the warmer seasons. Attributions to poultry were positively associated with WWTP discharge points in winter (β=5.24, 95%CI 0.90;9.59). For *C. coli*, significantly higher attributions to poultry were associated with high poultry density (β=2.51, 95%CI 0.10;4.91) and agricultural waters (β=2.14, 95%CI 0.69;3.58). Furthermore, the attributions to wild birds were negatively associated with high poultry density (β=-2.63, 95%CI -5.04;-0.21), and positively associated with recreational waters (β=1.95, 95%CI 0.07;3.83) and WWTP discharge points (β=2.13, 95%CI 0.66;3.61).

Fig. 5 shows the canonical correlation analysis (CCA) plots to visualize the results of the regression analyses for both *C. jejuni*

Table 5
Source attribution estimates of the *C. jejuni* and *C. coli* isolates in surface water per livestock density area, water type, and season based on cgMLST.

Species	Variable	Category	Wild birds	Meat-producing poultry	Laying hens	Adult cattle	Small ruminants	Veal calves
C. jejuni	Livestock density	Poultry high	59.2 (35.8-82.2)	24.9 (8.1-44.4)	0.3 (0.0-0.9)	15.4 (0.0-33.0)	0.2 (0.0-0.4)	0.0 (0.0-0.0)
		Poultry low	99.4 (99.0-99.7)	0.3 (0.2-0.5)	0.1 (0.0-0.2)	0.2 (0.0-0.6)	0.0 (0.0-0.0)	0.0 (0.0-0.0)
		Pigs high	40.6 (10.8-70.3)	23.2 (3.0-46.8)	10.1 (0.2-28.3)	5.6 (0.0-16.8)	20.6 (0.3-50.4)	0.0 (0.0-0.0)
		Pigs low	50.0 (12.6-87.3)	16.8 (0.0-0.4)	1.1 (0.0-3.2)	14.0 (0.0-37.6)	17.6 (0.1-47.5)	0.7 (0.0-2.0)
		Ruminants high	64.4 (44.3-84.5)	18.4 (3.8-33.9)	4.6 (0.0-13.9)	0.0 (0.0-0.0)	12.6 (0.5-27.5)	0.0 (0.0-0.0)
		Ruminants low	54.6 (28.9-78.5)	19.8 (3.4-39.8)	7.2 (0.0-21.4)	6.8 (0.0-1.9)	11.1 (0.0-28.5)	0.6 (0.0-1.9)
	Water type	Agricultural waters	53.5 (35.4-69.3)	21.3 (8.7-34.6)	3.8 (0.3-10.2)	10.3 (2.2-19.8)	10.7 (1.4-21.9)	0.3 (0.0-0.8)
		Recreational waters	92.3 (78.0-99.7)	7.2 (0.3-20.5)	0.4 (0.0-1.3)	0.0 (0.0-0.0)	0.1 (0.0-0.3)	0.0 (0.0-0.0)
		WWTP discharge	51.8 (34.2-69.6)	21.8 (10.0-36.5)	6.1 (0.0-15.2)	6.5 (0.0-16.3)	13.6 (3.6-25.5)	0.18 (0.0-0.5)
	Season	Spring	54.5 (27.2-81.7)	12.3 (0.3-33.1)	0.0 (0.0-0.0)	23.9 (0.0-50.4)	9.3 (0.1-27.5)	0.0 (0.0-0.1)
		Summer	40.2 (16.1-66.1)	36.2 (15.4-57.6)	1.1 (0.0-2.6)	5.2 (0.0-13.2)	17.3 (0.2-38.5)	0.0 (0.0-0.0)
		Autumn	66.4 (47.2-85.5)	12.1 (0.6-26.1)	8.9 (0.0-22.4)	4.6 (0.1-13.4)	7.6 (0.1-19.0)	0.4 (0.0-1.2)
		Winter	66.7 (47.4-85.4)	18.0 (0.8-26.2)	3.5 (0.0-22.4)	3.2 (0.1-13.3)	8.4 (0.1-19.4)	0.2 (0.0-1.2)
C. coli	Livestock density	Poultry high	87.0 (74.6-98.6)	12.8 (1.1-28.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
		Poultry low	93.2 (85.8-98.6)	4.1 (1.1-9.8)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	2.6 (0.0-7.6)	0.0 (0.0-0.0)
		Pigs high	90.9 (79.3-98.9)	9.0 (1.0-20.5)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
		Pigs low	99.1 (98.7-99.4)	0.9 (0.6-1.2)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)
		Ruminants high	93.3 (82.9-99.0)	6.5 (1.0-17.9)	0.1 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.3)	0.0 (0.0-0.0)
		Ruminants low	94.7 (88.6-98.8)	5.0 (1.0-11.2)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
	Water type	Agricultural waters	88.4 (81.6-95.3)	11.4 (5.4-19.1)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.1)	0.0 (0.0-0.0)
		Recreational waters	98.9 (98.5-99.3)	1.0 (0.0-1.4)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)
		WWTP discharge	95.0 (90.7-98.4)	3.4 (1.3-7.3)	0.1 (0.0-0.1)	0.0 (0.0-0.0)	1.5 (0.1-4.2)	0.0 (0.0-0.0)
	Season	Spring	92.2 (83.6-98.6)	7.7 (1.1-16.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
		Summer	93.0 (85.8-98.7)	6.9 (1.3-13.0)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
		Autumn	94.7 (89.6-98.8)	5.0 (1.2-10.5)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	0.1 (0.0-0.2)	0.0 (0.0-0.0)
		Winter	94.4 (88.0-98.7)	3.5 (1.0-7.8)	0.0 (0.0-0.1)	0.0 (0.0-0.0)	2.1 (0.0-6.0)	0.0 (0.0-0.0)

Table 6
Significant associations of *C. jejuni* and *C. coli* attributions based on cgMLST with livestock density, water type, and season.

Species	Source	Variable	Season	Beta	95% CI	P-value	Ref. category water	Ref. category livestock
C. jejuni	Poultry*	WWTP discharge points	Winter	5.24	0.90;9.59	0.02	Recreational waters	-
	Ruminants**	Ruminants high	Spring	-10.60	-20.42;-0.79	0.04	-	Ruminants low
		Agricultural waters	Spring	11.19	1.79;20.59	0.03	Recreational waters	-
		Recreational waters	Summer	-1.04	-20.05;-0.77	0.04	WWTP discharge points	-
		WWTP discharge points	Summer	0.10	0.77;20.05	0.04	Recreational waters	-
	Wild birds	Ruminants high	Spring	9.21	1.62;16.80	0.02	-	Ruminants low
		Agricultural waters	Winter	5.66	0.92;10.40	0.02	WWTP discharge points	-
		Recreational waters	Spring	15.31	8.04;22.57	0.00	Agricultural waters	-
		Recreational waters	Spring	10.93	3.67;18.20	0.01	WWTP discharge points	-
		Recreational waters	Winter	7.51	2.10;12.93	0.01	WWTP discharge points	-
C. coli	Poultry*	Poultry high	Spring	2.51	0.10;4.91	0.04	-	Poultry low
		Agricultural waters	Autumn	2.14	0.69;3.58	0.00	WWTP discharge points	-
	Wild birds	Poultry high	Spring	-2.63	-5.04;-0.21	0.03	-	Poultry low
		Recreational water	Winter	1.95	0.07;3.83	0.04	WWTP discharge points	-
		WWTP discharge points	Autumn	2.13	0.66;3.61	0.01	Agricultural waters	-

*It includes the attributions of meat-producing poultry (broilers and turkeys) and laying hens.
**It includes the attributions of adult cattle, veal calves and small ruminants.

and *C. coli*. The dots represent the attributions of the surface water isolates to the different sources and the arrows represent the different variables used in the linear regression analysis (i.e. livestock density area, water type or season) to test for differences in the attributions. The stronger the association of a variable with the attributions to a specific source, the longer the arrows. If an arrows points in the same direction as a particular source (dot), this means that there is a positive association between that source and the given variable.

4. Discussion

In this study, the prevalence, genotype diversity and animal origin of *C. jejuni* and *C. coli* strains isolated from surface water in the Netherlands were investigated. Furthermore, it was assessed whether the estimated contributions of the different animal sources varied significantly with the type of surface water (i.e. recreational waters, agricultural waters, and WWTP discharge points), season, and local livestock density.

C. jejuni and/or *C. coli* strains were detected in 66% of surface water samples, demonstrating the widespread presence of these pathogens in surface water, which is an indication of faecal contamination. In contrast to most animal sources, surface water was mainly contaminated with *C. coli*, with a *C. coli* to *C. jejuni* isolation ratio of about 3:1. This finding agrees with previous European studies (Mughini-Gras et al., 2016; Rosef et al., 2001; Shrestha, 2019). Prevalence of both *C. coli* and *C. jejuni* in agricultural water and water at WWTP discharge points was higher compared to that in recreational water. The relatively low prevalence in recreational water was anticipated, as the microbiological water quality at these official EU bathing sites has to comply with European guidelines for fecal contamination. The higher prevalence in agricultural water was also expected, as these water bodies are usually closer to farms, grazing fields, or fields fertil-

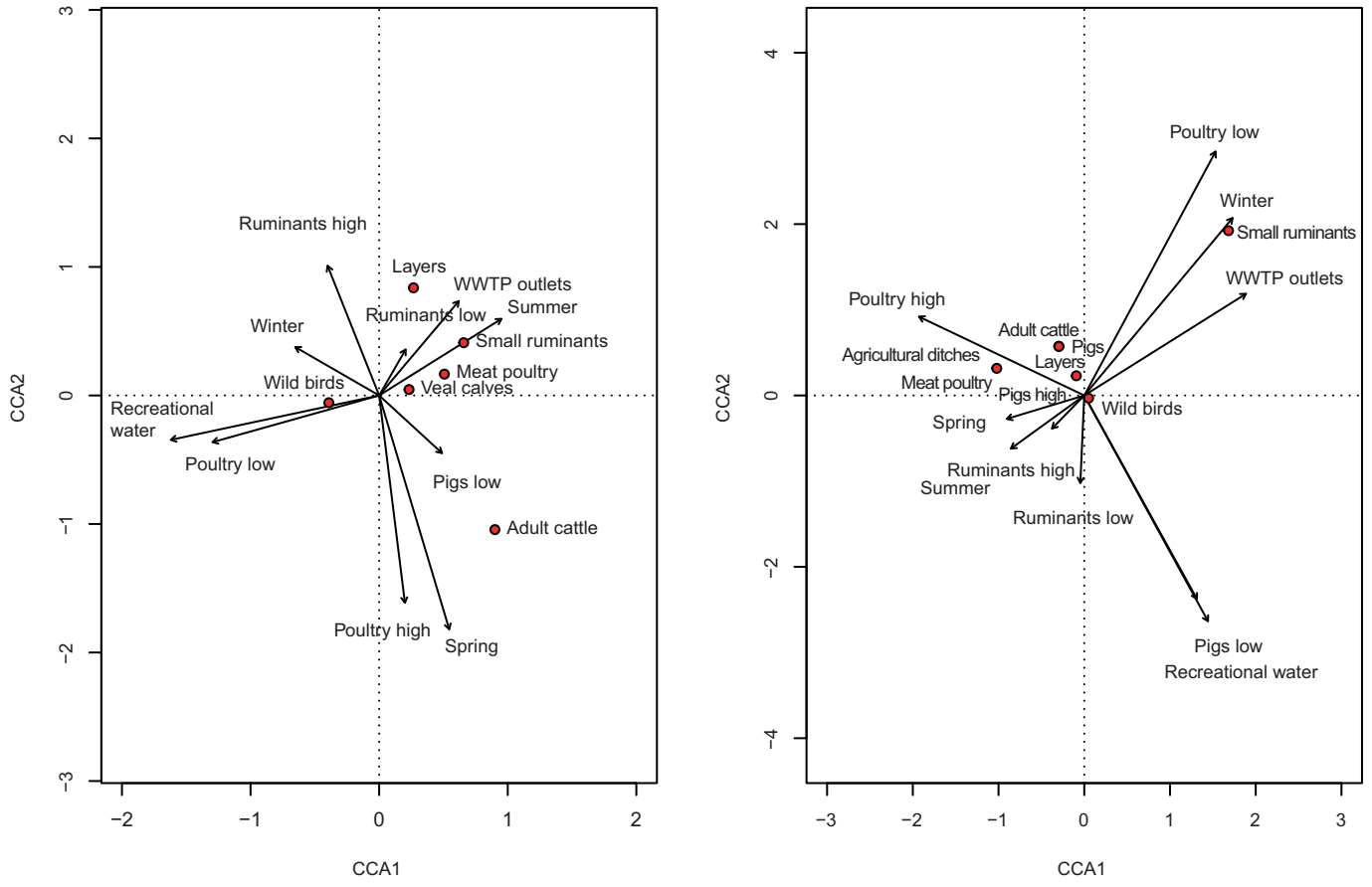

Fig. 5. Canonical correlation analysis plot of *C. jejuni* (left) and *C. coli* (right).

ized with manure where agriculture runoff is more likely to occur. Furthermore, the similarly high *Campylobacter* prevalence at WWTP discharge points was foreseen, as regular wastewater treatment does not completely remove bacteria (Rechenburg, 2009).

Campylobacter prevalence was higher during autumn and winter compared to spring and especially summer. This finding is in agreement with previous studies showing that *Campylobacter* prevalence in surface water is lower when there are more hours of sunshine (Jones, 2001), probably due to higher ultraviolet radiation levels and temperatures, which eventually lead to reduced *Campylobacter* survival in aquatic environments. Indeed, the summer season in the Netherlands has more hours of sunshine in comparison to the spring (KNMI, 2020), and thus higher levels of ultraviolet radiation and ambient temperatures, which might contribute to decreased *Campylobacter* presence in surface water. In agreement with this finding, an increased risk for human campylobacteriosis associated with swimming in surface water in spring compared to swimming in the summer was previously reported (Mughini Gras et al., 2012). Despite the clear difference in *Campylobacter* prevalence between the warmer (spring and summer) and the colder (autumn and winter) seasons, the differences between spring and summer and between autumn and winter were less prominent or absent. A possible explanation is that the water samples representing spring and summer in this study were taken in 2018, a year characterized by extremely dry spring and summer seasons in the Netherlands, including a drought record in July (KNMI, 2018). From the autumn of 2018 onwards, there was a reduction in precipitation deficit, with a recovery towards normal levels in the winter of 2018/2019. This shows that the weather conditions during sampling were quite similar in terms of precipitation for both the two warmer seasons (spring and summer) and the two colder seasons (autumn and winter) and suggests that the role of the aquatic environment as exposure route for humans to *Campylobacter* varies with the seasons and their weather conditions. Also, a higher frequency of recreational activities in surface water during summer compared to those in winter contributes to this variability.

Strain diversity in surface water as reflected by STs, was very high. There were also high numbers of surface water isolates with novel STs, mainly among *C. coli*. This may be due to *C. coli* being generally under-represented in human and animal samples that have been studied and typed previously, but they were also under-represented in the animal sources that were explored in this study. In agreement with a previous study conducted in the Netherlands and Luxembourg (Mughini-Gras et al., 2016), the most prevalent ST in surface water was ST45, which was most often detected in agricultural waters. ST45 has been recognized to be ubiquitous and to be more frequently found in the environment than other STs that are common in humans (French et al., 2005; Mughini-Gras et al., 2016; Sopwith et al., 2008). This has lent weight to the hypothesis that ST45 is a potential environmentally adapted ST that is able to survive advert conditions while being outside the host (Colles et al., 2011; French et al., 2005; Sopwith et al., 2008). Also the other STs prevalent in surface water, i.e. ST1766, ST137 and ST2654, have frequently been isolated from surface water and/or wild birds worldwide, as reported in the *Campylobacter* PubMLST database.

The population structure of both *C. coli* and *C. jejuni* from surface water showed predominance of wild bird-like strains compared to other sources. This was particularly the case for *C. coli*, where 94% of the water isolates were attributable to wild birds.

The remaining *C. coli* were predominantly attributable to poultry, in particular in areas with high poultry density. Interestingly, while *C. coli* were relatively frequently detected in pigs as well, the cluster of *C. coli* isolates from pigs was clearly separated from the surface water isolates. For *C. jejuni*, wild birds explained about 60% of all isolates in water, and livestock sources, particularly poultry, significantly contributed to water contamination as well. This was supported by the finding that *C. jejuni* prevalence was higher in the high poultry and ruminant density areas compared to the low poultry and ruminant density areas, while for *C. coli* it was the other way around, suggesting a more prominent role of these livestock groups in contaminating surface water with *C. jejuni* relative to *C. coli*.

Source attribution analysis confirmed that wild birds were the likely source of the majority of strains found in surface water, followed by poultry (broilers, turkeys and layers combined) and ruminants (cattle, sheep and goats combined). Similar results were found in studies performed in Luxembourg for *C. jejuni* and *C. coli* (Mughini-Gras et al., 2016) and in New Zealand for *C. jejuni* (Shrestha, 2019) in which about 61% of the surface water isolates originated from wild birds. As the wild bird isolates in this study mainly comprised isolates from aquatic bird species and only from one terrestrial species (common wood pigeon - *Columba palumbus*), this finding is highly plausible. Of note is that when repeating the source attribution analysis with the common wood pigeon isolates as separate group, the attribution to the terrestrial bird species was about 9% (data not shown), showing that aquatic wild birds remain the most likely source of strains found in surface water. With a larger collection of wild bird isolates it would be interesting to focus future studies on how *Campylobacter* prevalence and its attributions to different bird species differ according to their habitats, migration patterns and roosting behaviors (Ito et al., 1988; Waldenström et al., 2002; Whelan et al., 1988).

It was previously reported that *C. jejuni* and *C. coli* isolates from surface water in the Netherlands were mainly attributable to poultry (52%), followed by wild birds (37%). However, that study was performed in poultry-rich regions and results may therefore be explained by a relative high environmental dissemination of *Campylobacter* strains from poultry farms (Mughini-Gras et al., 2016). In agreement with this, the linear regression results in the present study show that surface water strains attributable to poultry were significantly more likely to be found in high compared to low poultry density areas and in agricultural diches. This supports the previously postulated hypothesis that geographical variation in the relative contribution of poultry as a source of surface water contamination with *Campylobacter* is associated with local differences in the magnitude of poultry production (Mughini-Gras et al., 2016). This could also explain the observed decrease in human campylobacteriosis incidence in areas where poultry farms and slaughterhouses were emptied (i.e. culled), thoroughly disinfected and closed to control the devastating H7N7 avian influenza epidemic in 2003 in the Netherlands (Friesema et al., 2012). Indeed, it is possible that this is a reflection of reduced environmental *Campylobacter* load due to the temporary inactiveness of poultry farms.

C. jejuni strains attributable to ruminants were more likely to be isolated from surface water in low vs. high ruminant density areas, which is counterintuitive. A possible explanation could be that in the low ruminant density areas, farming operations are less intensive (and more extensive) in nature, with differences being related to farm size (CBS, 2018a,b), grazing opportunities (e.g. use of pasture lands, time animals spent in pastures) (Van Den Pol-Van Dasselaar et al., 2015), and likely management of manure and distance to surface waterways as well. However, the attribution results of *C. jejuni* are more uncertain then the attribution results of *C. coli* strains, which could also influence the results.

Besides that livestock densities influence the relative contributions of *Campylobacter* of different sources in surface water, it was also shown that there are seasonal and water type-dependent variations in those contributions. Those variations may reflect different conditions facilitating access to, contact with, and discharge of fecal material, into surface water. An example is *C. jejuni* contamination in water at WWTP discharge points. Although contamination from sewage is mainly of human origin, water at WWTP discharge points had a significantly higher contribution of poultry-associated strains than other types of surface water. As poultry is the primary source of human *Campylobacter* infections (Mughini Gras et al., 2012), the *C. jejuni* contamination in water at WWTP discharge points is likely to reflect a pattern more similar to that of the (main) sources of human infections, i.e. poultry, as observed previously (Mughini-Gras et al., 2016), than that of other animal sources.

A few methodological considerations are called for. We used a no admixture model, meaning that each water isolate was assumed to come 'as is' from one of the animal sources. This model was appropriate for this study as we aimed to quantify the fraction of isolates found in surface water that is directly attributable to each of the animal sources, thereby considering only the last transfer step of the (potentially longer and more complex) *Campylobacter* transmission chains among hosts and the environment, i.e. the transfer step from animals to surface water. Indeed, *Campylobacter* is not able to grow outside the host, so its presence in the environment is only a matter of die-off rather than growth. This means that the isolates found in the environment originate as such from (the feces of) a specific host and are not generated in the environment itself. In the admixture model, on the other hand, the isolates are assumed to have mixed ancestry and this is modelled by saying that, for example, isolate i has inherited a given proportion of its genome from ancestors in population k (Porras-Hurtado et al., 2013; Pritchard et al., 2009). However, here we were interested in knowing the most likely animal origin of an isolate as a whole based on its genome and our goal was not to make evolutionary inference about the life history of strains.

The application of USEPOPINFO allowed for the inclusion of isolates of known origin (i.e. the animal isolates) as to attribute only the isolates of unknown origin (i.e. the water isolates) (Pritchard et al., 2009). Therefore, a potential bias derives from the assumption that the pre-defined (animal) populations are correct, while misclassification might occur. However, the use of AMOVA to (re-)define the groupings of animal isolates to be used as sources in the attribution analysis based on the genetic similarities of their isolates made it possible to consider the pre-defined populations as actual populations.

5. Conclusions

The results of this study led to the following conclusions:

- *C. coli* is the dominant *Campylobacter* species in surface water.
- *Campylobacter* prevalence is highest in agricultural waters and during the coldest months of the year and lowest in recreational waters and warmer months.
- Wild birds and meat-producing poultry are the main contributors to *Campylobacter* contamination of surface water, with water type, season, and local livestock (particularly poultry and ruminant) density being significant drivers of these contributions.
- Poultry-associated *Campylobacter* strains are mostly found in agricultural waters, water at WWTP discharge points, and in areas with high poultry density.
- Wild bird-associated *Campylobacter* strains are mostly found in areas with low poultry density, high ruminant density, recreational waters and WWTP discharge points.

- R-uminant-associated *Campylobacter* strains are mostly found in low ruminant density areas, agricultural waters and WWTP discharge points, mostly during the warmer seasons.

The above conclusions may have public health implications, because even if we can ensure that poultry meat is *Campylobacter*-free at the point of consumption, leading to a reduction in human campylobacteriosis cases, human exposure can also occur via environmental pathways and specifically the aquatic environment. This calls for interventions aimed at controlling environmental dissemination of *Campylobacter* at primary livestock production and WWTPs, provided that cost-benefit analyses show that the public health benefits outweigh the costs of such interventions. Conversely, virtually nothing can be done to control *Campylobacter* in wildlife. In this regard, the finding that >90% of *Campylobacter* strains from recreational waters are attributable to wild birds, and that the higher contribution of wild birds to recreational water contamination relative to other types of water is significant, implies that the risk of acquiring campylobacteriosis from, e.g., swimming in official recreational water sites in the Netherlands, is largely beyond human control.

Data statement

The livestock density data are available at Statistics Netherlands (https://www.cbs.nl/en-gb). The Campylobacter sequences are deposited in ENA Sequence Read Archive (project PRJEB38253).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Annemieke C. Mulder: Formal analysis, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Eelco Franz:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Sharona de Rijk:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Moyke A.J. Versluis:** Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. **Claudia Coipan:** Formal analysis, Formal analysis, Investigation, Project administration, Software, Writing - review & editing. **Ralph Buij:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Gerard Müskens:** Investigation, Writing - review & editing. **Miriam Koene:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Roan Pijnacker:** Formal analysis, Formal analysis, Investigation, Writing - review & editing. **Birgitta Duim:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Linda van der Graaf-van Bloois:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Kees Veldman:** Formal analysis, Investigation, Methodology, Writing - review & editing. **Jaap A. Wagenaar:** Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Aldert L. Zomer:** Formal analysis, Investigation, Methodology, Software, Writing - review & editing. **Franciska M. Schets:** Conceptualization, Funding acquisition, Investigation, Resources, Writing - review & editing. **Hetty Blaak:** Conceptualization, Funding acquisition, Investigation, Methodology, Resources, Writing - review & editing. **Lapo Mughini-Gras:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Writing - original draft, Writing - review & editing.

Acknowledgements

The authors would like to thank Menno van der Voort, Greetje A.A. Castelijn, Joke van der Giessen, Marieke Opsteegh and Angela van Hoek for support in isolate collection and logistics, and Ben Bom for support in selecting the study areas. This study was financially supported by the Netherlands' Organization for Health Research and Development (ZonMw) with grant number 50-52200-98-316 (project name: "DEPiCT - Discerning Environmental Pathways of Campylobacter Transmission") and the Dutch Ministry of Health, Welfare and Sport with grant number 9.2.09.E (project name: "Campylobacter source attribution"). The funding source had no involvement in the conduct of the research and/or preparation of the article. There are no conflicts of interest to declare for any of the authors.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116421.

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