



Quantifying potential sources of surface water contamination with *Campylobacter jejuni* and *Campylobacter coli*

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ABSTRACT

Campylobacter is the most common causative agent of human bacterial gastroenteritis and is frequently found in surface water, where it indicates recent contamination with animal faeces, sewage effluent, and agricultural run-off. The contribution of different animal reservoirs to surface water contamination with *Campylobacter* is largely unknown. In the Netherlands, the massive poultry culling to control the 2003 avian influenza epidemic coincided with a 44–50% reduction in human campylobacteriosis cases in the culling areas, suggesting substantial environment-mediated spread of poultry-borne *Campylobacter*. We inferred the origin of surface water *Campylobacter jejuni* and *Campylobacter coli* strains in Luxembourg and the Netherlands, as defined by multilocus sequence typing, by comparison to strains from poultry, pigs, ruminants, and wild birds, using the asymmetric island model for source attribution. Most Luxembourgish water strains were attributed to wild birds (61.0%), followed by poultry (18.8%), ruminants (15.9%), and pigs (4.3%); whereas the Dutch water strains were mainly attributed to poultry (51.7%), wild birds (37.3%), ruminants (9.8%), and pigs (1.2%). Attributions varied over seasons and surface water types, and geographical variation in the relative contribution of poultry correlated with the magnitude of poultry production at either the national or provincial level, suggesting that environmental dissemination of *Campylobacter* from poultry farms and slaughterhouses can be substantial in poultry-rich regions.

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1. Introduction

Campylobacter jejuni and *Campylobacter coli*, the most common causative agents of bacterial gastroenteritis in humans, are commonly found in surface water, where they usually give clues to recent contamination with animal faeces, sewage effluents, and agricultural run-off from nearby grounds (Jones, 2001). The fate of these bacteria in water is typically the one of survival and die-off

rather than growth; thus, surface water usually only serves as a vehicle for transmission and not as an amplifying reservoir (Thomas et al., 1998).

Although the primary route of infection with *Campylobacter jejuni* and *C. coli* in humans is contaminated food, source attribution studies have estimated that on top of the contributions of poultry, ruminants and pigs, the environment may account for a further 5–10% of human campylobacteriosis morbidity (Mossong et al., 2016; Mughini Gras et al., 2012), with exposure to recreational freshwater and to storm water overflows being an often overlooked potential source of human campylobacteriosis (Arnone and Walling, 2007; Sales-Ortells et al., 2015; Sales-Ortells and Medema, 2015). *Campylobacter* spp. survival outside the host

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depends on numerous exogenous factors (e.g. temperature, sunlight exposure, etc.) and *Campylobacter* spp. shedding from animals varies seasonally depending on factors such as stress, changes in diet, housing conditions, etc., and the raising period, particularly for poultry. Moreover, the pattern of human exposure to environmental sources (e.g. outdoor activities) is largely weather-dependent; thus, environment-borne human campylobacteriosis exhibits a strong seasonality (Bronowski et al., 2014; Jones, 2001; Mughini Gras et al., 2012). Studies in several countries have shown that heavy rainfall and failures in the treatment and proper management of drinking water and groundwater sources may lead to *Campylobacter* spp. being disseminated in the water supply system (Bartholomew et al., 2014; Holme, 2003; Kuusi et al., 2005; O'Reilly et al., 2007). Perhaps more importantly, environmental waters may also act as a source for *Campylobacter* spp. colonization in food-producing animals, including poultry, as *Campylobacter* spp. may enter the poultry flock horizontally via ground and surface water (Bull et al., 2006; Perez-Boto et al., 2010), which are frequently used as drinking water for farm animals. All this identifies the essential role of water within the *Campylobacter* spp. infectivity cycle, which has some of the hallmarks of a waterborne disease.

Although surface water cannot be considered as an amplifying host for *Campylobacter* spp., it represents a 'sink' that collects strains from different amplifying (animal) hosts, whose individual contributions are largely unknown, though wildlife and especially wild birds are often assumed to play a major role (Mughini Gras et al., 2012; Mullner et al., 2009a, 2009b; Smid et al., 2013). However, the devastating avian influenza outbreak (H7N7) hitting the Netherlands in 2003 showed that the massive poultry culling (involving predominantly egg-laying hens) and closure of poultry slaughterhouses undertaken to contain the outbreak was associated with a concurrent 44–50% decrease in human campylobacteriosis cases in the culling areas and in the areas where the slaughterhouses were closed (Friesema et al., 2012). Although the sales of poultry meat dropped too (~9% countrywide during the culling period), this drop was not proportional to the observed campylobacteriosis reduction, and sales recovered quickly after June 2003, whereas the campylobacteriosis reduction lasted until the end of the year. This provided suggestive evidence for a major role of environment-mediated spread of poultry-borne *Campylobacter* strains (Friesema et al., 2012).

Using a large collection of *C. jejuni* and *C. coli* strains typed with multilocus sequence typing (MLST), this study aims to quantify the contribution of four putative animal reservoirs, namely poultry, pigs, ruminants, and wild birds, to the surface water contamination with these two *Campylobacter* species.

2. Materials and methods

2.1. Water sample collection and processing

MLST was performed on 308 *C. jejuni* and 142 *C. coli* isolates from different types of surface water, including rivers ($n = 73$), ponds/lakes ($n = 27$), streams/canals/ditches ($n = 271$), and wastewater treatment plant outlets ($n = 79$) in various parts of Luxembourg ($n = 329$) and the Netherlands ($n = 121$) according to Dingle et al. (2001), with minor modifications (Ragimbeau et al., 2014; Schets et al., submitted).

Water samples were taken by submerging sterile glass bottles according to the NEN-EN-ISO 19458 standard procedure. The Dutch water samples were filtered using 0.45 μm cellulose-based membranes (Millipore) in volumes of 140–700 ml, depending on their turbidity. Filters were then placed in 20–25 ml Preston broth and incubated in microaerobic conditions using CampyGen sachets

(Oxoid) for 48 h at 42 °C. Samples were then streaked (10 μl) on CCDA agar and re-incubated at the same conditions. From each sample, a maximum of five colonies was inspected by light microscopy, and a maximum of three isolates per sample was analysed by PCR for genus confirmation and species identification as described elsewhere (Jensen et al., 2005; Keramas et al., 2003). Isolation of *Campylobacter* spp. from the Luxembourgish water samples was based on an optimised passive filtration protocol derived from the ISO 17995 standard. Volumes of 1–5000 ml of water samples, depending on their turbidity, were filtered onto 0.45 μm cellulose-based membranes (Sartorius). On average, five filters per sample were placed and retentate side up onto Campy-Count agar (Oxoid) and incubated under microaerobic conditions at 42 °C for 15–18 h. The filters were removed from the agar surface and the plates were re-incubated for another 48–72 h, depending on growth and colony development. Suspected *Campylobacter* spp. isolates were examined by light microscopy and confirmation at the genus level was achieved with an antibody-sensitised latex particle agglutination test (Dryspot *Campylobacter* test kit, Oxoid). Candidate *Campylobacter* strains were then isolated through two additional cultivation steps on mCCDA if they were not pure or, alternatively, on the non-selective chocolate PolyVitest agar (Bio-Mérieux). Colonies were stored at –80 °C in FBP medium. In parallel, DNA was extracted by using the QIAamp DNA Mini Kit (Qiagen). Identification at the species level was achieved by a duplex real-time PCR targeting the *hypO* and *gyrA* genes of *C. jejuni* and *C. coli*, respectively, as previously described (Ragimbeau et al., 2014).

2.2. Animal data collection

MLST-typed *C. jejuni* and *C. coli* isolates from poultry ($n = 774$), ruminants ($n = 115$), and pigs ($n = 97$) were obtained from different research and surveillance activities on farm, slaughterhouse, and retail in Luxembourg and the Netherlands. To enhance representativeness (Smid et al., 2013), ruminant and pig isolates were supplemented with others (108 from ruminants and 118 from pigs) from the neighbouring countries Germany ($n = 179$), France ($n = 39$), and Belgium ($n = 8$) available in the literature (Gripp et al., 2011; Ragimbeau et al., 2014) and in the PubMLST web repository (<http://pubmlst.org/campylobacter>). For wild birds, no local MLST data were available for Luxembourg and the Netherlands, so these were obtained from the literature (Colles et al., 2008a, 2008b, 2009, 2011) and from PubMLST based on those European countries belonging to the same biogeographical regions of Luxembourg and the Netherlands (i.e. continental and Atlantic regions) as defined by the official delineations used in the EU Habitats Directive (92/43/EEC) and for the EMERALD Network set up under the Bern Convention on the Conservation of European Wildlife and Natural Habitats (<http://www.eea.europa.eu/data-and-maps/data/biogeographical-regions-europe>). The available MLST-typed *Campylobacter* isolates from wild birds from the UK ($n = 463$), Germany ($n = 6$), and Denmark ($n = 1$) were then used.

2.3. Assessment of non-local and non-recent data

All isolates were collected during 2000–2014. A summary of the isolates used here is given in Table 1 and a breakdown per year of collection is provided as Supplementary Material S1. A breakdown of the water isolates per type of surface water and season is provided in Table 2 and in Supplementary Material S2. Part of the data used here has been used in previous studies in Luxembourg (Mosson et al., 2016; Ragimbeau et al., 2008, 2014) and the Netherlands (Mughini-Gras et al., 2014b; Mughini Gras et al., 2012; Mughini Gras et al., 2013; Smid et al., 2013).

Table 1Number of *Campylobacter jejuni* and *C. coli* isolates typed with MLST (and number of different sequence types therein) used in the source attribution analysis.

Country	Species	Surface water	Poultry ^a	Ruminants ^b	Pigs	Wild birds ^c	Reference
Luxembourg	<i>C. jejuni</i>	207 (107)	183 (89)	107 (30)	—	—	(Ragimbeau et al., 2014, Ragimbeau et al., 2008)
	<i>C. coli</i>	122 (77)	155 (67)	2 (2)	80 (47)	—	and original data
The Netherlands	<i>C. jejuni</i>	101 (52)	335 (103)	6 (6)	2 (2)	—	(Mughini-Gras et al., 2014b;
	<i>C. coli</i>	20 (13)	101 (44)	—	15 (11)	—	Mughini Gras et al., 2012; Smid et al., 2013)
Belgium	<i>C. jejuni</i>	—	—	—	—	—	(Schets et al., submitted)
	<i>C. coli</i>	—	—	—	8 (6)	—	(Ragimbeau et al., 2014)
France	<i>C. jejuni</i>	—	—	—	—	—	(Ragimbeau et al., 2014) and PubMLST
	<i>C. coli</i>	—	—	—	39 (30)	—	
Germany	<i>C. jejuni</i>	—	—	95 (40)	—	4 (4)	(Gripp et al., 2011) and PubMLST
	<i>C. coli</i>	—	—	13 (8)	71 (33)	2 (2)	
United Kingdom	<i>C. jejuni</i>	—	—	—	—	360 (133)	(Colles et al., 2011, Colles et al., 2008a,
	<i>C. coli</i>	—	—	—	—	103 (44)	Colles et al., 2008b, Colles et al., 2009) and PubMLST
Denmark	<i>C. jejuni</i>	—	—	—	—	1 (1)	PubMLST
	<i>C. coli</i>	—	—	—	—	—	
Total	<i>C. jejuni</i>	308 (145)	518 (152)	208 (56)	2 (2)	365 (137)	—
	<i>C. coli</i>	142 (86)	256 (91)	15 (10)	213 (111)	105 (46)	
Grand total	—	450 (231)	774 (243)	223 (66)	215 (113)	470 (183)	—

^a Broiler chickens (80%), turkeys (6%), egg laying hens (3%), quails (1%), and unspecified poultry (10%).^b Cattle (92%), sheep (5%), and goats (3%).^c Starlings and pigeons (62%), wild waterfowls (20%), gulls and passerines (18%).

Since animal isolates were not all from the same countries and years as the surface water isolates, and were supplemented with isolates from neighbouring countries within a 15-year time frame, we assessed potential sources of bias by calculating the proportional similarity index (PSI), a measure of the area of overlap between two frequency distributions (Smid et al., 2013). The PSI quantified the degree of (dis)similarity of the MLST frequency distributions of the local vs. non-local isolates from pigs and ruminants, as well as those from the water vs. animal isolates over time using five 3-year intervals as performed previously (Mughini-Gras et al., 2014a; Smid et al., 2013). The PSI was also calculated for the animal isolates from farm/slaughterhouse vs. retail to examine the potential differential selection of strains during food processing/storage.

2.4. Source attribution analysis

The asymmetric island model for source attribution (iSource) developed by Wilson et al. (2008) was used to estimate the proportion of surface water isolates attributable to (i.e. putatively originating from) each animal reservoir. Attribution analyses were performed separately for Luxembourg and the Netherlands, and the strains from sources other than poultry were pooled between countries to reach satisfactory statistical power (Smid et al., 2013).

Table 2Number of *Campylobacter* isolates typed with MLST from each type of surface water and their estimated percent relative probabilities (and standard deviation) to originate from poultry, ruminants, pigs and wild birds.

	Stream/ditch/canal	Pond/lake	River	Wastewater outlet
Luxembourg	<i>n</i> = 215	<i>n</i> = 2	<i>n</i> = 56	<i>n</i> = 56
Poultry	16.1% (26.5%)	14.6% (20.7%)	11.0% (27.3%)	36.5% (36.1%)
Ruminants	17.5% (29.7%)	0.2% (0.2%)	3.6% (13.2%)	22.5% (29.6%)
Pigs	3.1% (14.9%)	0.007% (0.001%)	0.5% (2.0%)	11.7% (26.0%)
Wild birds	63.3% (42.4%)	85.2% (20.9%)	85.0% (34.3%)	29.3% (40.9%)
Top STs (<i>n</i>)	45 (18), 1326 (18), 677 (6), 1766, 177 & 1002 (5)	5842 (1), 6283 (1)	1766 (9), 2269, 5658 & 5660 (2), 45 (1)	21 (3), 872 (3), 354, 1595, 48, 50, 61, 1142 & 6282 (2)
The Netherlands	<i>n</i> = 56	<i>n</i> = 25	<i>n</i> = 17	<i>n</i> = 23
Poultry	68.3% (29.8%)	13.3% (31.3%)	31.7% (40.3%)	72.7% (20.5%)
Ruminants	12.0% (16.0%)	1.6% (5.7%)	4.0% (10.6%)	15.2% (16.8%)
Pigs	0.1% (0.7%)	0.02% (0.08%)	0.002% (0.003%)	1.8% (8.3%)
Wild birds	19.6% (29.2%)	85.1% (34.4%)	64.3% (43.5%)	10.3% (10.8%)
Top STs (<i>n</i>)	45 (9), 230 (7), 827 (5)	1223 (6), 637 (4), 1292 (3)	45 (2), 41, 683 & 693 (1)	267 (4), 45 (3), 42, 1614 & 21 (2)

Attributions were also given for each type of surface water. Information on the season of sampling was only available for the Luxembourgish surface water isolates; thus, seasonality in attributions was assessed for Luxembourg only. Given the generally low poultry production in Luxembourg, within-country geographical variation in attributions was only assessed in the Netherlands. According to Statistics Netherlands (www.cbs.nl), most poultry production in the Netherlands is concentrated in three (out of 12) provinces, namely Gelderland, Limburg and Noord-Brabant, which account together for 62% of the national poultry production. We therefore assessed whether the surface water strains from these three provinces (*n* = 17) had a higher probability to originate from poultry than those from the other provinces (*n* = 76); for 28 surface water isolates the province of origin was unknown.

2.5. Minimum spanning trees

Minimum spanning trees (MSTs) based on the MLST profiles were built in BioNumerics 7.1 (Applied Maths) using the categorical coefficient to visualize the population structure of *C. jejuni* and *C. coli* strains included in this study. Distance between STs was calculated based on the number of different loci between profiles.

3. Results

3.1. Genotypes

An overview of the STs per source is given in Fig. 1. Of the 184 and 65 STs respectively identified among the Luxembourgish and Dutch surface water isolates, the most common (≥ 5 isolates) were ST-45, ST-1326, ST-1766, ST-677, ST-177, ST-1002, and ST-5964 (Luxembourg), and ST-45, ST-230, ST-1223, ST-267, ST-572, ST-827, and ST-42 (the Netherlands). The complete list of STs found in surface water in Luxembourg and the Netherlands is provided as [Supplementary Material S3](#). The top STs (accounting for at least 25% of the isolates) found in each type of surface water are reported in [Table 2](#). While ST-45 seemed to frequently occur in virtually all types of surface water in the Netherlands, in Luxembourg it was mainly prevalent in streams/ditches/canals ([Table 2](#)), and was relatively less prevalent during wintertime ([Table 3](#)).

Comparing local vs. non-local MLST data for pigs gave PSI values of 90.0% (Luxembourg) and 92.5% (the Netherlands), while the PSI values for ruminants were 77.5% (Luxembourg) and 63.3% (The Netherlands). In [Fig. 2](#), the PSI values for the temporal comparisons of MLST data between surface water and each of the animal sources are plotted as a function of the time difference between their years of collection. A linear function was fitted over these PSI values. Although water data tended to differ from the poultry data with increasing time between their years of collection (linear slope -0.013), this trend was not statistically significant ($p = 0.124$). No significant trends were also found for any of the other sources (wild birds, linear slope 0.007 $p = 0.461$; pigs, linear slope 0.002 , $p = 0.726$; ruminants, linear slope 0.001 , $p = 0.812$).

Of the 215 pig isolates, 97 (45.1%) were sourced at farms, whereas for the remaining 118 (54.9%) the sampling point was unknown; the PSI for pig data at farm vs. unknown sampling point was 92.6%. Of the 223 ruminant isolates, 114 (51.1%) originated from farm/slaughterhouse, 1 (0.4%) from retail, and the remaining 108

(48.5%) came from an unknown sampling point. The PSI for ruminant data at farm/slaughterhouse vs. unknown sampling point was 76.1%. All Dutch poultry isolates originated from farms/slaughterhouses. Of the 338 Luxembourgish poultry isolates, 66 (19.5%) were sourced at farm/slaughterhouse and 272 (80.5%) at retail; their PSI was 73.9%.

3.2. Attributions

In Luxembourg, most *Campylobacter* strains from surface water were attributed to wild birds (61.0%, 95% credible interval [CI] 54.9–67.0%), followed by poultry (18.8%, 13.0–25.0%), ruminants (15.9%, 11.0–22.0%), and pigs (4.3%, 1.9–7.4%). In the Netherlands, the attributions were as follows: poultry (51.7%, 95% CI 37.6–64.3%), wild birds (37.3%, 27.7–47.6%), ruminants (9.8%, 1.4–20.6%), and pigs (1.2%, 0.04–3.8%) ([Fig. 3](#)). The complete set of posterior probabilities per ST is provided as [Supplementary Material S3](#).

The specific attributions for each type of surface water are reported in [Table 2](#). In Luxembourg, wild birds were estimated to be the predominant contributor to *Campylobacter* contamination in streams/ditches/canals (63.3%), ponds/lakes (85.2%), and rivers (85.0%), but not in wastewater outlets, for which poultry was estimated as the primary source (36.5%). In the Netherlands, poultry was estimated to be the predominant contributor to *Campylobacter* contamination in streams/ditches/canals (68.3%) and wastewater outlets (72.7%), whereas wild birds predominated in ponds/lakes (85.1%) and rivers (64.3%).

Wild birds were estimated as the primary source of the *Campylobacter* strains contaminating Luxembourg's surface water at all seasons, but their contribution was highest in autumn (67.7%) and summer (63.5%) and lowest in winter (56.0%) and spring (57.8%). In contrast, poultry and ruminant contributions peaked in winter (26.8%) and spring (19.7%), respectively. The probability for the Dutch water isolates from the high poultry production provinces (61.7%, 95% CI: 46.4–76.9%) was significantly higher (Mann-

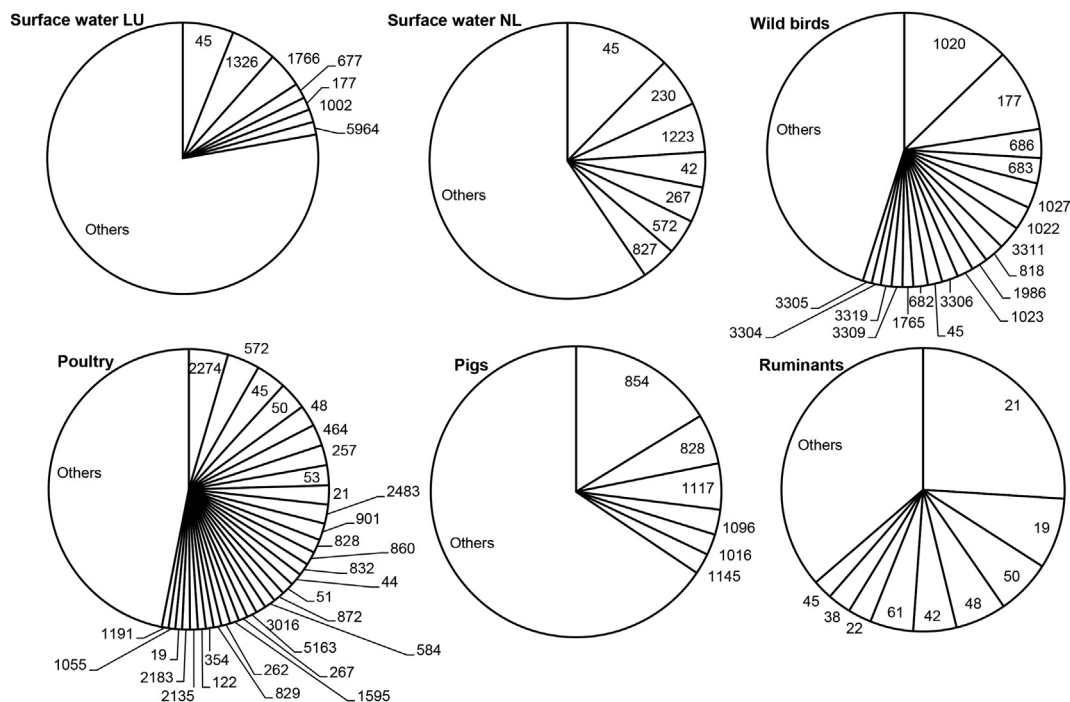


Fig. 1. Distribution of sequence types identified in the *Campylobacter jejuni* and *C. coli* isolates from surface water and from the different animal sources. The segment 'others' includes sequence types represented by less than 5 isolates.

Table 3
Number of *Campylobacter* isolates typed with MLST from Luxembourg's surface water per season and their estimated percent relative probabilities (and standard deviation) to originate from poultry, ruminants, pigs and wild birds.

	Winter <i>n</i> = 54	Spring <i>n</i> = 100	Summer <i>n</i> = 114	Autumn <i>n</i> = 61
Poultry	26.8% (34.7%)	17.6% (28.4%)	16.5% (26.6%)	17.5% (31.0%)
Ruminants	14.4% (25.2%)	19.7% (31.8%)	16.1% (27.9%)	10.5% (23.6%)
Pigs	2.7% (11.2%)	4.9% (18.6%)	3.9% (16.7%)	4.2% (16.4%)
Wild birds	56.0% (44.9%)	57.8% (44.4%)	63.5% (42.8%)	67.7% (44.2%)
	1766 (4), 5964	1326 (11), 45	45 (10), 1326 (7),	1766 (8), 45, 19,
Top STs (<i>n</i>)	(3), 5150, 5805, 5841 & 6282 (2)	(7), 1002 (4), 1142, 5979 & 6169 (2)	677 (5), 177, 267 & 583 (3)	1981 & 1595 (2)

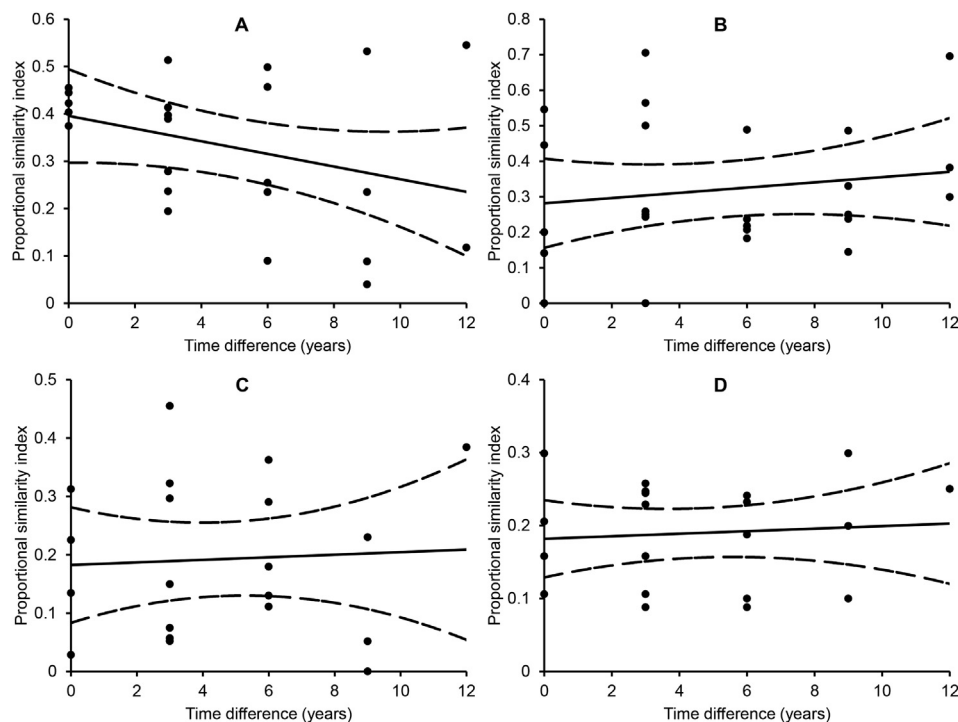


Fig. 2. Temporal similarity of MLST data of *Campylobacter* isolates from surface water vs. those from poultry (A), wild birds (B) ruminants (C), and pigs (D).

Whitney *U* test, $p = 0.0243$) than that for the isolates from the rest of the country (40.5%, 32.2–48.9%).

3.3. Population structure

The two MSTs displaying the MLST profiles of *C. jejuni* and *C. coli* strains are shown in Figs. 4 and 5, respectively. Each node represents a different ST, whose size is proportional to the number of strains with that ST. The colours within each node reflect the proportions of strains obtained from the different sources. Solid lines connect STs differing at only one locus; neighbouring STs differing at more than one locus are connected by a dotted line. We differentiated between Dutch and Luxembourgish surface water strains to better visualize the distribution of their respective STs among the different putative sources. The Dutch surface water *C. jejuni* strains were mainly distributed in generalist STs or in branches predominated by poultry (Fig. 4), and the Dutch surface *C. coli* strains were mainly clustered in an agricultural lineage, i.e. the distinct branch including all STs associated with domestic animals in the lower left part of the MST in Fig. 5. In contrast, for both *Campylobacter* species, most of the Luxembourgish surface water strains tended to cluster

in the same branches as the wild bird strains (see upper and lower branches for *C. jejuni* in Fig. 4 and the upper branches for *C. coli* in Fig. 5).

4. Discussion

This study showed that *Campylobacter* strains from surface water are mainly associated with poultry and wild birds, and to a lesser extent with ruminants and pigs. However, the relative contribution of poultry and wild birds to *Campylobacter* surface water contamination differed between Luxembourg and the Netherlands, with wild birds being inferred as the primary source of *Campylobacter* strains found in Luxembourg's surface water and poultry being so in the Netherlands'. This is probably related to the differences in average poultry density and slaughtering intensity between Luxembourg (circa 33 poultry/km² and <1 kg poultry meat produced/person-year) and the Netherlands (circa 2396 poultry/km² and 44 kg poultry meat produced/person-year) as derived from 2005 to 2010 Eurostat agricultural figures (<http://ec.europa.eu/eurostat>). Our additional analyses at the provincial level further supported the hypothesis of a relationship between

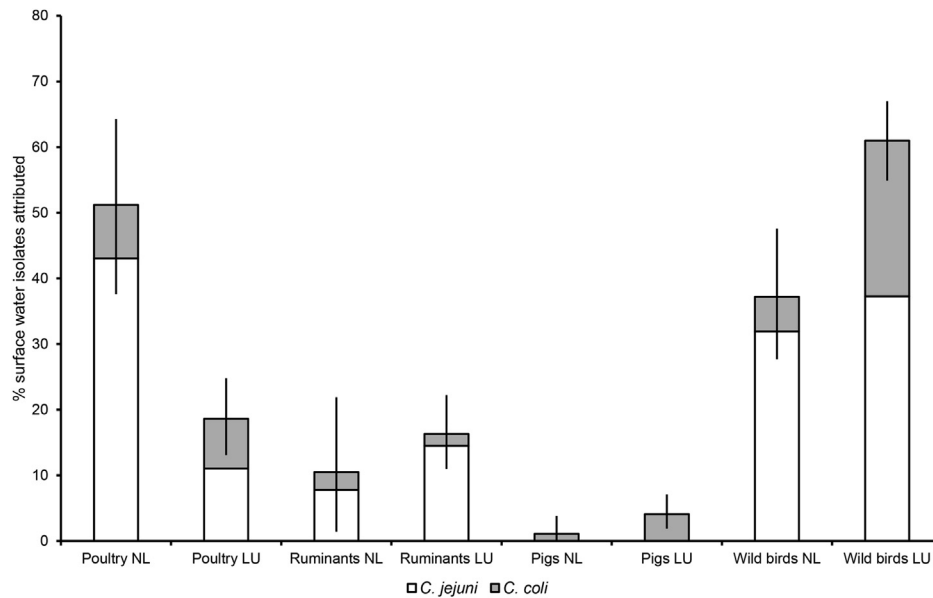


Fig. 3. Percent attributions of *Campylobacter jejuni* and *C. coli* isolates from surface water to putative animal sources in Luxembourg (LU) and the Netherlands (NL). Error bars indicate 95% confidence intervals.

the magnitude of poultry production and the environmental contamination with poultry-associated *Campylobacter* strains, as the surface water isolates from the largest poultry-producing provinces in the Netherlands were significantly more likely to originate from poultry than the isolates collected elsewhere. This would contribute to explain why when poultry farms and slaughterhouses were emptied, thoroughly disinfected and closed in response to the 2003 H7N7 avian influenza outbreak in the Netherlands, a concurrent drop in human campylobacteriosis incidence was observed in the areas where these measures were enforced, as this might be a reflection of a lower environmental load of *Campylobacter* usually disseminated from the temporarily inactive local poultry farms and slaughterhouses (Friesema et al., 2012). These findings are relevant to public health since there are many areas globally devoted to poultry farming. It follows, therefore, that wild birds would become the primary source of environmental contamination with *Campylobacter* spp. in relatively less poultry-rich areas. The application of source attribution models based on molecular typing and evolutionary modelling to estimate the relative contribution of different animal reservoirs to surface water contamination with *Campylobacter* spp. relevant to public health has also been performed in New Zealand to attribute water isolates from the Manawatu and Toenepi catchments (French et al., 2011). Interestingly, also these authors reported that most (~70%) water isolates were attributable to wild birds.

While the contribution of the main food-producing animals to human campylobacteriosis is well documented (Boysen et al., 2014; Mughini Gras et al., 2012; Mullner et al., 2009b; Sheppard et al., 2009; Strachan et al., 2009; Wilson et al., 2008), limited knowledge exists about their contribution to disseminating *Campylobacter* into the environment. *Campylobacter* is generally believed to survive poorly outside the animal host relative to other indicators of faecal contamination, such as *Salmonella* spp., coliforms and streptococci. Yet some specialist *Campylobacter* strains seem to have successfully occupied specific sylvatic (Hepworth et al., 2011; Williams et al., 2010) and farmland niches (Biggs et al., 2011; French et al., 2005), although such strains often lack some of the genes associated with the ability to colonize chickens and are uncommon among human infections (Hepworth et al., 2011). Indeed intensive

animal husbandry may be involved in generating reservoirs for *Campylobacter* strains associated with human disease (Colles et al., 2011; Griekspoor et al., 2013). As an example, a study found that >90% of *Campylobacter* strains from domesticated mallard ducks were commonly associated with human disease in contrast to <1% of strains from their wild counterparts (Colles et al., 2011). Besides the factors influencing *Campylobacter* shedding in animals mentioned in the introduction, seasonal and surface water sample-dependent variations in the relative contributions of different reservoirs may reflect different conditions facilitating access to, and discharge of faecal material into, surface water. For example, *Campylobacter* contamination in Luxembourg's wastewater outlets, which come from wastewater treatment plants that also treat human wastes, showed a relatively higher contribution of poultry than wild birds (unlike the other types of surface water), possibly reflecting a pattern more similar to that of the (main) sources of human infections, i.e. poultry. However, in developed countries like the Netherlands and Luxembourg with high infrastructural and sanitation standards, sewerage systems (or septic tanks in some rural areas) are well maintained and have total territorial coverage. Thus, except for confined accidents (e.g. sewage leakage or overflow from malfunctioning/overwhelmed sewerage systems), human sewage is always restrained to pipelines and subject to treatment before release. Moreover, regular wastewater treatment is quite effective against *Campylobacter* spp., and *Campylobacter* concentrations in wastewater outlets do not seem to increase downstream as more treatment plants discharge into it (Rechenburg and Kistemann, 2009). Considering all this and that humans are not a reservoir for *Campylobacter* spp. (so excretion is usually short-term), there is no major environmental dispersion of *Campylobacter* strains from human faecal material. For this reason, the potential contribution of humans was ruled out *a priori* in this study, but future research may benefit from considering this aspect if/when dealing with a different epidemiological setting. A way to address this would be, for instance, to use the isolates from wastewater treatment plants as a source.

A number of studies have investigated wild bird-associated *Campylobacter* strains and their contribution to human campylobacteriosis (Colles et al., 2011, 2008a, 2009; French et al., 2009;

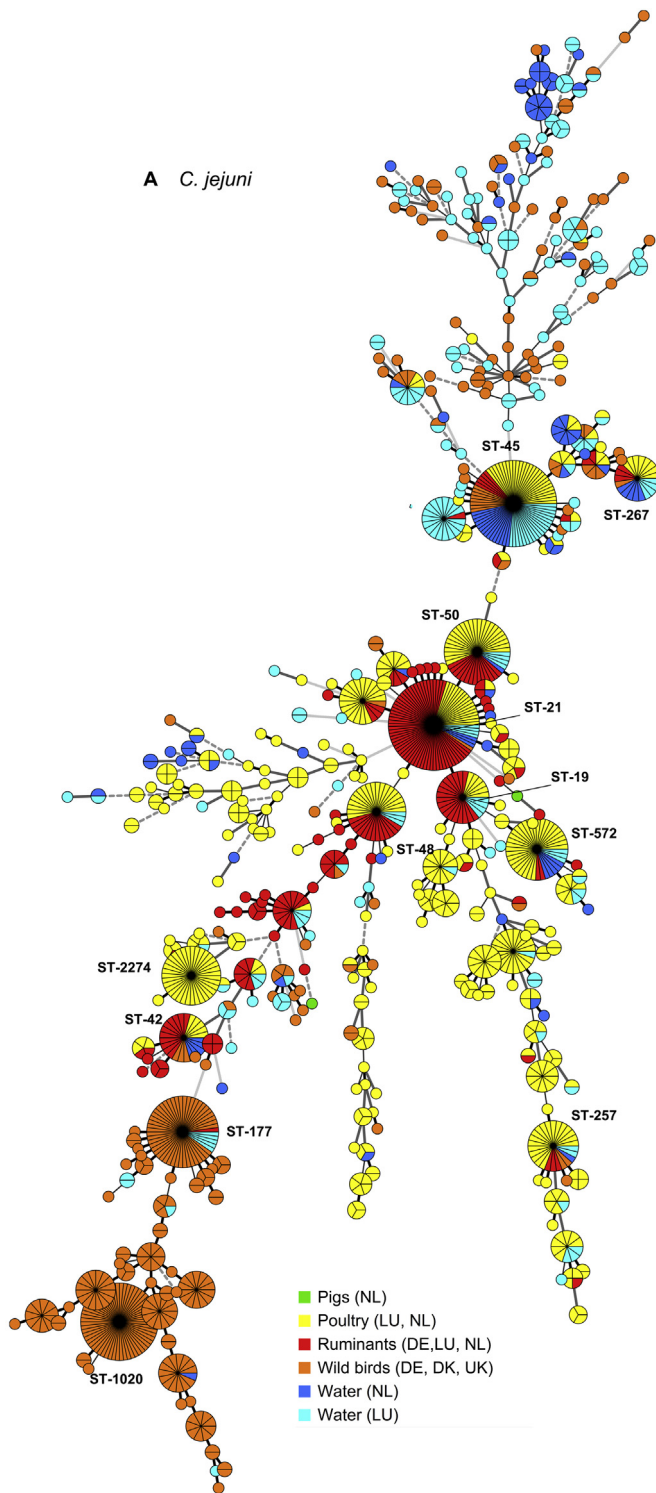


Fig. 4. Minimum spanning trees of *Campylobacter jejuni* strains typed with multilocus sequence typing from surface water and from potential animal reservoirs. The number of strains per source and country of origin is reported in Table 1, while the frequencies of sequence types are summarized in Fig. 1. Surface water *C. jejuni* strains were 207 and 101 from Luxembourg and the Netherlands, respectively. NL = The Netherlands; LU = Luxembourg; BE = Belgium; FR = France; DE = Germany; DK = Denmark; UK = United Kingdom.

Griekspoor et al., 2013; Strachan et al., 2009), and a recent study from the UK (Cody et al., 2015) has reported that wild birds may account for up to 3.5% of human campylobacteriosis cases annually, although this proportion varies seasonally, with wild bird-

associated strains being more represented among humans during the warm months (Cody et al., 2015). Moreover, a Scottish study reported that wild bird-associated strains may account for 24% of *Campylobacter* infections in <5-year-old children living in rural areas, and 6% in their urban counterparts (Strachan et al., 2009). Other routes besides surface water by which wild birds can contribute to human campylobacteriosis are via faecal material in children's playgrounds (French et al., 2009) or by environmental exposure during leisure or employment activities (Strachan et al., 2009).

The most prevalent ST found in both the Dutch and Luxembourgish surface water strains, ST-45, is known to have a widespread distribution and to be more frequently found in the environment than other generalist STs (French et al., 2005; Sopwith et al., 2008). This has long lent weight to the hypothesis that ST-45 is a potential environmentally adapted *Campylobacter* ST with increased fitness outside the host, making it a key driver of transmission between animals and humans via the environment (Colles et al., 2011; French et al., 2005; Sopwith et al., 2008), which may happen either through direct exposure to environmental water and outdoor activities or through pets (Mughini Gras et al., 2013). This could also apply to poultry, and it has been shown that *Campylobacter* colonization with ST-45 in a new flock can arise from a simple puddle outside a chicken house (Bull et al., 2006). The main sources of ST-45 in surface water differed between Luxembourg and the Netherlands (Supplementary Material S3). In Luxembourg, ST-45 was mainly associated with wild birds (42.1%), although a relatively high contribution from poultry was also found (34.5%), but in the Netherlands ST-45 was strongly associated with poultry (71.6%), confirming that this ST lacks of a strong host specificity.

The main limitation of this study is related to the availability of local data for wild birds, which were sourced from the UK and to a much lesser extent from Germany and Denmark. Inherent to this is the assumption that the data used are representative of the wild bird-associated STs circulating in Luxembourg and the Netherlands. However, following Smid et al. (2013), this potential bias was minimized by selecting strains from geographically close countries with comparable ecological conditions that could be expected to harbor wild bird populations similar to those in the countries under study. Moreover, even though geographical variation of *Campylobacter* STs may have an impact on the attributions (Smid et al., 2013), the association between host species and *Campylobacter* STs is expected to be stronger than that with geographical origin (Sheppard et al., 2010), and this has been proven true for wild birds whose *Campylobacter* STs exhibit a marked host specificity and lack of phylogeographic population structure (Griekspoor et al., 2013). Also livestock data were not all from the same countries and years as the surface water samples, and were supplemented with data from neighbouring countries within a 15-year time frame. Although this may be considered a major limitation, the work of Smid et al. (2013) does indicate that this may not result in major biases provided they are similar to the local and recent data. We therefore performed additional analyses to assess the degree of (dis)similarity between local and non-local data, and between water and animal data over time. Altogether these analyses confirmed that the non-local data were far more similar than they were different to the local data, thereby acting as a good surrogate. Moreover, no significant temporal trends were found in the similarity between water and animal data, indicating that temporal variation is unlikely to have caused significant bias. Indeed, Smid et al. (2013) had already showed that while MLST frequencies of two sources become increasingly dissimilar as the geographic distance and time interval between their collections increase, even when looking at a time lag of over a decade the potential temporal bias is minimal and far less problematic than the potential

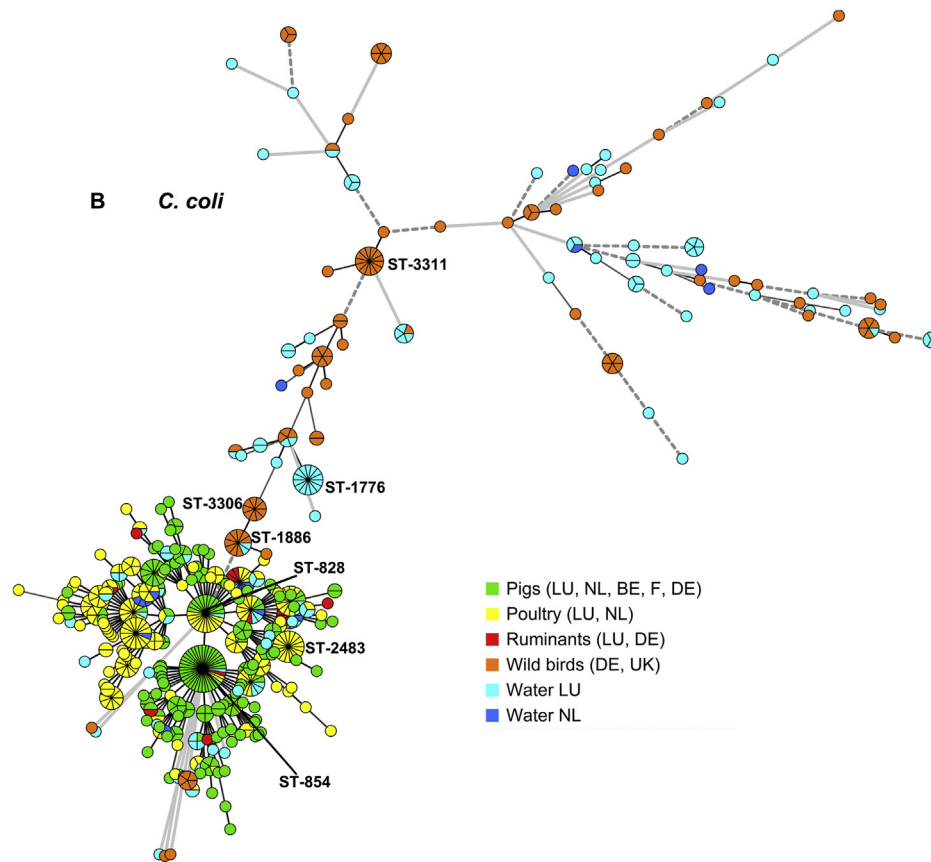


Fig. 5. Minimum spanning trees of *Campylobacter coli* strains typed with multilocus sequence typing from surface water and from potential animal reservoirs. The number of strains per source and country of origin is reported in Table 1, while the frequencies of sequence types are summarized in Fig. 1. Surface water *C. coli* strains were 122 and 20 from Luxembourg and the Netherlands, respectively. NL = The Netherlands; LU = Luxembourg; BE = Belgium; FR = France; DE = Germany; DK = Denmark; UK = United Kingdom.

geographical bias derived from using data from distant regions. Our non-local ruminant and pig isolates originated from neighbouring countries in Western Europe with which either Luxembourg or the Netherlands have intensive trading relationship of livestock and products thereof. Moreover, both Luxembourg and the Netherlands are relatively small countries and the non-local source strains used here were highly representative of what can be found domestically, providing an explanation of why the non-local data were so similar to the local data. Other limitations referred to the use of retail data despite our hypothesis that environmental contamination with *Campylobacter* spp. originates from farms and slaughterhouses. However, also retail data were much alike those collected at farm/slaughterhouse or those of unknown origin, so also the potential bias due to differential selection during food processing/storage was minimized. Although geographical variation was assessed at both the national (Luxembourg vs. the Netherlands) and provincial (within the Netherlands) levels, our approach essentially relies on aggregate data and it is prone to ecological fallacy; thus, further smaller-scale analyses based on areas with varying poultry productions may be more insightful. Other reservoirs, including non-avian wildlife species that are likely to play a role in spreading *Campylobacter* spp. in the environment, could not be assessed here. In addition, 20 of the Dutch surface water isolates were sourced from water bodies in proximity to poultry farms and were therefore likely to originate thereof. However, removing these strains from the analysis did not change the attributions (results not shown), so the same conclusions were drawn. Finally, analysing data from different studies called into question the representativeness of the genetic diversity in each source since these data derived from

different sampling and testing methods. Conventional protocols including an enrichment step might entail a bias due to selection of some *Campylobacter* strains to the detriment of others. In contrast, direct plating with or without a passive filtration may lift this selection, allowing for a better representativeness of genetic diversity. Recent studies demonstrated that both culture-based strategies are in fact complementary in representing the diversity of *Campylobacter* isolates (Vidal et al., 2016). Additionally, refining the analysis of these genotypes does not impact on the inferred source population structure, as their genetic profiles remain closely related (Vidal et al., 2016; Williams et al., 2012). Moreover, the representativeness of genetic diversity in a given source seems to rely more on the characterised number of isolates per sample and/or sampling location than on the culture method itself (Vidal et al., 2016). Similarly, Vidal et al. (2016) also showed that sample types (environmental vs. animal) are associated with smaller differences than the sampling locations. The large strain collection used in this study would have therefore minimised the potential impact of the different protocols followed to generate such data.

5. Conclusions

Surface water is a recognized key player in campylobacteriosis epidemiology and it is therefore crucial to assess the relative importance of the different animal reservoirs for *Campylobacter* spp. of public health significance. The results of this study allowed us to conclude that:

- Of the four putative animal reservoirs considered here, wild birds and poultry appear to be the most important contributors to surface water contamination with *Campylobacter* spp. in Luxembourg and the Netherlands, respectively.
- Attributions vary according to season and surface water type, possibly reflecting different conditions influencing *Campylobacter* shedding in animals and discharge of faecal material into surface water.
- There is suggestive evidence indicating that geographical variation in the relative contribution of poultry is linked to the magnitude of poultry production itself, supporting the previous hypothesis of substantial environmental dissemination of *Campylobacter* strains from poultry farms and slaughterhouses in poultry-rich regions like the Netherlands (Friesema et al., 2012). This has important public health implications because it means that even if poultry meat is *Campylobacter*-free at the point of consumption, human exposure may occur earlier via environmental pathways, calling for interventions aimed at controlling environmental dissemination of *Campylobacter* during production. Conversely, very little can be done to control *Campylobacter* in wildlife, and more research is needed to clarify the directionality of *Campylobacter* transmission between reservoirs and surface water and how *Campylobacter* spreads into the environment beyond farming boundaries.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2016.05.069>

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