

Effect of environmental parameters on the inactivation of the waterborne pathogen *Campylobacter* in a Mediterranean river

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ABSTRACT

Campylobacter is a major waterborne pathogen that can be found in rivers of the Mediterranean area. Characteristics of these rivers change throughout the seasons due to variations in environmental parameters. As these variations may affect water survival of *Campylobacter*, we analyzed it in the Llobregat River using three approaches whose complexity increase progressively: (i) river water microcosms in the laboratory subjected to varying temperatures; (ii) *insitu* experiments carried out in the river, in which bacteria were exposed to varying levels of environmental parameters; and (iii) monitoring of thermotolerant *Campylobacter* in the river over two years. *Campylobacter* was quantified using the most probable number (MPN) method. The results showed that an increase in water temperature accelerates *Campylobacter* inactivation, measured as the loss of culturability. *Insitu* experiments revealed that inactivation rates were also affected by sunlight, but not by pH, oxygen concentration or water conductivity. These observations are supported by the seasonality detected in Llobregat River. *Campylobacter* inactivation was fastest in spring and summer, when temperature and solar radiation were at their highest. The results highlight the importance of considering the inactivation rates in natural conditions to improve the monitoring of this pathogen and thus evaluate properly the health risk associated to water.

Key words | *Campylobacter*, environmental parameters, inactivation, Mediterranean, river water

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INTRODUCTION

Thermotolerant *Campylobacter* species are the main cause of bacterial gastroenteritis in developed countries. In fact, they are the most commonly reported cause of acute bacterial food poisoning in the European Union (EFSA 2007). In Spain, the incidence has increased in recent years (2000–2008) and is currently at levels comparable to that of *Salmonella*, which classically has been the most significant gastric pathogen in this country (Anon 2008). *Campylobacter* is a zoonotic pathogen that lives in the intestinal tract of homoeothermic animals, especially birds. Its major routes of transmission include foods derived from animal carriers, and polluted water (Koenraad *et al.* 1997; Savill *et al.* 2003).

Campylobacter species are widely distributed in aquatic systems. Numerous studies have reported their presence in

rivers, lakes, estuaries and even groundwater. Water is mainly contaminated by sewage inputs from urban areas, by faeces from wild birds or other animals, and by agricultural run-off. In the environment, campylobacters are unable to multiply outside host animals, so they are considered as a sign of recent faecal contamination (Jones 2001). The survival of *Campylobacter* in river water has been related to biotic and abiotic factors such as predation, dilution or temperature. However, to our knowledge, the effect of environmental parameters on *Campylobacter* has been only tested in laboratory-related systems (Obiri-Danso & Jones 1999; Eyles *et al.* 2003).

This study was carried out in the Llobregat River, which flows through a Mediterranean area of Spain, in southern

Europe. It has a temperate climate, with temperatures averaging 25 °C in summer and 12 °C in winter. Most of the precipitation occurs in autumn and spring; in the summer, hours of sunlight are highest and precipitation levels are lowest. Many studies indicate that southern Europe is the area of the continent most severely affected by climatic change (EEA 2004). This area is expected to become warmer and drier, posing new threats to its waterways, particularly river ecosystems and their microbiota.

The aim of this work is to study the effect of environmental parameters on the inactivation of *Campylobacter* in the Llobregat River. The research focused on the effects of temperature, sunlight and rainfall, which are the factors most affected by climatic change in southern Europe. *Campylobacter* inactivation was determined by culture methods, comparable with those recommended by ISO normative 17995:2005 (Anon 2005). So, in this study, inactivation is defined as the loss of culturability. Three types of experiment were performed and their complexity increased progressively. First, we measured the effect of temperature on the inactivation of *Campylobacter* in river water microcosms kept in the laboratory. Secondly, to study the behavior of the bacteria in the river, we carried out *in situ* experiments in which the pathogen was exposed to variations in several environmental parameters, as water temperature, pH, oxygen concentration, water conductivity and solar radiation. Finally, we studied the relationship between the

most characteristic environmental parameters of the Mediterranean climate and the *Campylobacter* counts in the river, over a two-year sampling period.

METHODS AND MATERIALS

Study site

The Llobregat River flows through Catalonia, in the north-east of Spain (Figure 1). It rises in the Pyrenees and flows over a distance of 170 km into the Mediterranean Sea, south of the city of Barcelona. It is a typical Mediterranean river, so its flow is characterized by a high variability, which is strongly determined by seasonal rainfall. The Llobregat is an overexploited river; it has been subjected to intense anthropogenic pressures, receiving extensive agricultural, urban and industrial waste-water discharges (Ginebreda *et al.* 2010).

Bacterial strains and culture

Inactivation studies were conducted on the thermotolerant strain *Campylobacter jejuni* DSMZ 4688, which was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). This species was chosen due to its significance in human disease (Butzler 2004). The strain was stored at -80 °C in Brain Heart Infusion (Difco) with 20% of glycerol.

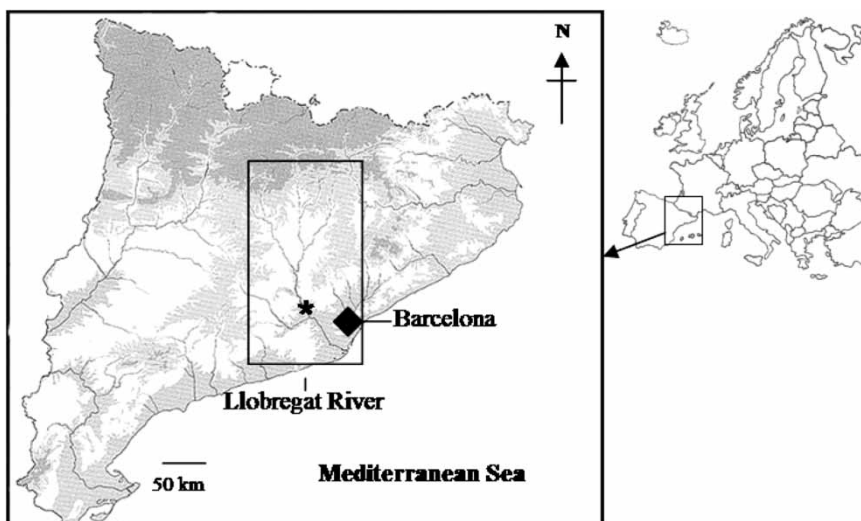


Figure 1 | Location of the Llobregat River in southern Europe. The region of Abrera is marked as an asterisk.

Campylobacter was cultured on Karmali agar plates (Scharlab) at 42 °C for 2 days under microaerobic conditions, generated in a jar by the CampyGen™ system (Oxoid). Cells were harvested by suspension in phosphate-buffered saline (PBS), which was adjusted to give a bacterial concentration of 10⁸ cfu/ml, using a DU 730 Coulter spectrophotometer (Beckman) with a wavelength of 450 nm.

River water microcosms subjected to varying temperatures

Water was collected from the Llobregat River in the area of Abrera (lat 41°30', lon 1°55') (Figure 1). Volumes of 200 ml were placed in 250 ml sterile Pyrex bottles and inoculated with suspensions of *Campylobacter* DSMZ 4688 to give a concentration of approximately 10⁷ cfu/ml. A duplicate set of bottles were incubated in the dark at each of the incubation temperatures studied: 4, 10, 20, 30 and 37 °C. Aliquots were taken at time 0, 6 and each 24 h. *Campylobacter* was enumerated by the most probable number method (MPN) with Preston enrichment broth, as explained below.

In situ experiments in the river

To study *Campylobacter* inactivation in the Llobregat River, dialysis tubes were placed in the river as follows: dialysis tubes were pre-treated to ensure a uniform pore size (molecular weight cut-off of 14 kDa) and sterilized before use. A suspension with a final concentration of 10⁷ cfu/ml of *C. jejuni* DSMZ 4688 was prepared in river water previously collected from Abrera (Figure 1), which is also where the inactivation experiments were carried out. The dialysis tubes were then filled with 40 ml of the *Campylobacter* suspension and sealed with a knot. They were then placed at a depth of 20–25 cm in a metal cage in the river. *Campylobacter* inactivation in dialysis tubes was measured under five experimental conditions: winter sunlight, winter shade, summer sunlight, summer shade, and spring sunlight. Shade conditions were achieved by installing a roof. Two dialysis tubes were taken daily for a week and *Campylobacter* was enumerated in both of them using the MPN method as explained below. Data on temperature, pH, oxygen concentration, water conductivity and solar radiation for each sampling day were supplied by the Water Treatment Plant

of Abrera, which was next to the place where the experiment was conducted.

Campylobacter monitoring in the Llobregat River over a two-year study period

Thermotolerant campylobacters were quantified in the Llobregat River using the MPN method as explained below. Samples were collected in two sampling sites near the location of Abrera over a two-year period (Figure 1). Forty-seven 3 L river samples were analyzed monthly from January 2005 to December 2006. Data on precipitation and solar irradiance for each day of the sampling period were supplied by the weather station nearest the sampling points (approximately 20 km away, in the location of Vacarisses), which belongs to the *Servei Meteorològic de Catalunya* ('Meteorological Service of Catalonia'). Water temperature was measured *in situ* at the time of sampling. The faecal indicator *Escherichia coli* was also quantified in water samples. The culture medium was RAPID *E. coli* agar (Scharlab) and was incubated at 37 ± 1 °C for 24 h.

Enumeration of *Campylobacter* using the MPN method in Preston broth

To quantify *Campylobacter*, water samples were analyzed using the MPN method in Preston enrichment broth (Hijnen *et al.* 2004). A three-tube MPN analysis was carried out and appropriate tenfold dilution series were tested. When necessary, the water samples were concentrated by centrifugation at 7,700 × g for 20 min. Each pellet was resuspended in 1 ml of PBS and inoculated in the enrichment Preston broth (Oxoid). For river samples, volumes from 1 to 1,000 ml of water were analyzed. In microcosms and dialysis experiments, volumes from 1 ml to 10⁻⁷ dilution were tested. Tubes were incubated at 42 °C for 48 h under microaerobic conditions. A subculture was started by streaking a loopful of growth from a Preston broth tube onto a plate of Karmali and incubated for 48 h at 42 °C under microaerobic conditions. Plates were examined for the presence of *Campylobacter* colonies. MPN values were obtained from MPN tables. Presumptive colonies of campylobacters were confirmed with the '*Campylobacter* test kit' of Oxoid.

Statistical data analysis

Graphs were plotted using Excel software (Microsoft® Excel XP). The inactivation of *Campylobacter* was calculated as the decrease in \log_{10} units ($\log N_o/N$), where N_o is the value at the beginning of the experiment and N is the value at the time indicated. The kinetics of the inactivation were fitted to a linear model ($Y = -ax$), where Y represents the logarithmic units of inactivation, a represents the slope of the line and x represents the time (hours). A correlation coefficient of r^2 was added. T_{90} is the time (hours) taken for the initial microbial population to reduce its culturability by 90% and it corresponds to the value of x in the equation when $Y = 1$. T_{90} was calculated for each experiment.

Statistical analyses were performed using the program STATGRAPHICS Plus 5.1. Nonparametric Spearman's rank correlation coefficients (r_s) and regression analyses were calculated for all the parameters analyzed. Correlation coefficients were tested at the 95% confidence level.

RESULTS

The effect of temperature on *Campylobacter* inactivation in river water microcosms

The inactivation of thermotolerant *C. jejuni* was analyzed in river water microcosm systems at varying temperatures in

the dark. Figure 2 shows the regression lines of *Campylobacter* reduction. The r^2 values obtained from these experiments are high, indicating robust results (Table 1). The time taken for the initial microbial population to reduce its culturability by 90% (T_{90}) progressively decreases from 98 h at 4 °C to 11.1 h at 37 °C. A regression analysis was applied to determine the role of temperature in T_{90} variations. In the dark, temperature explains 94.4% of *Campylobacter* inactivation ($p < 0.05$). These results confirm that the inactivation of microorganisms increases at higher temperatures.

The effect of environmental parameters on *Campylobacter* inactivation: *in situ* experiments

To effectively replicate the effect of differing environmental parameters on *Campylobacter* cells in river water, an *in situ* experiment was carried out in the Llobregat River during different seasons. *Campylobacter* inactivation is shown as regression lines in Figure 3. Equations of the line and T_{90} are shown in Table 2. Water conditions in each assay are also shown. Differences in *Campylobacter* were detected depending on the season and on the intensity of solar radiation. With regard to the effect of light, *Campylobacter* was inactivated faster in summer ($T_{90} = 10.3$ h) than in winter ($T_{90} = 18.5$ h) or spring ($T_{90} = 16.6$ h). In the shade i.e. in the absence of direct sunlight, the times for inactivation were longer. In the summer, time for inactivation in

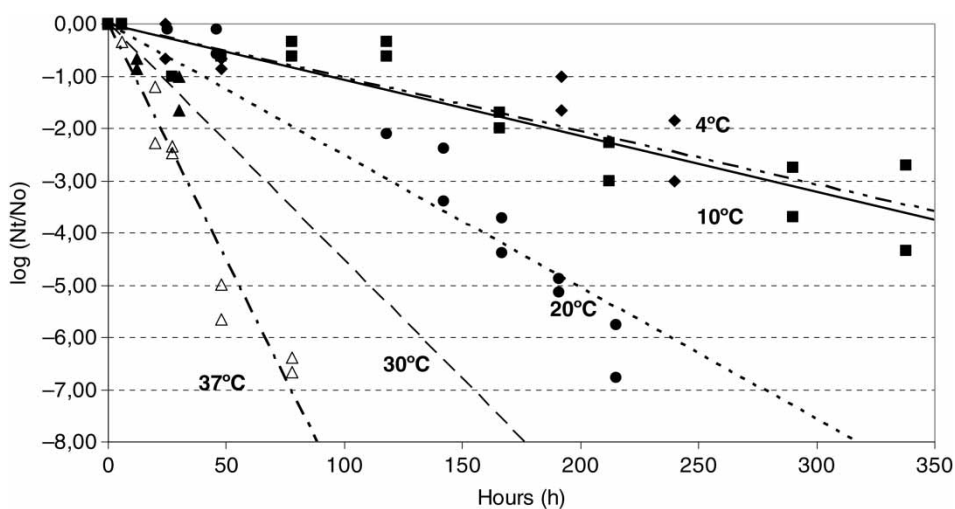


Figure 2 | Regression lines of *Campylobacter* DSMZ 4688 inactivation in microcosms in the dark and at varying temperatures. Empty triangle and (— · —): 37 °C, filled triangle and (— —): 30 °C, filled circle and (- -): 20 °C, filled square and (—): 10 °C and filled rhombus and (— · —): 4 °C.

Table 1 | Equations of inactivation models and T_{90} of *Campylobacter* DSMZ 4688 in microcosms at varying temperatures

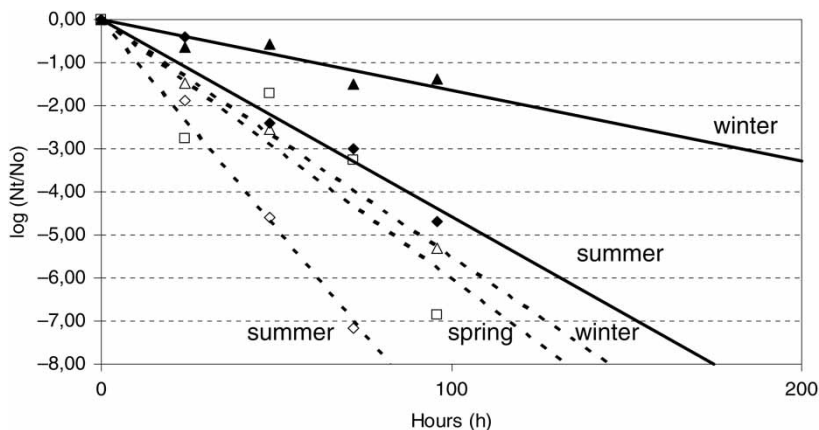
	Temperature (°C)	Equation	R^2	T_{90} (hours)
<i>C. jejuni</i>	4	$y = -0.0102x$	0.9472	98.0
DSMZ 4688	10	$y = -0.0107x$	0.8968	93.5
	20	$y = -0.0252x$	0.9159	39.7
	30	$y = -0.0454x$	0.7618	22.0
	37	$y = -0.0905x$	0.9498	11.1

the shade ($T_{90} = 21.8$ h) was approximately double that recorded in the light, whereas in winter it was three times higher in the shade ($T_{90} = 61.0$ h) than in the light.

To identify a possible correlation between the T_{90} obtained in the dialysis assays and water conditions (water temperature, pH, oxygen concentration, conductivity and solar radiation), a multiple correlation test was applied. Inactivation correlated strongly with solar radiation ($r_s = -0.97$), however, no other relationship was apparent between T_{90} and water conditions.

Campylobacter monitoring in the Llobregat River over a two-year period

Occurrence of thermotolerant campylobacters was analyzed in the Llobregat River. *Campylobacter* was isolated in 81% of the 47 samples collected over the 2 years of sampling. A seasonal trend was observed. Recovery rates were lower in summer and only 50% of the 12 samples were positive. In autumn, 75% of the 12 samples carried campylobacters, and in winter and spring campylobacters were recovered from 100% of the 23 samples. Seasonal fluctuations in *Campylobacter* numbers were also detected. The highest counts were detected in winter, with a geometric mean of 11.3 MPN/100 ml. In autumn and spring, the counts were 3.9 and 2.82 MPN/100 ml, respectively (the geometric mean was calculated from only the positive samples). The lowest counts were detected in summer, with a mean of 0.4 MPN/100 ml. An analysis of variance (ANOVA) test confirmed seasonal difference ($p < 0.05$) between *Campylobacter* counts.

**Figure 3** | Regression lines of *Campylobacter jejuni* DSMZ 4688 natural inactivation in *in situ* experiments in the River Llobregat. Empty rhombus: summer sunlight; empty squares: spring sunlight; empty triangle: winter sunlight; filled rhombus: summer shade and filled triangle: winter in shade. Linear models are shown for each experiment: Dotted lines show sunlight experiments and continuous lines show experiments performed in the shade.**Table 2** | Water conditions and equations of inactivation models and T_{90} of *Campylobacter jejuni* DSMZ 4688 in *in situ* experiments in the Llobregat River – Winter: February; Spring: March; Summer: July; T: water temperature; Cond: conductivity; SR: solar radiation; ND: non detected

<i>C. jejuni</i> DSMZ 4688	T (°C)	pH	O ₂ (ppm)	Cond (mS/cm)	SR (W/m ²)	Equation of the line	R^2	T_{90} (h)
Winter sunlight	6.8	8.5	8.6	1.3	113.2	$y = -0.055x$	0.998	18.5
Spring sunlight	14.1	8.1	7.2	1.8	147.6	$y = -0.060x$	0.788	16.6
Summer sunlight	22.3	8.1	8.1	1.5	241.7	$y = -0.097x$	0.992	10.3
Winter shade	7.40	8.2	7.2	1.8	ND	$y = -0.016x$	0.824	61.0
Summer shade	21.5	7.9	3.5	2.5	ND	$y = -0.046x$	0.954	21.8

Data of environmental parameters for the region were provided by the *Servei Meteorològic de Catalunya* and a summary is provided in Table 3. Correlation coefficients (r_s) between the bacteria and the environmental parameters were calculated for the monthly data. Solar radiation and water temperature parameters showed a significant negative correlation with *Campylobacter* counts of $p < 0.05$ (r_s of solar radiation: -0.62 ; and r_s of water temperature, -0.53). Precipitation was irregular in the area and was highest in autumn. Furthermore, a correlation was not found between this parameter and *Campylobacter* ($p > 0.05$).

E. coli was analyzed in parallel with the thermotolerant *Campylobacter*, and the data were as well compared with environmental parameters. *E. coli* counts did not show significant seasonal variation $p > 0.05$ (ANOVA) and did not depend on temperature or precipitation. However, a moderate correlation was revealed with solar radiation ($r_s = -0.3$). Moreover, a significant correlation within the pathogen *Campylobacter* and the faecal indicator *E. coli* was not found.

DISCUSSION

Persistence rates of microorganisms in the environment determine the way of monitoring them. Survival of the zoonotic pathogen *Campylobacter* in environmental waters depends on factors such as temperature, oxygen levels and sunlight. Several laboratory studies have examined model

aquatic systems (Obiri-Danso *et al.* 2001; Thomas *et al.* 2002), and others have sought to quantify campylobacters in the environment (Bolton *et al.* 1987; Eyles *et al.* 2003; Hörman *et al.* 2004), but to our knowledge no author has attempted to link the two of them. This study aims to fill this gap by combining laboratory-based inactivation systems with assays performed *in situ*, and then compare how environmental factors affected the results of the two study types.

Our results are consistent with other authors, who report that *Campylobacter* inactivation in microcosms is faster at higher temperatures, whereas the bacteria remains culturable longer at cool temperatures, especially around 4°C (Rollins & Colwell 1986; Buswell *et al.* 1998; Thomas *et al.* 1999, 2002; Talibart *et al.* 2000; Obiri-Danso *et al.* 2001; Cools *et al.* 2003). According to Buswell *et al.* (1998) and Thomas *et al.* (1999), the temperature threshold for *Campylobacter* survival lies between 16 and 22°C , which our studies confirm.

Although the experiments on river water microcosms allowed us to study the effect of temperature, they provided only limited information on the effect of other factors that may affect the inactivation of the bacteria in natural conditions. To solve this, we conducted an *in situ* assay in the Llobregat River in different seasons. The results showed that the inactivation rate was related to the environmental parameters of each season. Inactivation was slow in winter ($T_{90} = 18.5$ h) and fast in summer ($T_{90} = 10.3$ h).

Table 3 | Summary of statistics of *Campylobacter*, *E. coli* and physicochemical parameters by season in the Llobregat River – Winter: December, January, February; Spring: March, April, May; Summer: June, July, August; Autumn: September, October, November; n : number of samples; SR: solar radiation; G. mean: Geometric mean; SD: standard deviation; % positive: Percentage of positive samples. ^aArithmetic mean.

	Statistics	<i>Campylobacter</i> (MPN 100 ml ⁻¹)	<i>E. coli</i> (CFU 100 ml ⁻¹)	SR (W/m ² day ⁻¹)	Water temperature (°C)	Precipitation (mm month ⁻¹)
Winter $n = 12$	G. mean	11.0	1,500	93 ^a	5 ^a	37 ^a
	SD	330	1,300	26	4	44
	% positive	100	100	–	–	–
Spring $n = 11$	G. mean	2.8	730	195 ^a	16 ^a	16 ^a
	SD	44	5,400	55	5	13
	% positive	100	100	–	–	–
Summer $n = 12$	G. mean	0.4	980	235 ^a	25 ^a	22 ^a
	SD	6	6,300	27	1	18
	% positive	50	100	–	–	–
Autumn $n = 12$	G. mean	3.9	2,000	129 ^a	16 ^a	75 ^a
	SD	36	4,100	70	4	74
	% positive	75	100	–	–	–

Inactivation in the river and in microcosms followed the same trend, but it was faster in the *in situ* experiment. This indicates that *Campylobacter* inactivation is affected by factors other than just water temperature.

A correlation between *Campylobacter* counts and solar radiation was found in the *in situ* experiments even when results in the shade include the small effect of the scattering sunlight. In all the assays, inactivation was faster under sunlight conditions than in the shade. No relationship between *Campylobacter* inactivation and pH, conductivity or oxygen was found, Buswell *et al.* (1998) also did not find a relationship with oxygen concentrations. In the winter experiments, inactivation was approximately three times faster in light than in the shade. In summer, inactivation was twice as fast in the light as in the shade. The more rapid inactivation of *Campylobacter* is attributed to its high sensitivity to solar radiation. These results agreed with those obtained in other conditions by Obiri-Danso *et al.* (2001) who studied the effect of light on *Campylobacter* inactivation in water using Petri plates. Sinton *et al.* (2007) studied the same subject by exposing 100 L of river water inoculated with campylobacters to radiation with a light intensity comparable to that of the Mediterranean, but they maintained the experimental system at a constant temperature of 14 °C. Inactivation in our dialysis system was slower than that reported by these authors probably due to differences in experimental design.

The results obtained with microcosms and *in situ* assays were compared with environmental data from the Llobregat River. *Campylobacter* numbers in the river showed a seasonal pattern, being highest in winter and autumn. *Campylobacter* was clearly affected by seasonality, as other authors have described in New Zealand (Eyles *et al.* 2003) and in England (Obiri-Danso & Jones 1999). Some authors have explained seasonality in terms of seasonal increases in contamination inputs (Eyles *et al.* 2003; Hörman *et al.* 2004). We therefore considered this possibility in the Llobregat River and found that *E. coli* counts were constant throughout the study, indicating that faecal contamination was regular throughout the sampling time. We found that *E. coli* was not related to *Campylobacter* counts, so we can confirm that *Campylobacter* seasonality in the Llobregat is not attributable to variations in faecal contamination.

Previous studies performed by Bolton *et al.* (1987) in the UK and by Rechenburg & Kistemann (2009) in Germany

correlated high *Campylobacter* counts with run-off after rainfall. In contrast, in the Llobregat River, *Campylobacter* counts did not correlate with precipitation, so run-off is not an important input of campylobacters in this case, probably because the river flows through an urban area at the sampling point, and most of the few farm animals in the zone are kept in enclosures.

We found that *Campylobacter* seasonality in the Llobregat River is mainly due to changes in water temperature and sunlight, which is shown by the significant negative correlation between these parameters and *Campylobacter* counts. As the inactivation of the bacteria is faster in spring and summer, it may be difficult to detect *Campylobacter* inputs in water samples from areas with Mediterranean climates between May and September. This problem could be intensified by climate change, making routine monitoring less effective at detecting this important pathogen. Therefore this should be considered in any attempt to improve detection limits in such geographical areas.

CONCLUSIONS

According to the results of our research, water temperature and solar radiation are the most influential environmental parameters in *Campylobacter* inactivation in river water. The data obtained in laboratory and in real conditions in the Llobregat River support the argument that *Campylobacter* inactivation is faster in spring and summer, when temperature and solar radiation are highest in the Mediterranean area of southern Europe. We therefore conclude, that the health risk associated with the presence of *Campylobacter* in water depends on the persistence of the bacteria that in this case is directly related to the climate of the geographical area. This should be considered to properly monitor this pathogen in the environment.

ACKNOWLEDGEMENTS

This study was supported by the Generalitat de Catalunya (2009SGR01043) and the 'Xarxa de Referència en Biotecnologia (XeRBa)'. The authors especially wish to thank E. Ballestè and X. Bonjoch for their kind help in sampling.

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