Factors Influencing the Occurrence and the Fate of *E. coli* Population in Karst Hydrosystems

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Abstract

The persistence of *Escherichia coli*, a bacterial indicator of water quality, is relevant to assess the health risk associated with aquifer use for drinking water supplies. In order to investigate the fate of *E. coli* in a karst aquifer, populations of both viable and culturable *E. coli* were monitored, according to their settling velocities, for contrasting hydrological conditions. Solid-phase cytometry was carried out to quantify the viable *E. coli*, and both the genetic diversity and the resistance to antibiotics of *E. coli* were investigated. This study shows that: (i) at the sinkhole, the structure of the *E. coli* population varied with the hydrological conditions and land use; (ii) the input of *E. coli* strains resistant to antibiotics was linked to contamination of human origin during rainfall events; (iii) irrespective of the hydrological conditions, the karst system is a permanent reservoir of viable but non-culturable *E. coli* even when culturable *E. coli* became undetectable at the well; and (iv) following a rainfall event or during a dry period, both populations of culturable and viable but non-culturable *E. coli* are mainly associated with non-settleable particles, corresponding to organic or organo-mineral microflocs.

1 Introduction

Assessment of the microbial quality of the water environment, including the spread of antibiotic-resistant faecal bacteria and their corresponding genes, will be one of the major challenges of the next decades. Such assessment is vital to cope with the expected increase of human population and the related agricultural activities (Bartram and Cairncross 2010; Hales and Corvalan 2006). Among aquatic environments, karst aquifers represent one of the most important freshwater resources: water supplies originating from karst are used by 25% of the global population. In France, this resource supplies water for up to 33% of the population. Karst hydrosystems are complex and original aquifers: they are fissured aquifers, mainly in carbonate rocks, limestone, or dolomite, where surface water disappears in a conical depression named the sink, then flows until it emerges at a natural resurgence (the spring). Usually, drinking water is pumped out at the well through an artificial opening connected directly to the aquifer (Bakalowicz 2005). In the phreatic zone, there is a hydraulic connection through the karst aquifer between the surface water and the groundwater.

Consequently, owing to farming practices or the human use of karst watersheds, karst aquifers are particularly vulnerable to microbial contamination, mainly during rainfall events, which are frequently associated with an increase of turbidity (Bakalowicz 2005; Dussart-Baptista et al. 2003; Fournier et al. 2007; Nnane et al. 2011; Sinclair et al. 2009; Viau et al. 2011). Faecal bacteria such as *Escherichia coli* (*E. coli*), originating from septic tank systems, wild animals or livestock, can be introduced into groundwater by both surface water run-off and soil leaching (Mahler et al. 2000; Muirhead et al. 2006; Page et al. 2012; Pronk et al. 2007). Although *E. coli* is currently considered as a commensal

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bacteria from the intestinal tracts of humans and vertebrate animals (host-associated habitats), water and sediment are now recognised as secondary habitats (open or non-host-associated habitats), to which some strains could have become naturalised (Bergholz et al. 2011; Ishii and Sadowsky 2008; Vital et al. 2008). On the basis of epidemiological studies to link the abundance of *E. coli* to the risk of waterborne outbreaks of gastroenteritis, *E. coli* has been identified as one of the two bacterial indicators of faecal contamination used to monitor the microbiological quality of water, according to the World Health Organisation and European regulation (2006/7/EC). High genetic and phenotypic diversities were observed within the *E. coli* population, which could be divided into seven major phylogroups, to which *Escherichia* clades have been added (Tenaillon et al. 2010; Gordon 2010; Walk et al. 2009). While *E. coli* was the most abundant heterotrophic culturable bacteria in microbiota gut of human and animals, some *E. coli* strains were also important human pathogens (Kaper et al. 2004).

In groundwater, the fate of *E. coli* is mainly dependent on (i) grazing by protozoans, viral lysis, and competition with autochthonous microbial communities and (ii) their ability to overcome environmental stresses such as low temperature under oligotrophic conditions (Rozen and Belkin 2001; Van Elsas et al. 2011). Furthermore, in such environments, some bacteria can survive (viable bacteria including culturable bacteria) although some of them fail to grow on laboratory media (non-culturable bacteria); these bacteria have been designated as viable but non-culturable (VNC) bacteria (Oliver 2010; Trevors 2011). In this physiological state, the VNC bacteria, sometimes designated as latent or dormant cells, could be considered as an adaptive response in order to survive under hostile conditions, and their presence could lead to an underestimation of the pathogens in a water environment (Oliver 2010; Trevors 2011). Today, while the issue of the resuscitation of VNC *E. coli* in the environment, mainly the virulent strains, is still highly debated (Arana et al. 2007; Keep et al. 2006; Özkanca et al. 2009), detecting and counting the total quantity of viable *E. coli* mainly by a cytometry-based method has been to be an appropriate way to follow the presence of these bacteria at very low cell concentrations in water (Vital et al. 2012). However, in drinking water supplies, the monitoring of the total quantity of viable *E. coli*, including putative VNC cells, could be particularly relevant to estimate the return time—i.e. the time required for this aquifer to regain its original level of microbiological quality—following a rainy event.

There is a little understanding of the persistence of viable *E. coli* in karstic aquifers. The survival of *E. coli* in water environments, that is, the time during which bacteria maintain their viability—with or without culturability—has been shown to be greatly influenced by their association with particles (Garcia-Armisen and Servais 2009; Pachepsky and Shelton 2011; Trevors 2011). The percentage values of particle-attached bacteria reported in the literature are highly variable, ranging between 18 and 55% in estuary or river water and reaching 100% in karst aquifers after a storm event (Characklis et al. 2005; Garcia-Armisen and Servais 2009; Krometis et al. 2007; Mahler et al. 2000; Pronk et al. 2007). However, to date, water quality models have been developed on the assumption that 20–44% of *E. coli* are associated with particles in rivers (Jamieson et al. 2005). Indeed, while turbidity has proven to be a valuable proxy for microbial quality of water (Nnane et al. 2011; Viau et al. 2011), estimation of the relative contributions of particle-associated versus free-living *E. coli* still remains a significant parameter for assessing the main processes that govern the transport and fate of these bacteria in aquifers. In karstic aquifers, considering their hydrological properties, the fate of *E. coli* must take account the two main processes which control the particle transfer in this hydrosystem: (i) the direct transfer of particles from the inlet to the outlet of the karstic system (surface origin), and (ii) the re-suspension of previously deposited particles, i.e. mainly the adherent bacteria that settle with larger particles within surface bed sediments (karstic origin) (Dussart-Baptista et al. 2003; Fournier et al. 2007; Massei et al. 2003; Pronk et al. 2007).

The aim of the present study is to identify the main factors influencing the occurrence and the fate of *E. coli* population in a karst hydrosystem. We focused this study on a small karst system developed in chalk in Upper Normandy. This system has been extensively monitored for water, particle and solute transport during the past 15 years and belongs to the French national observatory network on karst (INSU/CNRS). It is a small binary system ($\sim$12.5 km^2) which comprises a surficial watershed on the chalk plateau drained by a permanent creek (the Bébec creek). The creek is swallowed by a sinkhole connected to the karstic spring located at the foot of the plateau. Finally, a well used for water supply is located 130 m downstream from the spring. The abundance of viable or culturable *E. coli,* depending on their settling velocities, was monitored on the surficial watershed (i.e. the upstream part of the system) and at the spring downstream for contrasting hydrological conditions (dry and wet). In addition, both the genetic diversity—i.e. phylogroups distribution—and the resistance to antibiotics of the *E. coli* population were investigated.

2 Materials and Methods

2.1 Study Site

This study was carried out on a karstic test site of particular importance as a source of drinking water, which was

included in an INSU/CNRS (National Institute for Earth Sciences and Astronomy/National Center for Scientific Research, http://www.insu.cnrs.fr/node/3973) national observatory network on karst hydrological systems. The study site is a chalky karst aquifer located in Haute Normandie (France), a rainy Atlantic temperate climate located on the north side of the Seine estuary (Fig. 1). This small rural karst hydrosystem has been extensively studied and its geographical limits and hydraulic connections are well known (Fournier et al. 2007; Massei et al. 2002, 2003). With a production capacity of 4000 m^3 d^{-1}, this aquifer provides drinking water for 2400 inhabitants, after treatment by chlorination. This karstic system is composed of a sink (surface water), a spring and a well (groundwater). The sink is a point of infiltration of Bébec Creek, draining a small watershed of about 10 km^2 of which 95% is classified as agricultural land. The land-use patterns are as follows: approximately 55% cropland, 30% pasture (42 beef cattle, 130 dairy cattle), 10% forest, and the remaining 5% has several other allocations. There were 213 households in the watershed (639 equivalent inhabitants), relying on on-site septic systems. Only 49 septic tanks (147 equivalent inhabitants) were located between 500 and 600 m from the creek. These could contaminate the water by soil leaching only during rainfall events. Moreover, untreated sewage of human origin (four equivalent inhabitants) was located 400 m away from the sampling point and contaminated the creek water by run-off. The elevation of the plateau is about 100 m. Soils on the plateau, consisting of silts approximately 10 m thick, are highly susceptible to crusting, compaction and erosion, particularly during autumn and winter sowing. Discharge in Bébec Creek is variable, from 3 L s^{-1} in summer dry periods to 15 L s^{-1} in winter, and close to 500 L s^{-1} in response to major winter storms, when turbidity can exceed 1000 NTU as a result of soil erosion (Massei et al. 2002). Water from the creek recharges the chalk aquifer via a swallow hole. When the discharge exceeds the infiltration capacity of the swallow hole (saturation), the creek water overflows its banks and floods the valley.

The spring discharging from the foot of a karstified chalk cliff is an overflow of the aquifer resulting from the fine semi-permeable alluvial deposits that cover the chalk in the Seine valley (Fig. 1). The spring is the major outlet for the waters introduced in the swallow hole, with a transit time of

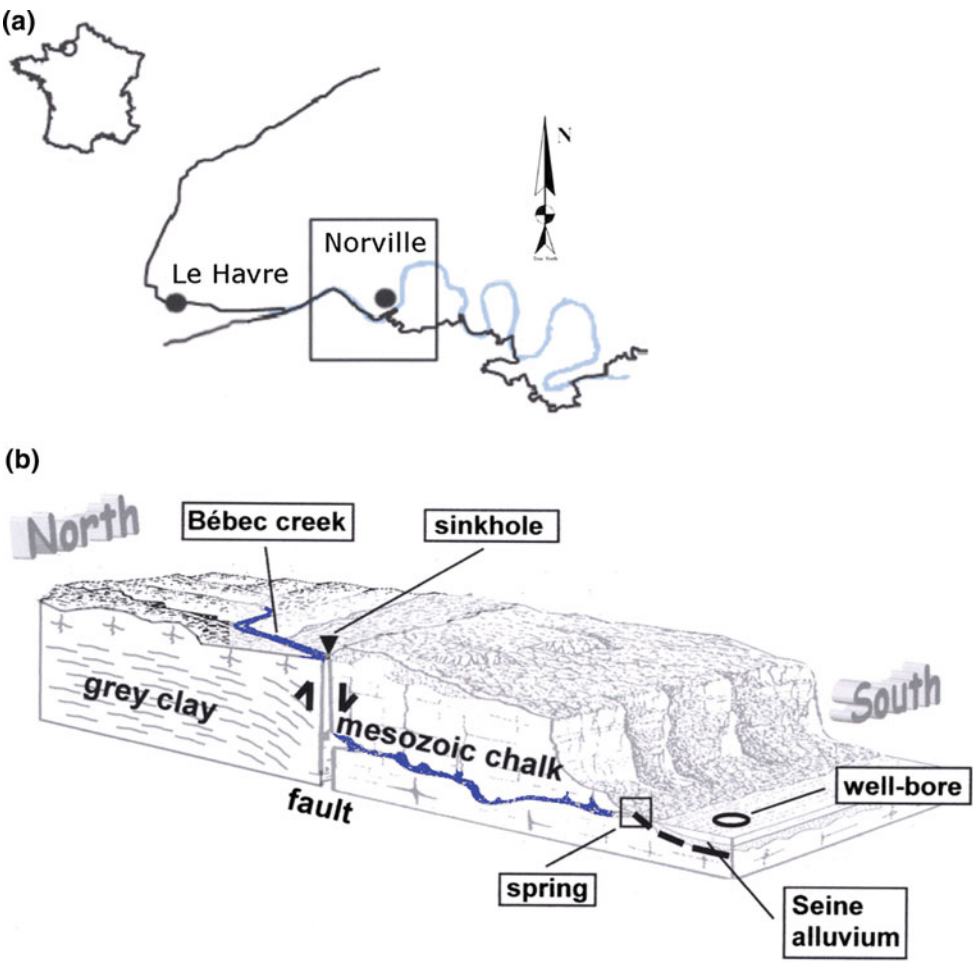

Fig. 1 **a** Location of study site, **b** sampling points on a 3D geomorphologic diagram of the Norville karst/alluvial system

less than a day (Massei et al. 2003). The spring water is, therefore, a mixture of surface water and groundwater. After storms, the turbidity of the water discharging from the spring can exceed 600 NTU, and this water consists of around 60% surface water. Because of the low microbial quality of the water according to the European standards, use of the spring as a drinking water supply was ceased in 1994 in favour of a well located 130 m away in the alluvial plain. The well (located 130 m from the spring) passes through the overlying alluvium and is screened to where it intercepts the chalk (Fig. 1b). During rainfall events, the well water can include as much as 10% rapidly infiltrated surface water, and the turbidity can reach 15 NTU (Fournier et al. 2007). The connections between the well and the spring are more complex than those between the spring and the swallow hole (Massei et al. 2002).

2.2 Materials and Sampling Method

Three sites were sampled, i.e. the sinkhole, the spring, and the well prior to chlorination (Fig. 1), with autosamplers (ISCO 6700) for five contrasting hydrological periods and land use: three campaigns during dry periods: (i) with pasture (May 2007), (ii) without pasture (February 2012) and (iii) in response to a heavy rainfall event (July 2007); and two campaigns during wet periods: (iv) without pasture (February 2007) or (v) following a rainfall event with pasture (March 2008). The sampling strategy at the spring and the well was defined on the basis of the transit time between the inlet (sinkhole) and the outlets (spring and well). Based on previous studies, the delays in spring and well response after recharge at the sinkhole were estimated at +30 h (dry periods) and 15 h (wet periods) at the spring, and +40 h (dry periods) and 20 h (wet periods) at the well, respectively (Fournier et al. 2007). During each sampling period (Table 1), about 1 L of water was collected every hour for 24 h, and about 300 mL of the hourly samples were mixed together in order to obtain a daily averaged sample. Water samples (100 mL) were filtered through pre-weighed Millipore filters (0.45 µm) to determine suspended matter concentrations. All samples were kept at 4 °C until the microbiological analyses were carried out, which occurred within 4 h.

2.3 Particle Settling

A water sample of 600 mL was placed in a separation funnel at room temperature to separate large particles from smaller ones on the basis of settling velocity. The sample was partitioned into three fractions collected through a tap positioned at the bottom of the funnel without sample perturbation. The fraction unable to settle within the 30-min experimentation time, referred to as "non-settleable" in this study, corresponded to the top water of the settling experiment and was mainly composed of particles with settling velocities ranging between 10^{-5} and 10^{-2} mm s^{-1}, with a dominant particle population of 8 µm size. The bottom fraction, referred to as "quickly settleable" in this study, was enriched with the larger particles with settling velocities ranging from 10^{-1} to 1 mm s^{-1}. This fraction was composed of two dominant particulate populations, with peaks in size distribution at 8 and 40 µm.

2.4 Microscopy

The filtered particulate material of each settling fraction was identified by electron microscopy (Au-Pd coating, secondary

Table 1 Hydrological characteristics and land use of the karst watershed for the sampling campaigns

Sampling period	Date	Turbidity NTU		SPM g L^{-1}		Flow rate m^3 s^1		Human input[a]	Head of cattle 172 ± 10 (40 ± 10)[b]	Pluviometry mm	
		Sinkhole	Spring	Sinkhole	Spring	Sinkhole	Spring			Day 1[c]	Day 5[d]
Dry period	03/05/2007	4	NA	0.011	NA	0.005	0.05	+	+ (+)	0	3.8
	16–17/02/2012	21	15	0.025	0.017	0.01	0.06	+	–	0	3.7
Heavy rainfall after a dry period	11/07/2007	33	16.5	0.075	0.025	0.025	0.065	+	+ (+)	50	8.4
Wet period	21/02/2007	15	NA	0.023	NA	0.02	0.075	+	–	2	27.8
Rainfall event during wet period	01/03/2008	105	NA	0.55	NA	0.02	0.045	+	+ (+)	14	17.4

[a]On the watershed (10 km^2) 639 inhabitants, 172 septic tanks for which there was one malfunctioning septic tank located 400 m from the sampling point; [b]for which cattle located at the vicinity of the sampling; [c]pluviometry on day of sampling, [d]cumulated rain 5 days before sampling; *SPM* suspended particulate matter; *NA* not analysed

electron-based method, voltage of 20 kV), and more than two hundred particles were observed. The proportions of particles were estimated by counting organic and mineral particles for 15 different microscopic fields for each particle class.

2.5 Bacterial Dissociation

The enumeration of culturable and viable bacteria and bacterial dissociation of particles were performed on 20 mL of each fraction by sonication for 30 s at 4 °C with an ultrasonic probe (20 kHz, 20 W). These values were obtained from a specific protocol which had been previously designed based on the counts of viable and culturable *E. coli* (see Sects. 2.6 and 2.7) according to the time of the ultrasonic treatment. The analysis of two distinct water samples—the bulk water sample and the corresponding settling fractions (see Sect. 2.3)—was carried out in triplicate for two contrasted water samples: (i) treated effluent from a waste water treatment plant (MES 1.94 mg L^{-1}, mainly organic flocs) and (ii) river water (MES: 16 mg L^{-1}). A control experiment was carried out on an exponential-phase culture of *E. coli* (2.2×10^8 CFU mL^{-1}). This treatment enabled the recovery of 80% of culturable *E. coli*, of which the membranes were undamaged.

2.6 Enumeration of Culturable *E. coli*

Escherichia coli were enumerated in duplicate using membrane filtration methods (0.45 µm HA047 Millipore, Bedford, MA, USA). *E. coli* strains were directly isolated from previously treated water samples with a selective chromogenic medium specific for *E. coli*, with the addition of a selective supplement for water samples (RAPID'*E. coli* 2 Medium and Supplement; Bio-Rad, CA, USA) and incubated for 24 h at 37 °C. To obtain a number of colonies ranging from 5 to 35 with a filter, 10 to 100 mL of water was filtered depending on the hydrological conditions. The threshold value for the enumeration of *E. coli* in water was 5 CFUs 100 mL^{-1}.

2.7 Enumeration of Viable *E. coli*

Water samples were filtered through a 25-mm-diameter 0.4-µm Cycloblack™-coated polyester membrane filter (AES Chemunex). Labelling of viable *E. coli* cells was prepared following the manufacturer's instructions (*E. coli* drinking water AES/Chemunex). *E. coli* cells were labelled with β-D-glucuronide substrate linked with fluorescein, and viable cells were enumerated by solid-phase cytometry (Chem*Scan*® RDI, AES Chemunex, Ivry-sur-Seine, France) as previously described (Lemarchand et al. 2001). The solid-phase cytometer consisted of a laser-scanning unit equipped with a 488-nm argon laser that scans a 25-mm-diameter membrane filter in 3 min. Each fluorescent event was confirmed by using an epifluorescent microscope (Nikon 50I Tokyo, Japan), equipped with an FITC filter block (500-nm dichroic mirror, a 450–490-nm band-pass excitation and a 515-nm cut-off emission filter). Enumeration of viable *E. coli* was performed in duplicate for each sample, VNC *E. coli* was evaluated by calculating the difference between the number of viable *E. coli* and the number of culturable *E. coli* growing on the selective media described above, which was then expressed as a percentage.

2.8 Determination of the *E. coli* Phylogroups

The phylogenetic group to which the *E. coli* isolates belonged was determined by the PCR-based method as proposed by Clermont et al. (2011). The identification of Escherichia clade strains, which are phenotypically undistinguishable from the *E. coli* sensu stricto strains, were performed by PCR as described in Clermont et al. (2011).

2.9 Antibiotic Resistance Testing of *E. coli*

E. coli antibiotic resistances to antibiotics were tested by the agar diffusion method according to the recommendations of the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM). *E. coli* CIP 7624 (ATCC 25922) was used as a control. The tested antibiotics (17) included the most commonly used in France for the treatments of *E. coli* infections in human and veterinary medicine: amoxicillin (AMX, 25 µg), amoxicillin + clavulanic acid (AMC, 20 + 10 µg), ticarcillin (TIC, 25 µg), ticarcillin + clavulanic acid (TIM, 75 + 10 µg), imipenem (IPM, 30 µg), cefoxitin (CEF, 30 µg), ceftazidime (CAZ, 30 µg), cefotaxime (CTX, 30 µg), gentamycin (GEN, 15 µg), kanamycin (KAN, 30 ui), streptomycin (STR, 10 µg), chloramphenicol (CHL, 30 µg), tetracycline (TET, 30 µg), trimethoprim–sulfamethoxazole (SXT, 23.75 + 1.25 µg), nalidixic acid (NAL, 30 µg), ciprofloxacin (CIP, 30 µg), and chloramphenicol (C, 30 µg).

2.10 Statistical Analysis and Data Transformation

Statistical analyses were performed using R software. The Pearson test was performed to determine whether the numbers of *E. coli* associated with each class of settling particles

were significantly different along the karstic aquifer, from the sinkhole to the well.

3 Results and Discussion

3.1 Vulnerability of a Karstic Aquifer to Contamination by *E. coli*

Before the infiltration of surface water into the karst aquifer (Fig. 1b), the Bébec Creek water drained a small, well-known watershed characterised by human pressure combined with pasture. Five campaigns were carried out for contrasting hydrological conditions and land use (Table 1). In order to monitor the occurrence of *E. coli* in the karst aquifer following a rainfall event, the sampling at the sinkhole, the spring and the well takes into account the time of transfer of the water body.

In the karst aquifer, a continual level of contamination by *E. coli* ranging from $(1 \pm 0.5) \times 10^2$ to $(4 \pm 0.3) \times 10^4$ was observed. Maximal values were observed following a heavy rainfall event during a dry period with pasture on the watershed. The lowest contamination was observed during dry period in the absence of cattle. In such dry period, it has been demonstrated that more persistent *E. coli* strains better adapted to the water circulate in karst aquifer (Berthe et al. 2013). The contamination of the water by culturable *E. coli* decreased by about one to three orders of magnitude from the sinkhole to the well, mainly due to the strong dilution of the surface water within the groundwater (Table 2). Once in the karst, as expected, the rapid mixing between ground and surface waters was the main explanation for the decrease in culturable *E. coli* densities previously introduced at the sinkhole, in addition to the disappearance of cells by die-off or grazing (Dussart-Baptista et al. 2003; Rozen and Belkin 2001; Van Elsas et al. 2011). However, no significant decrease of *E. coli* densities was observed from the sinkhole to the spring in the absence of cattle grazing.

Based on the proportion of the B1 phylogroup within the *E. coli* population, a change of the genetic diversity was observed, with the maximal ratio of *E. coli* belonging to the B1 phylogroup in water during dry period and pasture. Nevertheless, no significant change of the proportion of the B1 phylogroup within *E. coli* population occurred from the sinkhole to the spring for the five sampling campaigns. A greater proportion B1 phylogroups was detected here, suggesting the main faecal contamination was of bovine origin. Indeed, it has been demonstrated that the *E. coli* population structure of humans significantly differs from that observed in herbivorous animals such as cows, the B1 phylogroup being more prevalent in the gut microbiota of cows (Carlos et al. 2010). The percentages of *E. coli* isolates

Table 2 Contamination of karst water by *E. coli* for contrasting hydrological conditions and land use[a]

Sampling campaigns		*E. coli*	Sinkhole	Spring	Well
Dry period	With pasture 03/05/07	Culturable CFU 100 mL^{-1}	$(6.2 \pm 0.3) \times 10^2$	$(5.5 \pm 1.1) \times 10^1$	<5
		Antibiotic resistance % (n/N)[b]	0 (0/45)	5.7 (2/35)	$X < 10$
		Phylogroups B1 (%)	86.7%	77.1%	NA
	Without pasture 16–17/02/2012	Culturable CFU 100 mL^{-1}	$(9.8 \pm 2.2) \times 10^1$	$(5.6 \pm 0.9) \times 10^1$	<5
		Antibiotic resistance % (n/N)[b]	NA	NA	NA
		Phylogroups B1	7.4%	4.8%	NA
	Heavy rainfall event with pasture 11/07/2007	Culturable CFU 100 mL^{-1}	$(4.0 \pm 0.3) \times 10^4$	$(2.5 \pm 1.1) \times 10^3$	$(9.6 \pm 3.3) \times 10^1$
		Antibiotic resistance % (n/N)[b]	55.8 (18/34)	35.7 (5/14)	44.8 (13/29)
		Phylogroups B1 %	44%	28.6%	NA
Wet period	Without pasture 21/02/2007	Culturable CFU 100 mL^{-1}	10^2	10^2	<5
		Antibiotic resistance % (n/N)[b]	0 (0/44)	0 (0/39)	0 (0/28)
		Phylogroups B1 %	39%	41.1%	NA
	Rainfall event[c] with pasture 01/03/2008	Culturable CFU 100 mL^{-1}	$6 \pm 0.3 \times 10^2$	10^2	<5
		Antibiotic resistance % (n/N)[b]	54% (14/26)	41% (11/27)	30 (7/23)
		Phylogroups B1 %	15%	NA	NA

[a]According to Ratajczak et al. (2010) and Laroche et al. (2010), [b]N total number of *E. coli* isolates, n number of strain resistant to at least one antibiotic; [c]+6 h after the rain event

resistant to at least one antibiotic were the highest after a rainfall event, with no significant change along the karst aquifer. However, based on microcosm experiments it has been shown that some culturable *E. coli* enabled to persist in water less than two days, and are resistant to most antibiotics (Berthe et al. 2013).

3.2 Change of Structure of *E. coli* Population During a Rainfall Event

To have a better understanding of the variation of the structure of the *E. coli* population in water, Ratajczak et al. (2010) studied in detail the kinetics of the water contamination during a rainfall event after a wet period (March 2008) (Fig. 2). During this rainfall event, the turbidity increased, as the number of *E. coli* did. Thus, before the rainfall event, the level of contamination by *E. coli* was low. Six hours after the rainfall, the *E. coli* density increased up to a maximum value of 720 CFU 100 mL^{-1} and then decreased to a value close to that initially observed (+19 h) (280 CFU 100 mL^{-1}) (Fig. 2). In the most highly contaminated water (+6 h), there was an input of the *E. coli* phylogroup A0 (23%), phylogroups E/D (23%) and *Escherichia* clades (28%), showing a structure of the *E. coli* population that was significantly different from that observed in the less contaminated water 19 h after the rainfall event (χ^2 test $P < 0.001$). Six hours after the rainfall event, the resistance level to one or more antibiotics was significantly highest in the *E. coli* population isolated at the peak of high flow event (Laroche et al. 2010; Ratajczak et al. 2010). Some of these bacteria exhibited resistances to more than three classes of antibiotics, suggesting faecal contamination of human origin resulting from septic tank overflow. These strains were thus

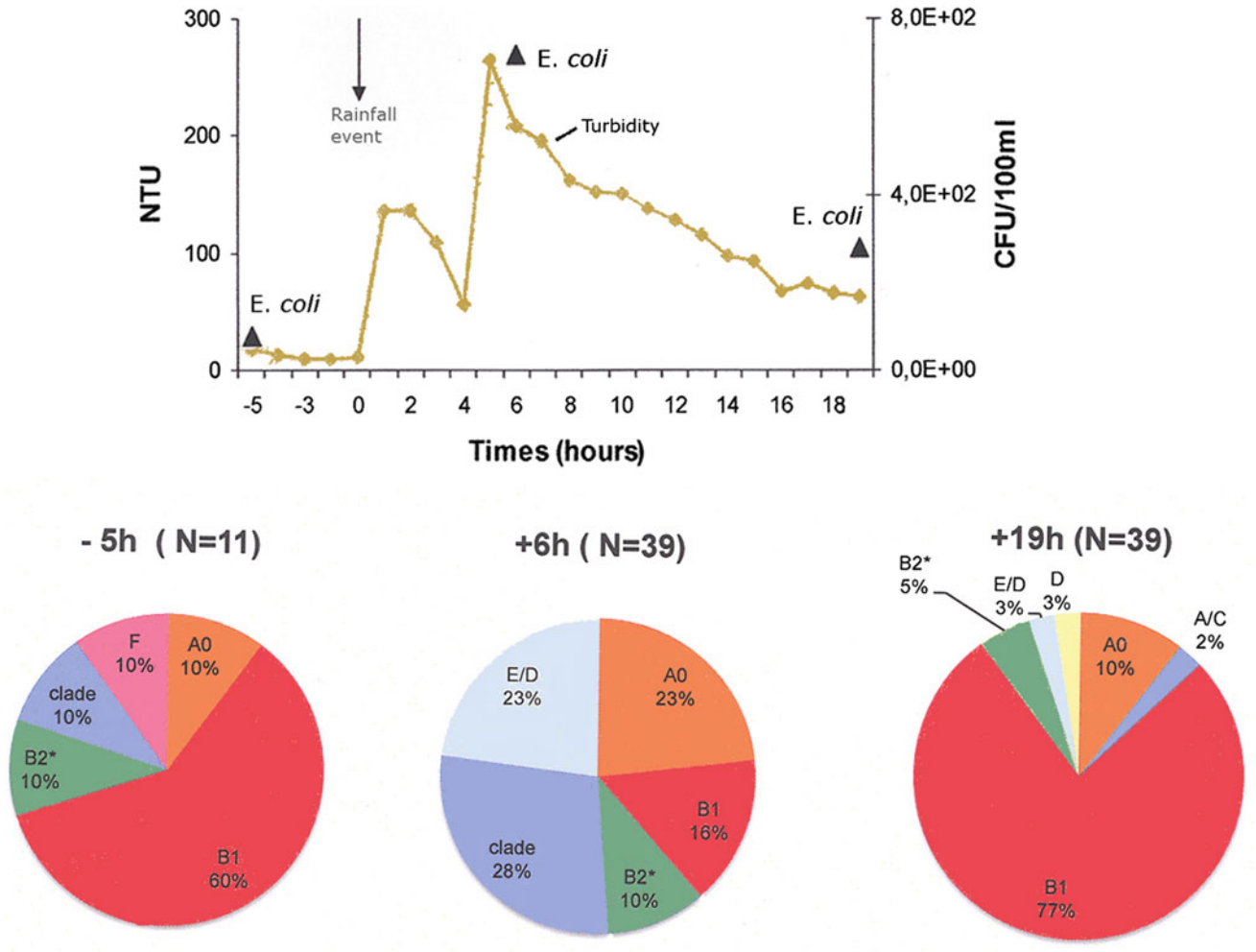

Fig. 2 Change of turbidity (*filled diamond*), density (*filled triangle*) and diversity of *E. coli* population at the sinkhole during a rainfall event following a wet period. The *arrow* indicates the beginning of 14-mm rain event. Diversity assessment of *E. coli* population has been carried out by estimation of the phylogroups distribution according to Clermont et al. (2013)

considered as multiple-resistant strains as defined by Magiorakos et al. (2012).

In contrast, in the less contaminated water (+19 h), *E. coli* B1 strains (77%) became the main *E. coli* phylogroup again. These isolates are mainly *hly* positive (72%), and antibioresistant (AR index = 5%) (amoxicillin, tetracycline, chloramphenicol and ciprofloxacin), suggesting a distant input of *E. coli* of bovine origin resulting from run-off and/or a leaching of the soil. These results showed that during the storm event, an increase in the contamination was accompanied by a change of the structure of the *E. coli* population, reflecting the faecal contamination of the watershed with a high percentage of phylogroup B1 in water contaminated by run-off of pasture land.

3.3 Transport of Culturable and "Viable but Non-culturable" *E. coli* in the Karst Aquifer

Interestingly, irrespective of the hydrological conditions, the prevalence of a population of viable *E. coli* greater than that of the culturable one was always observed, indicating that a population of VNC *E. coli* was always resident in this karst aquifer. Following a rainfall event (sampling campaign 11/07/07), in addition to the high input of culturable *E. coli*, a large proportion of VNC *E. coli* was introduced into the karst. From the sinkhole to the spring, the two sub-populations of viable *E. coli*, culturable and VNC *E. coli* populations, decreased (Fig. 3a). In the dry period (sampling campaign

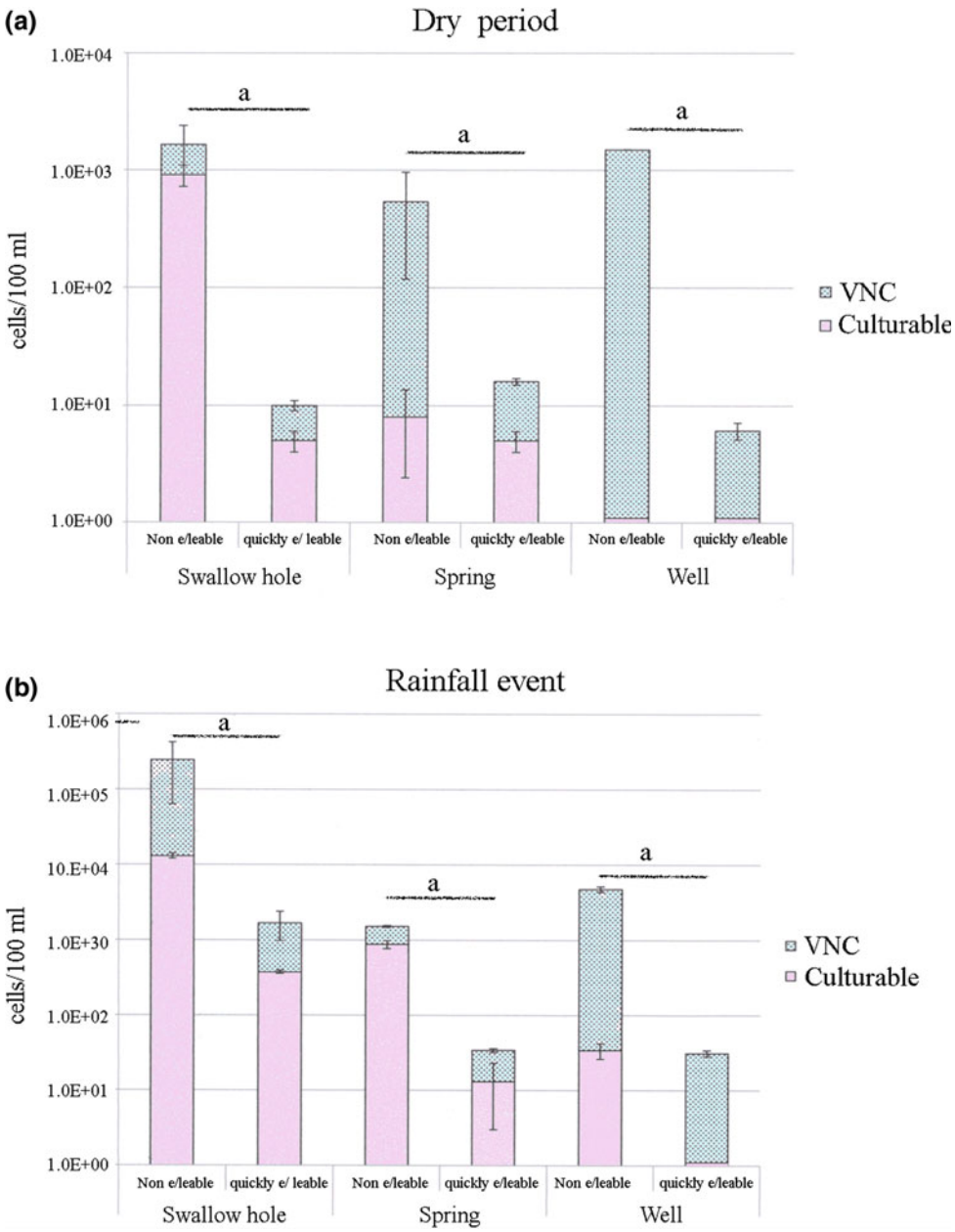

Fig. 3 Transport of culturable and viable but non-culturable *E. coli* related to their settling velocities **a** during dry period and **b** following a rainfall event. **a** *P* value < 0.01 compares VNC *E. coli* associated with non-settleable particles to quickly settleable particles; **b** *P* value < 0.01 compares culturable *E. coli* associated with non-settleable particles to quickly settleable particles

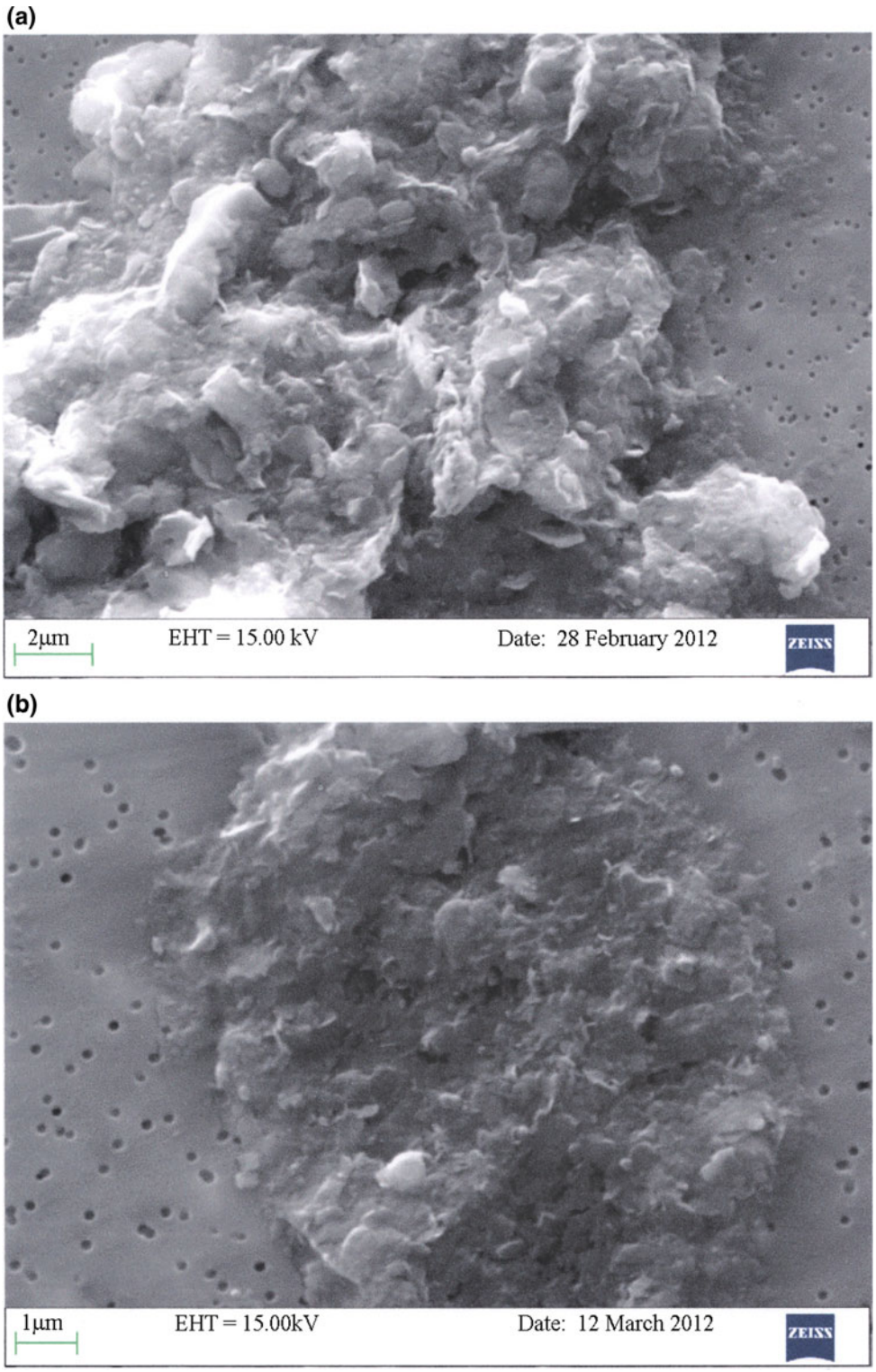

Fig. 4 Scanning electron micrographs of particles of organo-mineral microfloc from "non-settleable particles" fraction with settling velocities between 10^{-5} and 10^{-2} mm s^{-1} (**a**); and of floc dominated by mineral particles from the "quickly settleable particles" with settling velocities between 10^{-1} and 1 mm s^{-1} (**b**)

03/05/07), without an event of contamination by faecal bacteria, the density of culturable *E. coli* decreased in the karst aquifer, until they were not detectable at the well, while the proportion of VNC strains among total viable *E. coli* increased from the sinkhole to the spring. Irrespective of the sampling campaign, when water was pumped out at the well, the VNC *E. coli* were predominant among the viable *E. coli* population (Fig. 3b). As previously described, the occurrence of *E. coli* in the VNC physiological state—corresponding here to bacteria unable to grow on the selective medium used for the

enumeration of this bacteria in water—suggests that viable bacteria persist more longer than culturable ones in karst (Rozen and Belkin 2001; Van Elsas et al. 2011). Even if some of these bacteria could correspond to injured cells, here we show that the karst aquifer could be a reservoir of viable *E. coli* even outside a rainfall event.

The monitoring of viable, culturable and VNC *E. coli* was carried out according to their settling velocities *i.e.* according to their association with particles (Fig. 3). For each sample of water, a 30-minute settling experiment in a settling funnel led us to define two classes of particle-associated *E. coli*. Each class was characterised on the basis of its settling velocity combined microscopic analysis (Fig. 4). Following a rainfall event or during a dry period, both populations of culturable and viable but non-culturable *E. coli* are mainly associated with non-settleable particles, corresponding to organic or organo-mineral microflocs (Kruskal–Wallis $P < 0.05$, and with Bonferroni correction $P \leq 0.01$). At the well, in dry period, viable and non-culturable *E. coli* were also mainly associated with "non-settleable" particles. Culturable *E. coli* could not be detected at the well, while an increase of VNC *E. coli* associated with both "non-settleable" and "quickly settleable" fractions was observed, suggesting that when water was pumped out, an input of VNC bacteria associated with each class of settling particles occurred. These results are consistent with those previously described by estimating the fraction of free-phase and particle-associated organisms using filtration techniques, which demonstrated that a substantial fraction of bacteria are associated with particles circulating in the karst hydrosystem (Mahler et al. 2000). As previously reported in a similar aquifer by Pronk et al. (2006), this *E. coli* population, associated with the "non-settleable" particles corresponds to organic or organo-mineral microflocs, probably originated from the leaching of bacteria by surface run-off associated with aggregates or colloids originating from cowpats (Muirhead et al. 2006; Soupir et al. 2010).

4 Conclusions

- The abundance of culturable *E. coli* in the studied karst aquifer reflected the previous contamination of the creek by run-off and soil leaching, both depending on the hydrological conditions and the land use (pasture).
- The contamination of the water by culturable *E. coli* decreased by about one to three orders of magnitude from the sinkhole to the well, mainly due to the strong dilution of the surface water within the groundwater.
- At the sinkhole, the structure of the *E. coli* population in water—i.e. the distribution of phylogroups—varied with the hydrological conditions and the land use, with a higher proportion of B1 phylogroup of bovine origin during pasture.
- The input of *E. coli* strain resistant to more than three classes of antibiotics (i.e. a multiple antibiotic-resistant strain) was linked to contamination of human origin during a rainfall event.
- Irrespective of the hydrological conditions, the karst system is a permanent reservoir of viable *E. coli* even when culturable *E. coli* became undetectable in the drinking water pumped out at the well during dry periods.
- Following a rainfall event or during a dry period, both populations of culturable and viable but non-culturable *E. coli* are mainly associated with non-settleable particles, corresponding to organic or organo-mineral microflocs.

In 2015, the main conclusions drawn regarding the karst aquifer and rivers were reported to the stakeholders and to the French ministry of health, to enable assessment of the microbiological risk due to the spread of antibiotic-resistant bacteria, according to the DPSIR (Driving forces—Pressure-State-Impact-Response) concept.

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