MONITORING OF POTENTIAL POLLUTION SOURCES AND IMPACT OF PASTURE MANAGEMENT ON GROUNDWATER QUALITY

1. INTRODUCTION

Carbonate aquifers provide the main drinking-water resources in Southern Italy supplying an average volume of $4100 \cdot 10^6$ m^3 y^{-1} (Celico et al., 2000). Due to cattle grazing, these aquifers are often characterized by groundwater microbial contamination (Celico et al., 2004a, b).

Historically, fecal coliforms and *E. coli* have been utilized as indicator microorganisms of water quality (Clesceri et al., 1998). Although fecal enterococci have been traditionally utilized for monitoring marine bathing water (Kani and Mills, 2000), both fecal coliforms and fecal enterococci have been referenced as being equally acceptable to monitor freshwater (Abbott et al., 1993). Studies were carried out to evaluate the reliability of fecal enterococci as indicators of surface water in recreational areas (Kinzelman et al., 2003). As per the groundwater, fecal enterococci did appear a more reliable indicator than fecal coliforms for the detection of microbial pollution in water samples collected at a spring located within the carbonate Southern Apennines (Italy; Celico et al., 2004b). These results are in good accordance with some of the findings in a French karstic aquifer (Personné et al., 1998).

The main purposes of this research were to analyze the influence of a topsoil of pyroclastic origin on retention of fecal coliforms and fecal enterococci and verify their reliability as indicators of groundwater microbial contamination in carbonate aquifers. To

avoid uncertainties related to the role of hydrogeological features on groundwater microbial contamination in such complex settings, the research was carried out in the Acqua dei Faggi experimental field site whose hydrogeological behaviour was thoroughly analyzed in the last 6 years (Celico et al., 2004b; Celico et al., 2006; Petrella et al., 2007). The retention of bacteria within the topsoil was analysed in laboratory by carrying out column tests in intact soil blocks collected in the same site.

2. DESCRIPTION OF THE FIELD SITE

The Acqua dei Faggi experimental site (Longano, Molise; Fig. 1) mainly consists of limestone (Cretaceous-Oligocene). The rocks have very low primary permeability but are extensively fractured and subordinately karstified (Celico et al., 2004b). In pasture areas carbonate rocks underlie an *Epilepti-Vitric Andosols* (*Mollic*) which is characterized by a profile A/R. The horizon A generally ranges in thickness from 4 to 12 cm above the limestone (R). The pH ranges from 5.1 to 5.4, while the Total Kjeldahl Nitrogen from 0.5% to 0.6%. The test area is similar to other carbonate aquifers in Southern Italy where the groundwater preferentially flows through a fracture network (Celico et al., 2000). The aquifer's boundaries are fault zones that act as barriers to groundwater flow (Celico et al. 2006). Due to this discontinuous heterogeneity (*sensu* Freeze & Cherry, 1979), the aquifer looks like a basin-in-series aquifer system where seasonal springs can be detected along some fault zones depending on groundwater level fluctuations (Celico et al., 2006). The groundwater flows westwards to different springs.

Seasonal spring 2 (1065 meters a.s.l.) flowed several months a year during the observation period with a discharge ranging from 0 to 0.44 $m^3 s^{-1}$. The highest outflow (1 in Fig. 1; 1075 m a.s.l.) flowed a few weeks a year (December 2004, April 2005, and from December 2005 to January 2006) with a discharge ranging from 0 to 0.14 $m^3 s^{-1}$. Seasonal spring 3 (1014 m a.s.l.) flowed several months a year during the observation period with a discharge ranging from 0 to 0.03 $m^3 s^{-1}$. The only perennial spring of the basin-in-series aquifer system flows at 980 m a.s.l. with a discharge ranging from 0.02 to 0.07 $m^3 s^{-1}$.

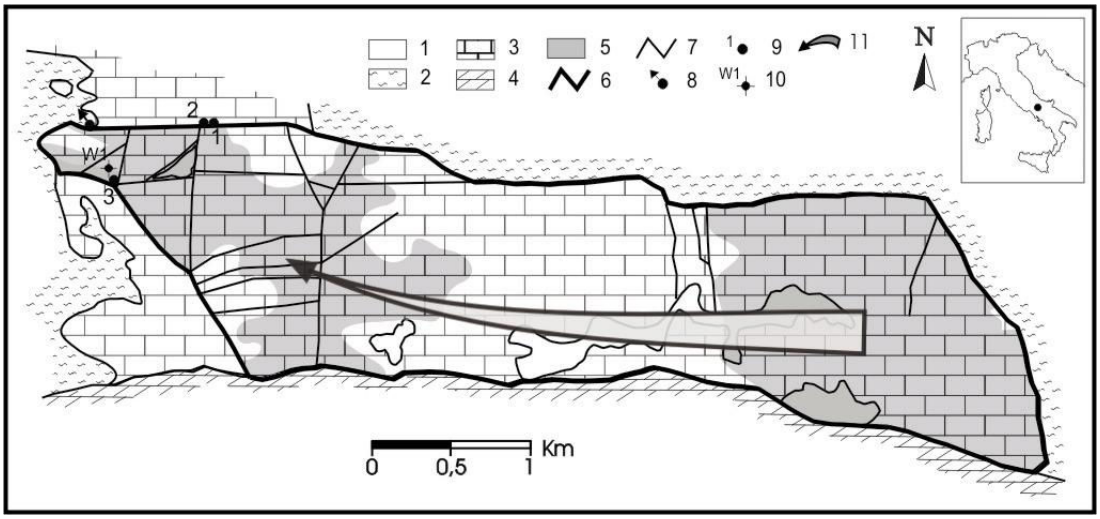

Figure 1 - Hydrogeological map (1: quaternary deposits; 2: siliciclastic rocks; 3: limestone; 4: dolostone; 5: pasture area; 6: aquifer boundary; 7: fault; 8: perennial spring; 9: seasonal spring; 10: well; 11: main groundwater flow direction).

Despite the existence of epikarstic zones, rainwater infiltration and then transport of microorganisms from the ground toward the groundwater is not influenced by neither the water storage in perched aquifer nor the "funnelling" of this water into larger shafts. In fact, the contrast in permeability at the bottom of the epikarst is not significant enough to cause percolation retention in the thin near-surface high conductivity layer of the carbonate bedrock (Petrella et al., 2007). Thus, the microorganisms are diffusely and rapidly transported within a topsoil of pyroclastic origin and then through a network of well interconnected fractures.

The only source of microbial contamination in the study site is cattle grazing (a few hundreds heads of cattle throughout the whole research period). Pasture areas are the 55% of the site, while a beech woodland covers the 45% (Fig. 1).

3. MATERIALS AND METHODS

3.1 Sampling and bacterial counting

Water samples for microbiological analyses were collected weekly from September 2004 to December 2006 (two hydrologic years) at the perennial spring and at seasonal springs 2 and 3. Water samples were collected in sterile 1000 ml bottles and transported in a refrigerated box to the laboratory. Filtration processes for bacteriological analyses were made within 2 hours after sampling. Indicators of microbial contamination were determined using classic methods of water filtration on sterile membranes filter with incubation on: (a) m-FC Agar (Biolife) for 24 h at 44 °C, for fecal coliforms and (b) Slanetz-Bartley Agar for 4 h at 37°C and 44 h a 44°C, for fecal enterococci.

3.2 Species identification

Taxonomic classification of fecal enterococci detected in spring water samples was performed using API 20 Strep fermentation strips (bioMérieux, Marcy l'Etoile, France) and by sequence analysis of the 16S rRNA genes amplified with universal oligonucleotides (BMR Genomics, Università di Padova, Italy). Classification was carried out to identify the main species in the study site and use it as tracer during the column tests.

Fecal coliforms were characterized only using API 20E fermentation strips because it is known that *E. coli* is the main species of the group (Krieg and Holt, 1984).

3.3 Soil block collection and column tests

Six intact soil blocks of *Epilepti-Vitric Andosols (Mollic)* were collected randomly from the test site in the pasture area. To minimize disturbance of samples, sod-covered blocks (181.36 cm^2 by 11 cm deep) were carved from undisturbed soil directly pushing permeameter cells used for column tests into the topsoil. All blocks were covered with plastic and transported to the laboratory where experimental procedure started immediately.

A diffuse interaction between bacteria and soil blocks was obtained carrying out column tests in a standard permeameter, to minimize lateral flow within the gap between soil block and metal cell. The water was applied on the top of blocks. The outflow was collected at the bottom using sterile plastic tubes. A peristaltic pump was used to constantly push the water through the soil.

In order to analyze retention of both microorganisms at medium term, it was poured 5400 ml of distilled water (corresponding to 300 mm of effective infiltration in the study site) at a velocity of 3 mm h^{-1}. Due to the clay content into the soil, a solution with 0.001 *M* $CaCl_2$ was used as rainwater to prevent dispersion of clays within the soil and the column plugging (McMurry et al., 1998).

The interaction between fecal bacteria and soil blocks was analyzed using of a collection strain of *E. faecalis* (ATCC 29212), nalidixic acid resistant, and a collection strain of *E. coli* (ATCC 10536), resistant to nalidixic acid and ampicillin. No fecal coliforms and fecal enterococci resistant to both antibiotics were observed in the natural background of soil blocks collected in pasture areas.

At the beginning of each experiment, $1.0 \cdot 10^{10}$ *E. faecalis* cells and $1.0 \cdot 10^{10}$ *E. coli* cells were applied on the top of soil blocks in a 0.001 *M* $CaCl_2$ solution. The cells were collected during the exponential growth phase. Soil block drainage was entirely collected beneath the outflowing holes. Concerning *E. faecalis*, 200 µl of each water sample and relative serial dilutions were plated in triplicate on Slanetz-Bartley Agar, supplemented with antibiotic (50

μg ml^{-1} of nalidixic acid) and incubated at 37°C. As per *E. coli*, 200 μl of each water sample and relative serial dilutions were plated in triplicate on m-FC Agar, supplemented with antibiotic (40 μg ml^{-1} of nalidixic acid and 50 μg ml^{-1} of ampicillin) and incubated at 44°C. The number of *E. faecalis* cells and *E. coli* cells was estimated as colony-forming units (CFU) using only plates where the number of colonies ranged from 30 to 300, after 48 hours and 24 hours respectively.

3.4 Soil characterization

Physical and index properties of topsoil samples were analysed by means of standard laboratory tests: water content, organic matter, grain size and specific gravity of particles. Water content and organic matter tests were sequentially and respectively performed through weight measurements of water loss, after oven drying, and organic matter loss, after muffle furnace heating. Accordingly to the ASTM standard procedures, soil samples drying was carried out by means of 24 hours oven drying at 60°C, while organic matter by means of loss-on-ignition technique, consisted in 72 hours heating at 450°C in a muffle furnace. Specific gravity tests were carried out using a 50 cm^3 picnometer, applied to the 0.075 mm passing fraction. Grain size tests were performed by means of wet sieving, with the ASTM standard sieves series and sedimentation procedure based on density measurements performed with hydrometer.

4. RESULTS

4.1 Field monitoring

The perennial and the seasonal springs are characterized by a discontinuous distribution of microbial contamination over time (Figg. 2, 3 and 4). The main peaks are observed during rainfall events which cause significant effective infiltration and a piezometric head rise or a slow-down of its depletion (Figg. 2, 3 and 4), in accordance with the findings of Celico et al. (2004b). At the perennial spring the contamination ranged from 0 to 794 CFU 1000ml^{-1} of fecal coliforms and from 0 to 2216 CFU 1000ml^{-1} of fecal enterococci. At seasonal spring 2 fecal coliforms ranged between 0 and 898 CFU 1000ml^{-1} and fecal enterococci between 0 and 740 CFU 1000ml^{-1}, while at seasonal spring 3 the range was 0 to 280 CFU 1000ml^{-1} for fecal coliforms and 0 to 1456 CFU 1000ml^{-1} for fecal enterococci. At all springs maximum concentrations were detected during the beginning of each hydrologic year when the great piezometric head rise causes the flow of the seasonal springs (Celico et al., 2006).

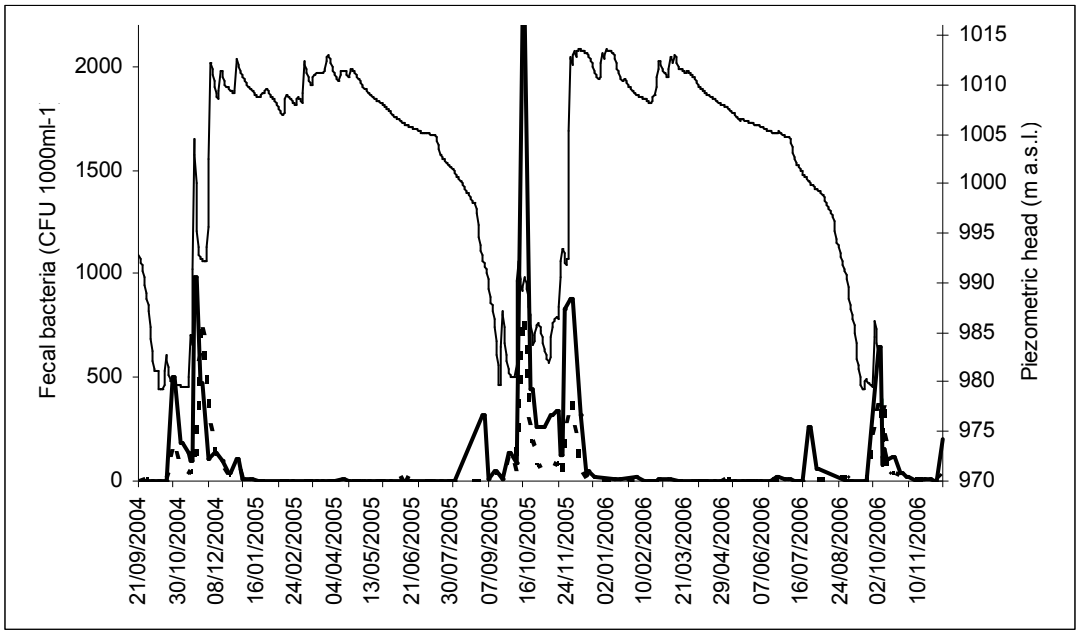

Figure 2 - Distribution of fecal coliforms (thick dashed line) and fecal enterococci (thick line) over time at the perennial spring and piezometric head fluctuations (thin line).

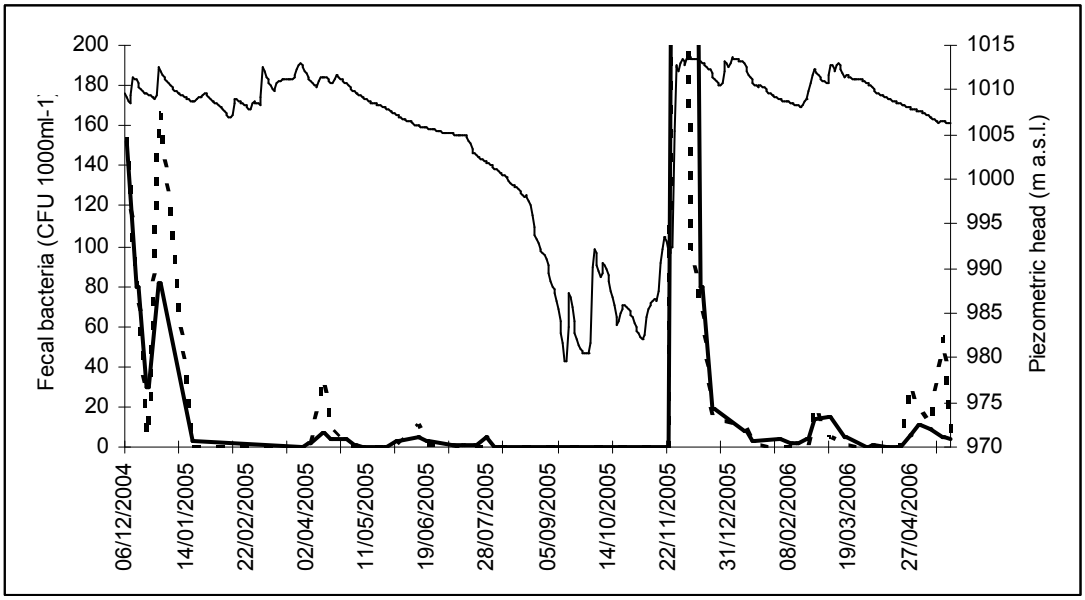

Figure 3 - Distribution of fecal coliforms (thick dashed line) and fecal enterococci (thick line) over time at the seasonal spring 2 and piezometric head fluctuations (thin line). The main peaks detected in late November 2005 are not shown to emphasize the lower peaks.

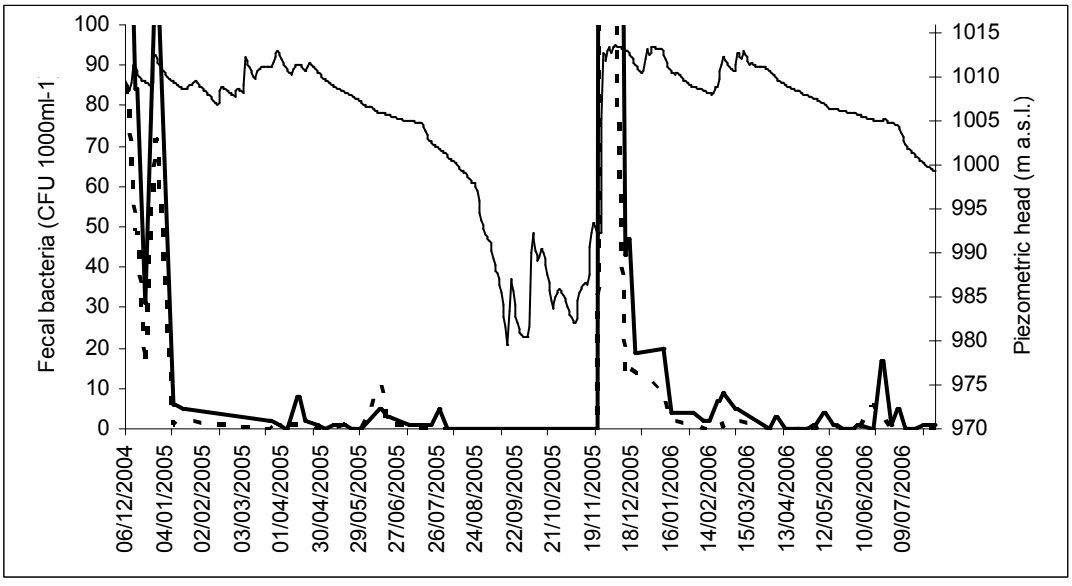

Figure 4 - Distribution of fecal coliforms (thick dashed line) and fecal enterococci (thick line) over time at the seasonal spring 3 and piezometric head fluctuations (thin line). The main peaks detected in late December 2004 and in late November 2005 are not shown to emphasize the lower peaks.

At the perennial spring the comparison of fecal coliforms to fecal enterococci suggests, on the whole, a comparable number of water samples affected by contamination. (Tab. 1). On the contrary, a significant difference was observed in spring water samples collected at springs 2 and 3 (Tab. 1).

	Perennial spring		Seasonal spring 2		Seasonal spring 3	
	Fecal coliforms (%)	Fecal enterococci (%)	Fecal coliforms (%)	Fecal enterococci (%)	Fecal coliforms (%)	Fecal enterococci (%)
0 CFU $1000ml^{-1}$	20.8	19.8	30.2	9.3	49.1	23.6
≥1 CFU $1000ml^{-1}$	79.2	80.2	69.8	90.7	50.9	76.4
Samples (n)	101	101	43	43	55	55

Table 1 - Occurrence (percent of samples) of fecal coliforms and fecal enterococci in springs' water from September 2004 to December 2006.

Difference in detection between the two bacterial indicators may be due to different factors such as (a) different decay of the two microorganisms in groundwater and / or (b) higher retention of fecal coliforms within the topsoil and / or the aquifer.

Die off kinetics for fecal coliforms and fecal enterococci in groundwater were determined and, on average, have respective decay rates of 4.2 and 11.6 days for a 90% reduction (Hanes and Fragala, 1967; McFeters and Stuart, 1974; Keswick et al., 1982; Bitton et al., 1983). For a 99% reduction fecal coliforms have a decay rate of about 50 days (Peckdeger and Matthess, 1982). Therefore, decay may be a factor to consider, even though its influence should be significant only for bacteria transported from the more distant pasture area (Fig. 1).

The hypothesis of a different retention of the two microorganisms within the topsoil and/or the aquifer is in agreement with the knowledge that fecal coliforms and fecal enterococci vary considerably in terms of their size, morphology, motility and surface chemistry, which leads substantive differences in their propensities for attachment to solid surface (Becker et al., 2003; Harvey and Garabedian, 1991). Due to the high aperture of

fractures in the carbonate bedrock (generally more than 0.2 mm in the study site), retention should be significant within the topsoil. In order to verify the effectiveness of this hypothesis several column tests were carried out.

4.2 Species identification

Fecal enterococci and fecal coliforms were analyzed using colonies isolated after membrane filtration of different samples collected from all springs. These colonies were characterized with API 20 fermentation strips showing that, out of 120 enterococcal isolates, 46 were identified as *E. faecalis*, 33 as *E. faecium*, 26 as *E. gallinarum*, the last 12 were unidentified. As per fecal coliforms, out of 100 isolates, 93 were identified as *E. coli*, 4 as *Citrobacter* spp., 2 as *Klebsiella* spp. and 1 was unidentified.

Chromosomal DNA was extracted by 10 strains of each *Enterococcus* group, PCR was performed to obtain amplification of the ribosomal 16S DNA genes. Sequence of the 16S genes revealed, after BLAST comparison with the DNA GenBank (Altschul et al., 1990), a good agreement (90%) with the identification performed with API system. Hence, *E. faecalis* is the most representative species of the *Enterococcus* group in contaminated water samples, while *E. coli* is the most representative species concerning fecal coliforms.

4.3 Column tests

The total number of *E. faecalis* cells recovered after 300 mm of infiltration water (50% of mean annual effective infiltration in the study area) ranged from 0.11% to 8.13% of inoculated cells. The total number of *E. coli* cells recovered after the same amount of water ranged from 0.08% to 0.41% of inoculated cells. Retention of *E. coli* cells was always more efficient than retention of *E. faecalis* cells, even though the ratio of *E. faecalis* to *E. coli* was different in different soil samples. In four blocks the ratio was slightly higher than unit,

ranging from 1.1 to 1.4 (Tab. 2). In other two blocks it ranged from 14.5 to 42.0 (Tab. 2). The increase of the ratio above 10 mostly depend on the different percent of *E. faecalis* cells recovered within the column tests. In fact (Tab. 2), *E. coli* showed a lower spread of the data about the mean value (0.25%), as confirmed by the standard deviation (δ 0.12%). On the contrary, *E. faecalis* cells were characterized by a higher spread of the data (δ 3.56%) about the mean value (2.57%). A detailed analysis of the data set shows that the ratio was above 10 when *E. faecalis* cells recovered at the end of the column test were higher than 6% of inoculated cells (mean 7.09% and δ 1.48%; samples 5 and 6), while it was slightly higher than unit when *E. faecalis* cells recovered at the end of the column test were less than 0.5% of inoculated cells (mean 0.31% and δ 0.18%; samples 1 to 4). The application of the *t*-test suggests that in blocks 1 to 4 the mean concentrations of the two strains were not significantly different using a 0.05 level, whereas in the other two blocks the mean concentrations of the two microorganisms were significantly different at the same significance level.

Soil sample	*E. faecalis* cells recovered (%)	*E. coli* cells recovered (%)	Ratio of *E. faecalis* to *E. coli*
1	0.46	0.32	1.4
2	0.11	0.08	1.3
3	0.21	0.18	1.1
4	0.45	0.33	1.4
Mean	*0.31*	*0.23*	
Stand. Dev.	*0.18*	*0.12*	
5	6.04	0.41	14.5
6	8.13	0.19	42.0
Mean	*7.09*	*0,30*	
Stand. Dev.	*1.48*	*0.16*	
1 to 6			
Mean	*2.57*	*0.25*	
Stand. Dev.	*3.56*	*0.12*	

Table 2 - Percent of *E. faecalis* and *E. coli* cells recovered at the end of each column test.

A control experiment suggested that the cell death did not play a significant role on the results of column tests. The same amount of bacteria that were utilized for column charge was incubated for 4 days in a mix of $CaCl_2$ solution and column soil. At the end of this experiment the number of bacterial cells was not significantly changed.

Hence, one or more factors will cause a different retention of *E. faecalis* cells within the topsoil, at core scale, while they do not cause the same effect on *E. coli* cells.

4.4 Soil blocks properties

Experimental results of laboratory tests put in evidence the high content in organic matter ranging from 20% to 35% (Tab. 1) that, according to the fibrous structure, allow to classify them as organic soils (Pt) (USCS). Specific gravity values ranging from 2.06 g/cm^3 to 2.36 g/cm^3 (Tab. 3) result to be compatible with values of alkali-potassic volcanic minerals typical of such soils of pyroclastic origin derived from eruptive centres of Campanian district. Grain size analyses show a global homogeneity testified by a narrow envelope of grain size curves with an uniformity coefficient (U) which ranges between 3 and 9 (Tab. 3) and by a prevailing sandy loam texture (USDA).

Soil Samples	Organic Matter (%)	Specific Weight (g/cm^3)	Sediment Type (%)			U (D_{60}/D_{10})
			Sand	Silt	Clay	
1	27.12	2.06	80.8	15.9	3.3	3.60
2	32.50	2.32	75.0	23.0	2.0	3.21
3	19.78	2.36	83.9	11.1	5.0	9.00
4	22.69	2.23	81.0	17.0	2.0	2.90
5	29.33	2.28	78.9	19.1	2.0	3.33
6	33.80	2.21	73.0	25.0	2.0	4.86

Table 3 - Soil block properties.

5. DISCUSSION

The research demonstrated that cattle grazing causes a significant, discontinuous and diffuse microbial contamination of groundwater in the Acqua dei Faggi experimental field site with breakthrough curves significantly influenced by distribution of rainfall over time. Fecal coliforms and fecal enterococci were both detected during the research period at all springs. Nevertheless, at springs 2 and 3 fecal enterococci were detected in several water samples not contaminated by fecal coliforms, in agreement with the findings of Celico et al. (2004b) at seasonal spring 3 during the hydrologic year 2002/2003. Taking into consideration the results obtained at field scale during the whole research, we can suggest that the reliability of the two bacterial indicators is comparable at the perennial spring, while fecal enterococci are an indicator better than fecal coliforms at the seasonal springs. Thus, one or more factors which influence migration of the two indicators from the ground towards the springs are not homogenously distributed within the experimental field site.

Within the column tests, the simulation of the 50% of the mean annual effective infiltration in the study site caused the outflow of less than 10% of inoculated *E. faecalis* cells and less than 0.5% of inoculated *E. coli* cells. *E. coli* cells were always more retained than *E. faecalis* cells, even though only in 2 out of 6 soil samples this difference can be considered statistically significant. Different retention of *E. faecalis* cells at core scale is not significantly influenced by soil texture and organic matter which have comparable characteristics in all soil blocks. Thus, there should be significant differences in terms of soil structure and distribution of macropores at block scale in agreement with the findings of other studies (Dexter, 1993; Wildenschild et al., 1994; McMurry et al., 1998).

As a matter of fact, taking into account the random extraction of soil blocks from the experimental site, the column tests demonstrated the existence of a uniform retention of *E.*

coli cells at field scale, while *E. faecalis* cells are characterized by a non-uniform retention at the same scale. Hence, the comparison of field data with the results of column tests in intact soil blocks suggests that the topsoil of pyroclastic origin significantly influences migration and retention of both fecal coliforms and fecal enterococci and can cause a different reliability of these indicators to detect microbial contamination of springs which flow in a same basin-in-series aquifer system.

Taking into account that topsoils of pyroclastic origin often overlie limestone rocks in Southern Italy, a similar behaviour is expected within a wide portion of carbonate Apennines.

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