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Microbial groundwater pollution in Italian carbonate aquifers

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ABSTRACT. A research is in progress to analyze the transport of fecal bacteria through carbonate aquifers and to verify the effectiveness of DAC method for groundwater vulnerability assessment in the same aquifers. The research was developed through the monitoring of microbial contamination of surface runoff and spring water for several years, and through column tests in intact soil blocks, by using collection strains.The diffuse infiltration of rainwater through soil and fractured limestone causes microbial pollution, while the infiltration of surface runoff causes microbial contamination just in case of low dilution into the groundwater. In all cases, the microbial contamination shows series of peaks, which are irregularly distributed and conditioned by the distribution of precipitation versus time. The comparison of the vulnerability map obtained by applying the DAC method to the results of the microbiological experiments demonstrated the effectiveness of the method as predictor of groundwater microbial contamination in carbonate aquifers.

Key terms: Carbonate aquifer, Groundwater, Microbial pollution

Introduction

The carbonate aquifers, which provide one of the main drinking-water resources of Italy, may be divided into three groups: (a) mainly dolomitic aquifers, (b) mainly limestone aquifers and, finally, (c) aquifers made up of alternating limestone, cherty limestone, marly limestone and, subordinate marl. The main springs of the carbonate aquifers generally occur at the contact with Miocene rocks or volcanic, marine and continental Pliocene-Quaternary deposits, which represent relative aquicludes. Such aquifers are characterized by the existence of basal groundwater and several, generally small (mean annual discharge generally <0.1 m^3/s), perched water tables. The latter are usually generated by the presence of marly intercalations within the succession. Fast interactions between surface and groundwater are often observed. Therefore, significant quantities of contaminants can be rapidly transported into the subsurface.

The activity widely existent within these aquifers is cattle grazing, above all in mountainous carbonate aquifers. Manure is thus spread in karst surfaces, but pasture and manure spreading often cause bacterial contamination of drinking water (CELICO *et alii*, 1998 and 2003). A high spatial and temporal variability of pollution is generally generated by the interaction of several factors, such as temporal distribution of precipitations (CELICO *et alii*, 2003) infiltration mechanisms, length of transport, dilution, dispersion (MATTHESS & PEKDEGER, 1981), type of soil and its degree of saturation (LANCE & GERBA, 1984), filtration (IWASAKI, 1937; YAO *et alii*, 1971; GANNON et al., 1991), adsorption (MAIER et al., 2000) and retardation factors (MATTHESS *et alii*, 1985).

The purpose of the present paper is to synthesize the recent finds of researches focused on the microbial contamination of groundwater in Italian carbonate aquifers. Details will be shown concerning (a) the influence of precipitation, infiltration modalities, dilution and soil cover on both the entity and the temporal variability of microbial contamination.

The paper shows results obtained through field (daily or weekly monitoring) and lab (column tests in intact soil blocks) experiments, during the last 3 years.

Methodology

Water sampling criteria

Surface water was collected where (swallow hole and / or karst depression) runoff infiltrated into the aquifers. The underground water samples were collected at springs, which were chosen to satisfy the conditions described below. The water resources had to be (a) monitored in a territory characterized by both kinds of activity which cause microbial contamination (grazing and manure spreading), (b) fed through both kinds of infiltration mechanisms (infiltration of the rainfall and infiltration of runoff), (c) monitored in a territory characterized by a representative pedological diversity, (d) the outflows of groundwater characterized by a different water budget, to emphasize the role of dilution of microbial pollution and, finally, (e) interactive with microorganisms coming from different distances.

Microbiological Monitoring

The 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 or less from collection. Indicators of microbial contamination were determined by using classic methods of water filtration (1000 ml and 100 ml) on sterile membranes filter (GN-6 Metricel, pore size 0.45 μm, Pall), with incubation on: (a) m-Endo Agar LES (Biolife) for 24 h at 35 °C, for total coliforms, (b) m-FC Agar for 24 h at 44 °C, for fecal coliforms and (c) Slanetz-Bartley Agar for 4 h at 35 °C and 44 h at 44.5 °C, for fecal enterococci..

Strain and medium

A collection strain of *E. faecalis* (American Type Culture Collection 29212), nalidixic acid resistant has been aerobically cultured at 37 °C in Luria-Bertani (LB) liquid or solid medium supplemented with antibiotic (20 μg/ml of nalidixic acid) (SAMBROOK *et alii*, 1989).

Speciation of enterococcal isolates from spring and rRNA genes amplification

The choice of the most representative strain for the simulation of transport of fecal bacteria through soil blocks required the identification of the main species existent in study area. Taxonomic classification of fecal enterococci detected in the water samples was performed by use of API 20 Strep fermentation strips (bioMérieux, Marcy l'Etoile, France) and by sequence analysis of one of the 16S rRNA genes amplified with two universal oligonucleotides: P1 (5'-GCGGCGTGCCT AATACATGC) and P2 (5'-CACCTTCCGATACG GCTACC), annealing to nucleotides 40 to 59 and 1532 to 1513, respectively, of *B. subtilis rrnE*.

Soil Block Extraction

Intact soil blocks of *Epilepti-Vitric Andosols (Mollic)* were extracted from study sites, in pasture areas. To minimize the disturbance of samples, sod-covered blocks (181.36 cm square by 11 cm deep) were carved from undisturbed soil directly utilizing permeameter cells used for column tests. All blocks were covered in plastic and transported to the laboratory, where experimental procedures started immediately.

Simulation of Bacterial Transport through Soil Blocks

A diffuse interaction between bacteria and soil blocks was obtained by developing column tests in a standard permeameter (MaTest S248, Italy), to prevent lateral flow within the gap between soil block and metal cell. The rainfall was applied on the top of blocks. The outflow was collected at the bottom. A peristaltic pump (Watson Marlow 505S/RL USA) was used to sustain a constant flow through the blocks.

The real precipitations monitored in the field were simulated. As rainwater, a solution with 0.001 *M* $CaCl_2$ was used to prevent dispersion of clays within the soil and the column plugging (MCMURRY *et alii*, 1998).

Due to the results of the field monitoring, that showed the higher reliability of fecal enterococci as indicators of microbial contamination in the study site (see below), the interaction between faecal bacteria and soil blocks was analyzed through the utilization of a collection strain of nalidixic acid resistant *E. faecalis* (ATCC 29212). No nalidixic acid resistant bacteria were observed in the natural background of soil blocks collected in pasture areas.

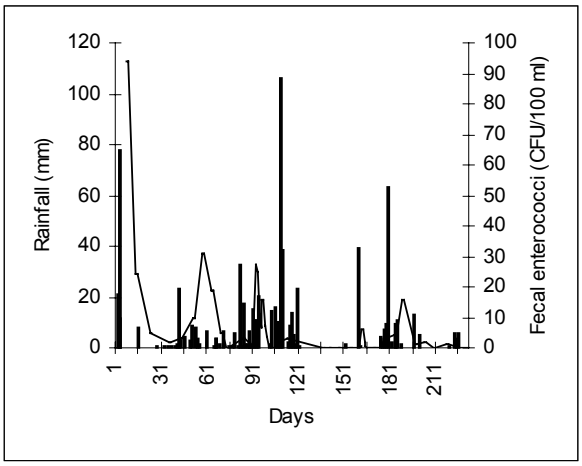

Figure 1. Example of concentration of fecal enterococci at spring (line) and rainfall (bars) versus time.

At the beginning of the experiments, 0.75 x 10^9 *E. faecalis* cells (during the exponential growth) were applied at the top of each block in a solution with 0.001 *M* $CaCl_2$.

Soil block drainage was collected in 10-ml sterile plastic tubes beneath the outflow holes. Two hundred microliters of each water sample and its serial dilutions were plated in triplicate on LB solid medium, supplemented with antibiotic (20 μg/ml of nalidixic acid) and incubated at 37 °C. After 24 h the number of *E. faecalis* cells was estimated as colony-forming units (CFU), by utilizing only the plates where the number of colonies ranged from 30 to 300.

Results and Discussion

Field Monitoring

Cattle grazing and manure spreading cause microbial pollution of groundwater in carbonate aquifers. The time dependence of fecal contamination shows irregularly distributed series of peaks (contamination events) (FIG. 1; CELICO *et alii*, 2003). Each contamination event is characterized by a different kind of breakthrough curve as a function of the distribution of precipitation versus time. The detailed analysis of different contamination events, through a daily monitoring, shows that a discontinuous distribution

of precipitation versus time produced articulated breakthrough curves (FIG. 2), while a single event, without dry intervals, caused a sort of Gaussian breakthrough (FIG. 3).

The effective infiltration of rainwater caused different effects on microbial pollution of springs as a function of the infiltration modalities (rainwater through

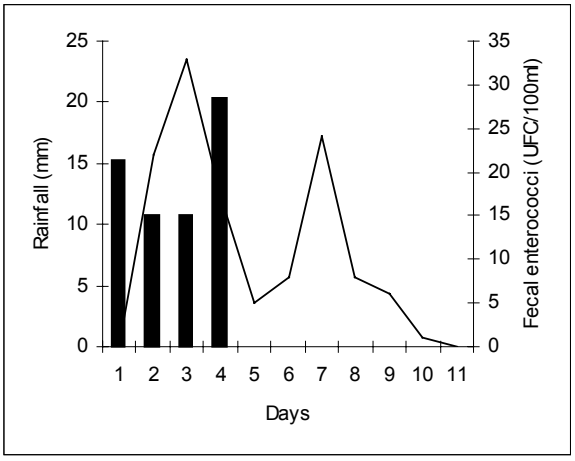

Figure 2. Example of daily concentration of fecal enterococci at spring (line) and rainfall (bars) versus time, after a discontinuous precipitation.

soil and fracture network, and/or surface water through karst conduit) and the dilution of polluted water into groundwater. The infiltration of rainwater through soil and fractured limestone produced significant microbial contamination when there was a stable near the springs and manure spreading at a short distance (a few hundred meters) from the natural receptor. In other words, the infiltration of rainwater causes microbial contamination of springs when large quantities of dung are concentrated in a small area close to the receptor.

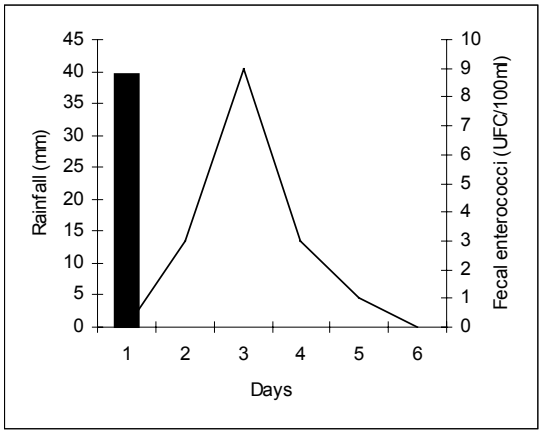

Figure 3. Example of daily concentration of fecal enterococci at spring (line) and rainfall (bars) versus time, after one day of precipitation.

The interaction between microbial contamination and karst conduit does not allow a significant self purification of polluted water. In the case of a mix between surface and groundwater, low contamination of springs is detected when the infiltrated runoff represents more than 10% of the groundwater discharge, although it interacts with a soil medium superimposed on karst conduits. On the contrary, no contamination of springs is detected when the infiltrated runoff is less than 1% of the groundwater discharge.

No fecal coliforms are observed in many contaminated water samples (fecal enterococci ≥1; Table 1; CELICO *et alii*, 2003). Hence, fecal enterococci are more reliable than fecal coliforms to detect microbial pollution in the study sites. These differences may be due to different factors: (a) animal feces are characterized by a fecal coliforms / fecal enterococci ratio of less than 0.7 (GELDREICH & KENNER, 1969), (b) fecal enterococci are more resistant than fecal coliforms

Table 1. Example of distribution (percent of samples) for fecal coliforms (FC) and fecal enterococci (FE) data at a spring.

(CFU/100ml)	FC	FE
0	78.0	42.4
≥1	22.0	57.6
Number of samples	59	59
Range	0-100	0-94

in the environment (GLEESON & GRAY, 1997), (c) fecal coliforms and fecal enterococci vary considerably in terms of their size, morphology, motility, and surface chemistry, which lead to substantive differences in their

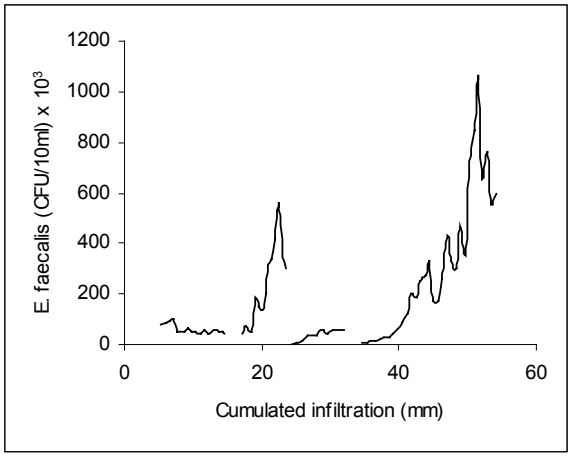

Figure 4. Example of *E. faecalis* breakthrough curves from a soil block.

propensities for attachment to solid surfaces within soils and aquifers (HARVEY & GARABEDIAN, 1991; BECKER *et alii*, 2003)

Identification of enterococcal Species

The speciation with API 20 Strep system showed that of the bacteria isolates, 38% were *E. faecalis*, 30% were *E. faecium*, 23% were *E. gallinarum*, and 9% were unidentified. These results were confirmed by sequencing ribosomal DNA and confronting the obtained sequences with those present in gene banks.

Flow-through Column Tests

Simulated "pulse" infiltration (discontinuous distribution of precipitation versus time) causes a sequence of branches characterized by different concentrations of *E. faecalis*. Each branch represents the breakthrough referred to each infiltration event (FIG. 4). Hence, the temporary stopping of infiltration and the presence of dry intervals (several hours in the case study) during a rainfall period caused different breaks of microbial transport through the soil. A new rapid increase in concentration for *E. faecalis* coincided closely with the beginning of each rainy step. The concentration at the start of each branch was significantly lower than that observed at the end of the previous breakthrough curve. On the whole, "pulse" infiltration produced a significant variation of the number of transported cells versus time and, then, an intermittent transport of bacteria to the "groundwater".

The column tests also confirmed that the soil cover of the carbonate rocks is characterized by a significant retention of bacteria. The high storage capacity of microorganisms has been already verified by several Authors, by utilizing different soils and bacteria (i.e. GANNON *et alii*, 1991; TREVORS et al., 1990).

Concluding Remarks

Microbial contamination of springs in carbonate aquifers is detected after precipitation events that produce effective infiltration. The time dependence of pollution shows series of peaks irregularly distributed, conditioned by the distribution of precipitation versus time. The "pulse" infiltration, produced by the presence of dry intervals (several non-rainy hours) during a precipitation period of a few days, will cause an intermittent transport of bacteria to the groundwater. Probably due to the short lengths of transport in study area, this kind of migration produced an articulated breakthrough at springs, detectable through a daily monitoring. Effective infiltration caused different effects on microbial pollution of springs as a function of the infiltration modalities (rainwater through soil and fracture network, and/or surface water through karst conduit) and the dilution of polluted water into groundwater.

The comparison of groundwater quality data with vulnerability maps obtained through the utilization of the DAC (DRASTIC-based; ALLER *et alii*, 1987) method (CELICO, 1996) demonstrates the effectiveness of the method for vulnerability assessment in carbonate aquifers (CELICO *et alii*, 2004). In those extensively fractured and karstified, the most significant weakness of the unmodified DRASTIC is the wrong interpretation of some hydrogeologic factors (depth to groundwater, recharge, soil media, topography and impact of the vadose zone media) where both diffuse infiltration of precipitation and concentrated infiltration of surface runoff can transport contaminants to the groundwater. In endorrheic areas the higher contamination of groundwater produced by the concentrated infiltration of runoff water into swallow holes and/or topographically low zones can be predicted through a reinterpretation of the five parameters mentioned above, as defined in the DAC. The same methodological approach can be also used in other kinds of aquifers, to assess vulnerability induced by the interaction between surface and groundwater. DAC could not be effective for chemical contaminants, because its effectiveness was verified using microorganisms as indicators of groundwater pollution.

Groundwater vulnerability maps developed by this new method will better identify areas of greatest potential for microbial contamination in carbonate aquifers and then they can be used to focus pollution prevention programs on areas of greatest concern, developing a sustainable land use, with emphasis on grazing and manure spreading.

Acknowledgements

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