

## WINTER SURVIVAL OF MICROBIAL CONTAMINANTS IN SOIL

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### Abstract

*The aim of this study was to evaluate, at site scale, the influence of freezing and freeze/thaw cycles on the survival of fecal coliforms and fecal enterococci in soil, in a climate change perspective. Viable cells of both fecal indicators were counted in ten different soil profiles and at different depths, before and after a winter season. Before the winter period and during grazing, viable cells of fecal coliforms and fecal enterococci were detected only in the first 10 cm below ground, while, after the winter period and before the new seasonal grazing, a lower number of viable cells of both fecal indicators was detected only in some of the soil profiles, and within the first 5 cm. Thus, a significant decrease in viable cells was observed in all soil profiles, due to cold shock, freezing, prolonged exposure to subzero temperatures, and alternating freezing and thawing periods, even though this decrease was not uniform at site scale. Taking into consideration the results of specific investigations, we hypothesised that the non-uniform spatial distribution of grass roots within the studied soil could play an important role in influencing this phenomenon, while several abiotic factors, such as organic matter, grain size and specific weight of particles, total porosity, and relative density, did not play any significant role. In a climate change perspective, taking into account also the local trend in air temperature, a different distribution of microbial pollution over time is expected in spring waters, and a higher risk of transmission of infections is expected throughout the year. The progressive increase in air temperature, with emphasis on the minimum one, will cause a progressive decrease in freezing and freeze/thawing at higher altitudes, therefore minimising cold shocks on microbial cells, and causing spring water pollution also during winter, differently from the actual observations.*

**Key words:** Climate change, Cold shock, Fecal indicator, Freeze/thaw cycle, Soil

### INTRODUCTION

Winter survival of microorganisms in soil can be negatively influenced by cold shock, freezing, prolonged exposure to subzero temperatures, and alternating freezing and thawing periods [7, 9]. Because survival times of microorganisms in the environment can be influenced by different abiotic and biological factors [4], and some of them can be non-uniformly distributed within a soil medium [13], specific investigations at site scale are necessary to analyse the role of the scale effect on relationships between winter stresses and the survival of fecal microorganisms in soil. In a broader context, climate change, with emphasis on temperature increase, can significantly influence the survival of fecal microorganisms and pathogens in soil causing a progressive

increase of the risk of infection transmission by different routes, such as water and contaminated soil. The present work has been carried out in a test site in the Campania region, southern Italy, to study in situ the winter survival of two usual indicators of microbial contamination of fecal origin, analysing both air and soil temperatures, the latter at different depths, and enumerating viable cells at different depths along several soil profiles, before and after winter stresses. The test area is a mountainous carbonate aquifer where cattle grazing is not allowed from mid November to late April, and a former study showed the existence of an anomalous distribution of microbial contamination of spring water over time, tentatively associated to winter stresses on microbial contaminants [1]. The statistical analysis of temperature time series was carried

out, using data recorded at a meteorological station located at high elevation.

## MATERIAL AND METHOD

### Site description

The aquifer has an extension of about 0.85 km<sup>2</sup>, and consists predominantly of calcareous deposits (Cretaceous in age). The rocks have very low primary permeability but are extensively fissured. The unconfined aquifer is bounded by normal faults that act as barrier and partially impede groundwater flow [1]. The hydraulic head slopes southwards to different springs.

### Soil characterisation

Some physical and index properties of soil were analysed by means of standard laboratory tests, to verify if the analysed abiotic factors can influence the microbial resistance to winter stresses. Soil samples were collected along the same 10 profiles where soil cores were collected to carry out the microbiological investigations, at 10 and 50 cm below ground. In fact, it is known that, during freezing and thawing, aggregates are broken and, shearing forces can lyse cells and hyphae, resulting in the selection of tolerant microbes (e.g., [6]). Organic matter, grain size and specific weight of particles, total porosity, relative density were analysed in order to verify the distribution of such features at site scale. Organic matter tests were performed through organic matter loss after muffle furnace heating. Accordingly to the ASTM standard procedures, the organic content of soil samples was measured by means of loss-on-ignition technique, consisting in 72-h heating at 450 °C in a muffle furnace. 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. Specific gravity tests were carried out using a 50 cm<sup>3</sup> pycnometer applied to the 0.075 mm passing fraction through the sieve n. 200 ASTM. Starting from these physical and volumetric parameters it was possible to obtain the total porosity (n) and the relative density (Dr). These are a-dimensional parameters and they are expressed in percentage. These are indirectly estimated, starting from the existing

relationship between weights and volumes of the different phases.

The vertical distribution of grass roots within the 10 soil profiles was analysed through some numerical analysis techniques for image processing. These were applied to the digital photo of the soil samples. In particular, we homogenized the resolution of the images and cropped them to show from 0 to 10 cm below ground, then we converted the images from RGB (color) to grayscale and used a sharpening filter (Sobel filter) to enhance the edges of objects and adjust the contrast. Then, we applied a numerical filter for the edge detection based on a gaussian and on a gradient filter (Canny edge detector method) of the function  $f(x,y)$  describing the image [5]. The next step was to count the pixels showing the grass roots, from black and white previous images.

This approach has been used to analyse in a detailed manner the vertical distribution of roots within the upper 10 cm of the investigated soil profiles, where viable cells of fecal coliforms and fecal enterococci were widely detected before winter, and where subzero temperatures were recorded during winter.

### Microbiological investigations

The enumeration of viable cells of both fecal indicators was carried out (a) in early November 2009, before the freeze/thaw period and during grazing, and (b) in April 2010, after the freeze/thaw period and before the new seasonal grazing. For the enumeration of soil bacteria, soil samples were collected at different depths (2, 5, 10, 15, 20, 30, 40, 50, 100 cm below ground) close to the temperature probes and along other 9 soil profiles within the same pasture area. The 10 profiles were randomly distributed over a 80 x 60 m area.

According to described method [12] soil cores (5 cm in diameter and 5 cm long) were sampled in ethanol-sterilized sleeves, capped, and stored on ice before being transported to the laboratory. One gram of soil from each sample was added to 3 ml of eluent buffer (0.1% Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 0.05% polyvinylpyrrolidone [pH 7.2]) and vortexed thoroughly for 5 min. Serial dilutions were made from the resulting supernatant in phosphate-buffered saline (0.1 M NaCl, 0.02 M sodium phosphate [pH 7]). Coliform and enterococci cells enumerations

were performed by direct plating on m-FC agar (Biolife) and SB agar (Biolife), respectively. Previous studies in this test area and in other comparable sites in southern Italy demonstrated that *Escherichia coli* is the most representative species within fecal coliforms, while *Enterococcus faecalis* is the most representative species of the *Enterococcus* group [1, 2, 10, 11].

### **Local air and soil temperature measurements**

Both the air and the soil temperatures were measured from February 2008 to June 2010 on a hourly basis, through a meteorological station and two probes with data-logger, respectively. Soil temperature was measured at 8 and 50 cm below ground. The deeper probe was used until May 2009 to analyse the vertical distribution of soil temperature during a winter season.

### **Historical temperature of mountain zones**

To analyse the historical temperatures of the ground-elevated areas, the series of Montevergine station (1270 m a.s.l.) has been considered, where data are available since 1884. This station is located inside a monastery, far from any urban area and, thus, data records are not affected by local thermal pollution [3]; these characteristics appear notable when comparing temperature over long period of time and, in particular, temperature of recent decades to the previous one.

## **RESULTS AND DISCUSSIONS**

With respect to all the analysed abiotic features, the soil medium is characterised by an overall homogeneity at both the investigated depths. Viable cells of fecal coliforms and fecal enterococci were detected only in the first 10 cm below ground, before the winter period and during grazing. After the winter period and before the new seasonal grazing, no viable cells of fecal coliforms were detected in 8 out of 10 soil profiles. Conversely, in other 2 investigated profiles viable cells of fecal coliforms were detected at -2 cm and at -5 cm. During the same sampling campaign, viable cells of fecal enterococci were not detected in 6 out of 10 soil profiles. Conversely, in other 4 investigated profiles viable cells of fecal enterococci were

detected at -2 cm and at -5 cm. On the whole, in 2 out of 10 soil profiles viable cells of both fecal coliforms and fecal enterococci were detected in April 2010. When detected after winter, viable cells were always detected within the first 5 cm of soil below ground.

Between November 2009 and April 2010, 7 freeze/thaw cycles were recorded in the upper soil horizon and the density of viable cells significantly decreased (~4 orders of magnitude) in several soil profiles, while a slighter decrease (<2 orders of magnitude) was observed in a few profiles.

The observed decrease in viable cells in the studied soil can not be significantly influenced by cells removal during effective infiltration of percolating water, because of the high retention capacity of this type of soil with regard to fecal coliforms and fecal enterococci [10, 11].

The observations made within this study therefore suggested that (a) the survival time of fecal coliforms and fecal enterococci in soil can be non-uniform at the decametric scale, and (b) in some soil portions, the survival time in soil, of both fecal indicators, can be longer than expected, despite freezing and freeze/thaw cycles.

Taking into consideration the overall homogeneity of the abiotic soil features that were analysed at the study site, the observed non-uniform survival time of these fecal indicators, as well as their higher resistance to winter stresses in the upper 5 cm of soil, seem to be more probably influenced by soil structure and/or biotic factors.

Concerning biological factors, it was found that *E. coli* O157 can survive longer in soil containing rooted grass (decline of less than 2 orders of magnitude in 130 days; [8]). As a matter of fact, when analysing the roots density within the upper 10 cm within the investigated soil profiles, that is to say where viable fecal indicators were detected before winter stresses, a good agreement can be observed between the results of this study and Maule's findings [8].

The statistical analysis of the air temperature series presents a downward trend during the 1936-1946 and 1952-1956. The beginning of the general warming has been recorded mainly since the eighties.

## CONCLUSIONS

Distribution of grass roots within the studied soil can play an important role in influencing the non-uniform survival time of fecal indicators, as well as their higher resistance to winter stresses in the upper five cm of soil, at the study site.

In a climate change perspective, taking into account the results of the microbiological investigations, and the local trend in air temperature, a different distribution of microbial pollution over time is expected in spring waters, and a higher risk of transmission of infections is expected throughout the year. As a matter of fact, the progressive increase in air temperature, with emphasis on the minimum one, will cause a progressive decrease in freezing and freeze/thawing at higher altitudes, therefore minimising cold shocks on microbial cells. With reference to spring water contamination, microbial pollution is expected also during winter, differently from the actual observations [1]. This effect will be the results of huge effective infiltration, and transport of a higher number of viable cells from the ground towards the groundwater and the springs.

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