Fate and transport of faecal contamination microbial indicators, pathogenic protozoa and *Campylobacter* in the artificially recharged fractured aquifer of Salento, Italy

R. La Mantia, C. Masciopinto, C. Levantesi and V. Tandoi

**ABSTRACT**

The study investigates the fate and transport of microorganisms introduced by artificial groundwater recharge at the Nardo` fractured aquifer in Salento, Italy. Microbial indicators of faecal contamination, parasitic protozoa (*Giardia* and *Cryptosporidium*) and pathogenic bacteria (*Campylobacter* spp.), were monitored into injected water and groundwater to test the efficiency of the “natural disinfection” into the fractured aquifer. A remarkable decrease of microbial indicators and pathogens was observed suggesting that pathogens removal or inactivation may be possible during water flow in fractured aquifer. The recently described PNA probe CJE195 (Lehtola et al. 2005) was utilised for the rapid and specific detection of *Campylobacter* spp. by fluorescence *in situ* hybridization (FISH) after enrichment. FISH results were consistent with those of traditional cultural method (ISO 17995) applied in parallel: time required for *Campylobacter* identification was reduced of 4 days.

**Key words** | aquifer recharge, *Campylobacter*, *Cryptosporidium*, *Giardia*, pathogens indicators

**INTRODUCTION**

The artificial recharge of exploited aquifers with treated wastewater has been often proposed as a valuable option for addressing water shortage problems, focusing on the recycled water microbiological quality. *Giardia* and *Cryptosporidium* are the most common parasitic protozoa transmitted with waters in industrialized countries and numerous water-borne outbreaks of giardiasis and cryptosporidiosis have been documented mainly in the USA, Europe and Australia (Rose et al. 2002 and Slifko et al. 2000). *Campylobacter* spp. are reported as new emerging waterborne pathogens, the vehicles of these microorganisms are usually foods and animals but they play a role in water borne infections as well (Horman et al. 2004). Thermotolerant *Campylobacter* spp. are important pathogens causing gastroenteritis: they can cause mild to severe diarrhoea, and several species such as *C. jejuni* are occasionally invasive. *C. jejuni*, *C. coli*, and *C. lari* account for more than 99% of the human isolates (*C. jejuni* 90%). In this study the fate and transport of various enteropathogens and different microbial parameters were investigated, in a site of direct aquifer recharge with treated wastewater in Nardo`, Southern Italy Apulia region, that suffers severe problems of water shortage (Lopez & Vurro 2008). At present the National Decree D. Lgs., 2003, fixes for agricultural reuse a series of chemical–physical parameters and maximum values for two microbiological parameters: *E. coli* (cfu/100 mL): < 10 and *Salmonella* (cfu/100 mL): absent, (Lopez & Vurro 2008).

The Nardo` aquifer is a fractured limestone formation where the groundwater is loaded by a channel that collects superficial untreated waters due to rainfalls and effluents derived from municipal waste water treatment plants. The collected water is directly injected in the aquifer trough a sinkhole with an average flow rate of about 150 L/s. The sinkhole is basically a vertical cave with a large diameter (about 6 m). The continuous artificial injection produces...
a mound of the water table of about 2 m around the injection site by increasing the piezometric head surface from the initial 2 m up to 4 m above the sea level. As a consequence, the water velocity in the fractures is very high (due to high hydraulic gradient) and ranges from 50 to 200 m/d. The aquifer was previously characterised by iodine and chlorophyll tracer tests; the spatial variability of the mean fracture aperture of the Nardò aquifer was determined by a geostatistical procedure and local aperture measurements evaluated by pumping borehole field tests (Masciopinto 2005). Furthermore by groundwater monitoring and mathematical transport model the apparent pathogenic micro-organisms pathways in fractures were determined: they flew only along two canalized directions around the injection site (Masciopinto et al. 2007). Five sampling points comprising the municipal waste water secondary effluent, the water entering the sinkhole and three wells located at different distances from the sinkhole, were selected to study the fate and transport of microorganisms in the aquifer. The presence and abundance of eight microbial indicators of faecal contamination (total bacterial count, total coliform, faecal coliforms, E. coli, faecal streptococci, enterococci, somatic coliphages and Clostridium spores) and three pathogens which may cause water borne diseases, namely Giardia, Cryptosporidium and Campylobacter were monitored in Nardo’ aquifer during 4 to 6 sampling campaign in 2006 and 2007. The goal was to follow the natural microorganisms decay during the passage through the limestone in the investigated area.

**METHODS**

**Sampling sites**

Water samples for microbial indicator analysis were collected during six sampling campaigns in the different seasons of the years 2006 (January, April, July, November) and 2007 (February, May). Five sampling points were monitored: wastewater secondary effluent (SP1), sinkhole (SP2), two wells located at progressive distance form the sinkhole, respectively at 320 (SP3) and 500 (SP4) metres, and one lateral well, unaffected by the apparent plume of the injected wastewater, and used as groundwater quality background (SP0). Enterobacteria were analysed only from July 2006. The presence of pathogenic protozoa and bacteria was analysed during respectively four and three sampling campaigns as described in details later.

**Microbiological parameters**

For the determination of microbial faecal contamination indicators, 100 ml sample were utilised for the quantitative detection of: total coliform, faecal coliform and E. coli (by MPN, APAT–CN–IRSA 2003), faecal streptococci, enterococci (by MF, Bonadonna & Ottaviani 2007), Clostridium spores (tube inclusion technique, Bonadonna et al. 2002). Bacterial counts were determined by pour plates (APAT-CN-R-IRSA 2005) method by using 1 mL of water sample. The presence and abundance of somatic coliphages was investigated by the double layer technique on 50 mL sample (direct inoculation or concentrated water sample from 20 L) by using 1.5% tryptic soy agar (TSA) as bottom layer and soft TSA (0.7% of agar) as top layer (EPA method 1601, 2001) on 5 large Petri dishes (140 mm) at different dilution factors. The mutant strain of E. coli ATCC 13706, that is nalidixic acid resistant, was utilised as host bacteria stock culture to detect somatic phi-X 174 stock coliphages (ATCC 13706-B1). After incubation at 37°C for 16–24 h, the Petri dishes were examined for the presence of lyses zones which were expressed as plaque forming units (PFU/100 mL) by the plaque method (Bonadonnae Ottaviani 2007).

For the recovery of Giardia and Cryptosporidium cysts and oocysts, water samples were filtrated at the field with “IDEXX Filta-Max Foam” Filters, filtering volumes of 5–120 L. Filter modules were eluted with “IDEXX Filta-Max xpress” automatic station according manufacturer instructions. The eluate was then concentrated to 10 mL by centrifugation (15 minutes, 2,000 rpm). Cysts and oocysts were finally enumerated by epifluorescence microscopy (Olympus BX-51) and phase contrast observation.

Campylobacter jejuni subsp. jejuni (DSM 4688) and Campylobacter coli (DSM 4689), obtained from the DSMZ...
(German Collection of Microorganisms and Cell Cultures) were used as positive controls for both traditional and FISH detection protocols. The ISO 17995 method was applied for the detection of thermotolerant *Campylobacter* spp. in 100 or 1,000 mL. Biomass fixation was performed as described in *Levantesi et al.* (2004) and FISH analysis was performed on 10 µL of the fixed biomass according to the protocol described in *Lethola et al.* (2005).

**RESULTS AND DISCUSSION**

**Microbial indicator results**

To predict the fate of the various classes of pathogenic microorganisms (bacteria, viruses and protozoa), faecal contamination indicators characterized by different resistance to wastewater treatments and environmental stress were analysed during 6 campaigns in 2006 and 2007 (Figure 1).

A reduction of the amount of microbial indicators was observed, from the effluent of WWTP (SP1) and the influent water into the sinkhole (SP2) to sampling points in the aquifer (SP3 and SP4), though during rainfalls an increase of bacteria and total coliform counts were also detected along the pathogen pathways into the subsoil, that were derived from model simulation results.

*Figure 2* shows the percentage of removal rate of microbial indicators due to filtration in the fractured aquifer, from SP2 to SP4, on the basis of the detected counts in water samples during the sampling campaigns. Somatic coliphages and *Clostridium* spores showed the highest percentage of inactivation (90–95%) in three samplings, though the coliphages reduction resulted 50% in July 2006 and C. spores 40% in April 2006. On the contrary *E. coli* showed the lower rate of inactivation on July 2006, together with total coliforms and faecal streptococi.

The inactivation constant rate of three microbiological parameters namely somatic coliphages, *Clostridium* spores and faecal coliforms, was estimated by using the first-order process (*Anders & Chrysikopoulos* 2006):

\[
\log \frac{C(t,x,y)}{C_0} = -\lambda t
\]  

The inactivation rate λ estimation is reported in *Table 1*, at assigned distance of 320 m (SP3) and 500 m (SP4) wells.

The inactivation constant λ can also be estimated roughly as a function of groundwater temperature in the range 12.5°C ≤ T_e ≤ 26°C by the following expression (*Yates & Yates* 1987):

\[
\lambda = -0.66 + (0.054 \times T_e)
\]  

\(2\)

It should be considered that groundwater temperature measured into the well was practically constant during year and ranged with depth from 14.8 to 15.5°C at a depth of 30 m below the water table: inactivation constant rates calculated by Equation (2) give values 0.14 – 0.18 d⁻¹ (for SP3 and SP4 wells). These values are in good agreement with those calculated by Equation (1).

The λ values have been estimated by Equation (1), assuming the arrival time of pathogens into well equal to 2 d and 6 d for SP3 and SP4, respectively. Moreover the dilution factor operated by groundwater on the influent water was 42.2% into the well SP3 and 13.3% into the well SP4. These values have been determined by a model simulations carried out at the Nardò site.

Although the coliphages inactivation rate can be considered almost constant with distance form injection and according to *Yates & Yates* (1987), a reduction of the inactivation constant vs. distance was noted for faecal coliforms and C. spores. This might be explained by a remarkable detachment of coliform and C. spores that were previously attached to the fracture walls during wastewater filtration. On the contrary, the attachment/detachment phenomena poorly affected the phages transport in the fracture: their inactivation was mainly determined by groundwater temperature.

**Giardia cysts and Cryptosporidium oocysts**

The presence of these protozoa was monitored during four sampling campaigns (January 2006, November 2006, February 2007 and May 2007) and 14 samples in total were analyzed. A quasi complete removal of these parasites was observed from SP1 to SP4; though both parasites were detected, *Giardia* count prevailed in all samples with respect to Cryptosporidium. *Giardia* cysts were detected in 85.7% of water samples (*Figure 3*). They were detected in a large amount in the WWTP effluent, with a count of
Figure 1 | Microbial indicators detected at Nardo during six sampling campaigns; (0): SP0 sampling, background groundwater well; (1): SP1, WWTP secondary effluent; (2): SP2, sinkhole; (3): SP3, well at 320 m from sinkhole; (4): SP4, well at 500 m from the sinkhole.
12 cysts/L, on average and ranging from 5 to 27 cysts/L. The cysts number was reduced to 2 cysts/L, on average, and ranging from 0.2 to 6 cysts/L, before the injection in the aquifer (SP2). A 1 order of magnitude removal (i.e. 10 times) has been observed in the aquifer filtration along the flow directions. Indeed at SP3, the average count of cysts was 0.9/L (i.e., from range 0.3–1.8 cysts/L) and this parasite was detected at 1 cysts/100 L only in 1 water sample derived from SP4, 500 m from sink-hole (SP2). Cryptosporidium oocysts were sometimes observed (36% of samples) and were always present in very low amount (from 0.1 to 0.4 oocysts/L). Differently from Giardia spp., it was most commonly found in the sinkhole samples (75% of SP2 samples) and only once in the WWTP secondary effluent and in the groundwater aquifer (SP3 Well). By a comparison with previous Italian surveys (Cacció et al. 2003; Briancesco et al. 2005) low concentrations of Giardia cysts have been detected in the WWTP effluent at Nardò. Briancesco et al. (2005) did not detect any Giardia and Cryptosporidium oocysts in groundwaters samples, even though they sampled groundwater utilized for drinking purposes.

The higher count of Giardia in wastewaters and river samples was also detected in Brazil and Italy (Cacció et al. 2003; Briancesco et al. 2005; Cantusio et al. 2006). Both Cantusio and Briancesco works suggested that methodological problems, such as the difficulty to detect the small oocysts especially in dirty waters samples might affect the lower count of Cryptosporidium detected. Such problems could explain the lack of Cryptosporidium oocysts in the SP1 water samples. However, in the Nardò site, a different source of Cryptosporidium contamination at the SP2 sampling point should be considered, i.e. the runoff transporting animal faeces which can convey this protozoa in the channel and in the sinkhole.

Table 1: Presence and inactivation rate of faecal coliform, somatic coliphages and Clostridium spores in Nardò artificial recharge site

<table>
<thead>
<tr>
<th></th>
<th>Faecal coliform°</th>
<th>Coliphages°</th>
<th>C. spores°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MPN/100 mL</td>
<td>PFU/100 mL</td>
<td>CFU/100 mL</td>
</tr>
<tr>
<td>SP0 Sampling well for background</td>
<td>106</td>
<td>132</td>
<td>172</td>
</tr>
<tr>
<td>SP1 WWTP effluent</td>
<td>11598</td>
<td>9492</td>
<td>7625</td>
</tr>
<tr>
<td>SP2 Sinkhole</td>
<td>4369</td>
<td>6168</td>
<td>913</td>
</tr>
<tr>
<td>SP3 Well at 320 m</td>
<td>511</td>
<td>0.642</td>
<td>0.117</td>
</tr>
<tr>
<td>SP4 Well at 500 m</td>
<td>453</td>
<td>0.038</td>
<td>0.120</td>
</tr>
</tbody>
</table>

° Average values derived from 6 samplings. The inactivation rate for faecal coliform at SP4 well was determined by eliminating anomalous high concentration detected in the last sampling of May 2007. Similarly, for C. spores at SP3, July 2006 and Feb 2007 anomalous high counts were eliminated.
Campylobacter

Two methods, the traditional cultural method (ISO 17995 2005) and a new PNA-FISH approach, adapted from Lehtola et al. (2005), were applied for the detection of thermotolerant Campylobacter spp. in Nardo’s aquifer. The PNA-FISH approach with the probe CJE195 (Lehtola et al. 2005), specific for the pathogenic Campylobacter spp. C. jejuni, C. lari and C. coli, requires a shorter time for Campylobacter detection and increases the identification specificity. In our case an enrichment step was introduced to overcome the limit of low count (i.e. density) and pour viability of Campylobacter in water samples.

Figure 4 shows the microscopic images of Campylobacter cells detected by PNA-FISH in the enrichment broth of SP2 sample (November 2006).

C. coli, C. lari and C. jejuni positive cells with their characteristic shape appeared bright fluorescent upon probe hybridization and could be clearly differentiated despite the presence of a background red fluorescence due to the residual blood cells present in the enrichment broth. The presence of thermotolerant Campylobacters was monitored during three sampling campaigns (November 2006, February 2007 and May 2007): the results are reported in Table 2.

As reported in Table 2 only one of the analyzed samples was positive. Although smaller and larger volumes of sinkhole samples were analysed, respectively 500–1,000 mL to increase the chance of recovery of rare Campylobacter cells and 10 mL to reduce the competition of background bacteria (Abulreesh et al. 2005), no increase in detection was observed. At present consistent results were obtained by cultural method and PNA-FISH analysis.

Table 2 | Campylobacter spp. presence in Nardo artificial recharge site

<table>
<thead>
<tr>
<th></th>
<th>May 07</th>
<th>February 07</th>
<th>November 06</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ISO17995</td>
<td>PNA-FISH</td>
<td>ISO17995</td>
</tr>
<tr>
<td>SP0 Sampling well for background</td>
<td>&lt;10 cfu/L</td>
<td>nd</td>
<td>&lt;10 cfu/L</td>
</tr>
<tr>
<td>SP1 WWTP effluent</td>
<td>&lt;10 cfu/L</td>
<td>nd</td>
<td>&lt;10 cfu/L</td>
</tr>
<tr>
<td>SP2 Sink hole</td>
<td>&lt;2 cfu/L</td>
<td>nd</td>
<td>&lt;1 cfu/L</td>
</tr>
<tr>
<td>SP3 Well at 320 m</td>
<td>&lt;10 cfu/L</td>
<td>nd</td>
<td>&lt;10 cfu/L</td>
</tr>
<tr>
<td>SP4 Well at 500 m</td>
<td>&lt;10 cfu/L</td>
<td>nd</td>
<td>&lt;10 cfu/sL</td>
</tr>
</tbody>
</table>
CONCLUSIONS

The estimated inactivation constant for somatic coliphages (0.12 d⁻¹) is in agreement with the values proposed by Yates & Yates (0.14 – 18 d⁻¹), as a function only of the groundwater temperatures. On the contrary attachment/detachment phenomena strongly affected the count of faecal coliforms and C. spores which were transported in the fractures. In particular, by increasing the length of wastewater filtration in the fractured aquifer the detachment of bacteria and spores caused a reduction of the estimated inactivation rates from 0.6 to 0.05 d⁻¹, however 1 Campylobacter could still be detected in 100 litres sample at the sampling point SP4 indicating a possible health risk associated to this groundwater well. While both Campylobacter and Cryptosporidium were present in the inlet flow at sinkhole sampling point, they were not detected or only rarely present in the groundwater samples. The PNA/FISH method, adapted from Lehtola et al. (2005), was successful for the analysis of Campylobacter spp. Although the results of PNA-FISH were consistent to those of ISO 17995, only one positive sample was detected and further research is still required to confirm the effectiveness of the PNA-FISH method. The groundwater quality already at 500 m distance from the sinkhole is fine for one of the two compulsory limit: E. coli. (less than 10 cfu/100 mL). Work is in progress to determine Salmonella in the various wells. The field data and model results suggest that for the municipal wastewater injected into the Nardo site a quasi complete pathogen inactivation may be possible during filtration into the fractures, though appropriate set back distances have to be imposed before utilization wells.

ACKNOWLEDGEMENTS

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