Unsafe tap water in households supplied from groundwater in the Salento Region of Southern Italy

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ABSTRACT

Although the fractured aquifer of the Salento supplies over 80% of the drinking water requirements of the local population, its exposure to pollution has recently increased. In recent years, owing to the arid climate and droughts, the spreading of wastewater on soil for irrigation has become much more frequent. Consequently, hazardous and pathogenic microorganisms released with wastewater have been transported into the subsoil and have contaminated groundwater. An elaboration of epidemiological data has shown that the local population has the highest exposure to endemic gastroenteritis in Italy. In order to reduce human exposure to unsafe groundwater, the setback distance for drinking wells necessary to achieve the 'natural disinfection' criteria, has been determined experimentally at the Nardò aquifer (Salento region), supported by groundwater monitoring results and a mathematical transport model able to determine the apparent pathogenic microorganism pathways in fractures. The results also provided valuable inactivation constants of cultural indicators (coliforms, enterococci, *Clostridium* spores and somatic coliphages) and viruses in the wastewater that have been injected into the fractured aquifer since 1991. Furthermore, the efficacy of chlorine to remove viral indicators from water in a well 500 m from wastewater injection was tested. Hypochlorination reduces somatic coliphages and *Clostridium* spores in groundwaters but did not achieve complete inactivation in all tests. Complete disinfection of groundwater samples was possible only when there was an initial *Clostridium* spores count of $\leq 10\,\mathrm{CFU}\,100\,\mathrm{ml}^{-1}$.

Key words | natural water quality, pathogenic microorganisms detection, pathogens inactivation constants, waterborne disease outbreaks

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LIST OF SYMBOLS

C	aqueous phase concentration or pathogen count ($\mathrm{PFU\,l^{-1}}$ or $\mathrm{CFU\,l^{-1}}$ or $\mathrm{MPN\,l^{-1}}$ or $\mathrm{IU\,l^{-1}}$)
C_0	initial aqueous phase concentration or pathogen count ($\mathrm{PFU\,l^{-1}}$ or $\mathrm{CFU\,l^{-1}}$ or $\mathrm{MPN\,l^{-1}}$ or $\mathrm{IU\,l^{-1}}$)
$C*$	count of pathogen sorbed onto fracture surfaces per unit of fluid volume ($\mathrm{PFU\,l^{-1}}$ or $\mathrm{CFU\,l^{-1}}$ or $\mathrm{MPN\,l^{-1}}$ or $\mathrm{IU\,l^{-1}}$)
I_{cw}	percentage of wells contaminated in the community distribution system ($-$)
$1 - \mathrm{I_m}$	percentage of reduction of disease due to population immunity ($-$)
P_{Daily}	daily probability of HAV infection ($-$)
P_{Annual}	annual probability of HAV infection ($-$)
Pop	population exposed
M_P	morbidity factor for primary route of illness as a result of infection ($-$)
M_s	morbidity factor for secondary route of illness as a result of infection ($-$)
M_D	morbidity factor for additional cases due to treatment failure in disinfected system and private distribution system sources ($-$)
N	daily (IU) of HAV ingested with water drunk ($\mathrm{HAV\,l^{-1}}$)
U	frequency of HAV underreporting cases in the database of notified infectious disease ($-$)

r_1, r_2	forward and reverse rate coefficients for pathogens attachment/detachment on fracture walls, respectively (t^{-1})
$\mathbf{x}$	particles pathway function $(-)$
β	inverse constant probability of initiation of infection by a single HAV per 1 l of water drunk (infections per HAV l^{-1})
$\lambda, \lambda*$	pathogen inactivation constants for suspended and adsorbed particles (t^{-1})
Ω	pathogens decay constant (t^{-1})

INTRODUCTION

Water shortage is one of the principal problems of the Salento (Figure 1) as well as other water deficient regions of the Mediterranean, which are characterised by low economic prosperity. In these regions the lack of water resources has led to frequent spreading on the ground of untreated wastewater for soil irrigation. Moreover the reduction in natural groundwater replenishment, owing to the absence of precipitation in arid regions, increases seawater intrusion by reducing exploitable groundwater. As a result, in the area studied, during drought periods (Hunter 2003) the quality of the groundwater may be considerably compromised because of the high vulnerability of the Salento aquifer

formation, which is characterised by numerous rock fractures and joints, by increasing risks for human health.

In the Salento peninsula recent Italian regulations (D. Lgs. 152, 1999 and 258, 2000) have prohibited direct untreated (or treated) wastewater injection into the subsoil. However, the spreading of minor volumes of partly treated domestic wastewater into unsaturated subsoil for sub irrigation is still permitted as are indirect stream waste outflows in sinkholes. In contrast, the State of California's Water Recycling Criteria (2000) require water treatment and a more stringent set of conditions for wastewater impoundment, including monthly analyses for *Giardia*, enteric viruses and Cryptosporidium, during the first 12 months of operation and use. The standards of microbiological quality for wastewater reuse in agriculture or groundwater recharge (Masciopinto *et al.* 1991; Asano *et al.* 1992; Tanaka *et al.* 1993; Asano & Cotruvo 2004) have not yet been properly defined by Italian law (DM 185 2003) which considers only *Escherichia coli* (<100 CFU $100\,\text{ml}^{-1}$) as a cultural indicator of pathogenic microorganisms in water, whereas viral indicators such as bacteriophage or *Clostridium perfringens* (Health Canada 2004) and protozoa (*Giardia* and *Cryptosporidium*) are neglected. The World Health Organization guidelines (WHO 1989) for the use of wastewater in agriculture and aquaculture
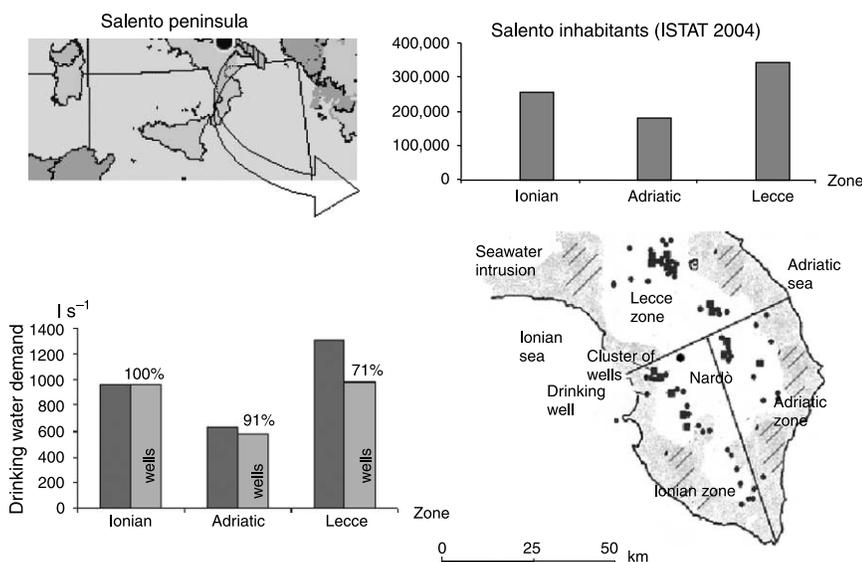

Figure 1 | Current drinking water demand of the Salento population and percentage of the local requirement supplied by wells in the Ionian, Adriatic and Lecce zones. The high percentage is due to shortage of surface water (i.e. lakes or rivers) whereas the extent of seawater intrusion is proportional to the shortage of groundwater replenishment and heavy groundwater withdrawals.

recommended a monitoring of intestinal nematodes together with faecal coliforms during irrigation periods whereas a new (2nd) edition of the WHO guidelines (technical report) is in preparation (see online, WHO 2005).

As the Salento groundwater supplies 80% of the total drinking water requirement of the local population (about 800,000 inhabitants (ISTAT 2004)) with 126 million $m^3 yr^{-1}$, (Masciopinto *et al.* 1999; Master Plan 2002), concern has arisen regarding groundwater quality and public health. Indeed the average frequency of the risk factor for drinking wells (or spring water) determined by Mele *et al.* (1997) among all the hepatitis A virus (HAV) cases recorded in Italy from 1986 to 1994 was classified as second only to mussel consumption.

At the end of 1997, as a consequence of the increase in outbreaks of gastrointestinal disease in the region, which showed an incidence of HAV cases several times the national average value, the Italian Government initiated the emergency plan for public health protection, which is still in effect.

In general high incidence levels of such diseases reflect multiple routes of primary and secondary exposure, one of which may be contaminated drinking water. This work explores the latter possibility, focusing on the potential relation between tap water and groundwater quality in the Salento region. In fact, the groundwater monitoring results presented in this paper reveal the presence of pathogenic microorganisms in some wells at the Nardò aquifer.

In the Salento area, the drinking water distributed by the local community is drawn from wells and conveyed in large tanks (150,000 m^3) where it is mixed with minor volumes derived from artificial lakes. Then the water is chlorinated prior to distribution in households. However numerous houses along the coastal area of the Salento use private wells directly for drinking water, without disinfection.

In the last 5 years, the regional emergency government completed 13 municipal wastewater tertiary treatment plants (their total production has been estimated to be 76,876 $m^3 d^{-1}$ during winter and 70,516 $m^3 d^{-1}$ in summer) but their real efficacy in terms of the reduction of groundwater contamination has not yet been verified. The integration of the treatment plants was also recommended by the regional Master Plan (2002). Epidemiological data, which considered the principal oral-faecal disease outbreaks

in three sub regions of the Salento peninsula, are reported in Figure 2. The data were collected by the Virological Department of Bari (Italy) University (Lopalco 2002) in the period 1999–2002 and by the Regional Epidemiological Observatory (OER 2004) in the period 1996–1998. The data were grouped to consider the whole Salento population, which has a homogeneous alimentary diet; in fact, in general the local population not only eat raw shellfish (mussel) but also drink water withdrawn from wells. All diseases recorded by the local health agencies (LHA) were arranged by considering the city of residence of every patient. Each LHA transmitted the number of cases to the regional coordinator office on a weekly (or monthly) basis. The sensitivity of the collected hepatitis data reported in Figure 2, ranges from 73.1% to 89.9% for the period 1997–1999 (Lopalco *et al.* 2001). A possible underestimation of 20% (on average) of cases reported should be considered, though Fiore (2004) suggested that the percentage of underreporting may rise by several orders of magnitude for HAV cases, as the disease can be asymptomatic. In order to obtain the specific disease incidence per 100,000 inhabitants, the sum of the pathologic cases recorded was divided by population for each zone; that is, for all 26 cities of the Ionian zone, 39 of the Adriatic Zone and 32 of the Lecce zone. The error bars in Figure 2 consider the standard deviations of the data collected. The hepatitis A outbreaks involving the whole Salento region in 1996–1997 were identified in epidemiological studies (Germinario *et al.* 2000; Chironna *et al.* 2002), though the reported HAV incidence in the peninsula (see Figure 2) fell rapidly below the national average value (20 per 100,000 inhabitants) in successive years. In fact, the number of cases of HAV infection in the Salento region fell in only two years from 10.1 to 1.2 per 100,000 inhabitants during the period 1997–1999. Although in recent years, the incidence of the infection cannot be viewed as a problem in the Salento region, it can be inferred from these results that the infecting particles may have been potentially transmitted by groundwater as one of the primary routes of exposure. This result was enhanced by higher incidences of endemic gastrointestinal diseases (typhoid fever) and HAV infections in the Ionian zone of the Salento peninsula supplied solely by wells (100%) (see Figure 2) with respect to the other sub domains of the same region, which were supplied by minor groundwater volumes.
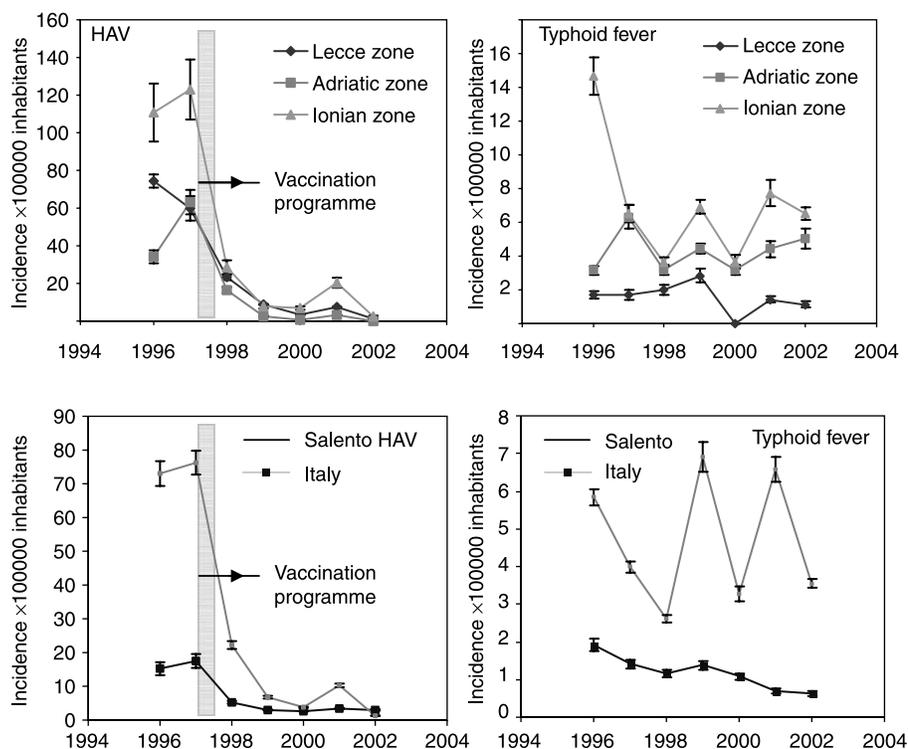
Figure 2 | Incidence of HAV and typhoid fever in the Salento sub regions (above) and comparison of the regional incidences with national values (bottom). The sensitivity of the collected HAV data in the Salento (southern Italy) was estimated to be equal to 80% by Lopalco (2002).

In order to ensure that established setback requirements for drinking water supplies i.e. a particle count inferior to 2×10^{-7} virus l^{-1} (Yates & Jury 1995) or similar (Regli *et al.* 1991; EPA-GWR 2000; Health Canada 2004), can be achieved by local community or private wells, adequate groundwater treatments should integrate chlorination before tap water distribution in the households of the Salento. Alternatively, appropriate setback distances must be imposed before pumping to ensure that natural filtration in subsoil, by means of soil aquifer treatment (Schijven *et al.* 2003), avoids failure of groundwater chlorine disinfection prior to distribution.

An experimental study has been carried out in the Salento peninsula to define the frequency of occurrence and number of pathogens in the drinking water that is potentially heavily influenced by untreated surface waters. The purpose of this work is to determine the appropriate distances between a sinkhole used for wastewater injection and consumer wells. As the average setback distance used by drinking wells at Salento is 500 m, laboratory tests in this work provide an assessment of the tap water quality when

groundwater is derived from a well 500 m from wastewater injection, and then hypochlorinated. Indeed, even though the inefficacy of using chlorine for wastewater disinfection treatment is documented in the literature (Tyrrell *et al.* 1995; Gehr *et al.* 2003; Jo *et al.* 2005), as well as its toxicity due to disinfection by-products, chlorination is still used by the local community as a disinfectant for drinking water distribution. Moreover, the minimum extent of setback distance that together with hypochlorination ensures safer tap water production in the houses supplied by wells has been determined at the Nardò aquifer on the basis of mathematical model simulations and groundwater monitoring results.

THE STUDY AREA

In the Nardò aquifer, at 8,000 m from the Ionian Sea coast (Figure 3), the groundwater is loaded by a channel (Asso) which collects superficial untreated waters due to rainfall (approximately 0.6 million $m^3 yr^{-1}$) and 3–4 million $m^3 yr^{-1}$ of secondary effluents derived from municipal wastewater treatment plants (Galatone and Galatina). The
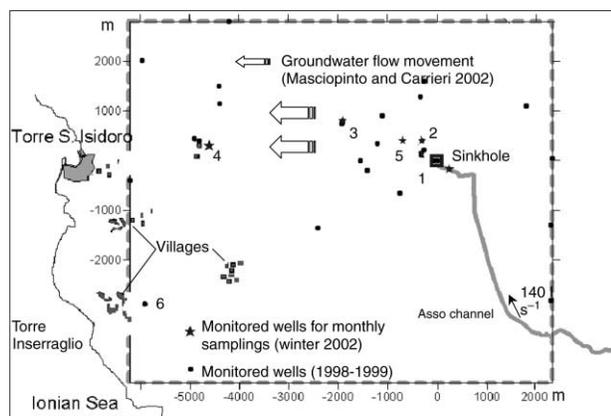
Figure 3 | Asso Channel, sinkhole and location of the affected community in the villages and Torre S. Isidoro town. The monitoring wells #1, #2, #3, #4 and #5 were selected for monthly sampling during winter 2002. Well #2 was used for the hypochlorination test. Well #6 was used for village tap water tests.

Asso channel has outflowed into a sinkhole since 1991 and artificial injection has succeeded in reducing the extent of seawater intrusion (Masciopinto & Carrieri 2002).

Hydrogeology

In the carbonate rock formation at Nardò (Cotecchia 1977; Del Prete & Caggiano 2003) the groundwater flows, under low pressure, inside the fractured Cretaceous (Senonian) limestone and the saturated aquifer thickness is equal to 30 m, on average. Along the coasts (e.g. Ionian and Adriatic) of the Salento region, the Calcare di Altamura (the fractured limestone) is onlapped by 5–10 m of Tertiary (Miocene) deposit known as Calcareniti di Gallipoli (sandstone) and, occasionally, is intercalated by lenses of terra rossa (calcspar) and loamy sand. The carbonate platform (limestone) contains freshwater floating on seawater of continental intrusion. The Nardò aquifer is very permeable everywhere because of great number of joints and fractures, caused by tectonic movements, that are karstified. The average ground level is about 35 m above sea level, approximately 8,000 m from the coast. The depth of the water Table is about 32 m below ground surface with a piezometric head equal to 3 m above the mean sea level. Groundwater salinity measured with a multi parametric probe (Ocean Seven) in wells, increased with depth below the water Table from 0.2 to 1.5 g l^{-1}, on average.

Numerous domestic wells in the houses and villages located along the coast, which accommodate many tourists during the summer season, draw water from the Nardò aquifer.

METHODOLOGY

In the Nardò aquifer sampling was carried out from June 1998 to March 1999 in order to monitor chemical constituents in the injected water and groundwater (Table 1).

Table 1 | Chemical constituents in injectant water and groundwater at the Nardò aquifer: mean values from eight wells (averages of monthly samplings from June 1998 through March 1999) at a distance less than 4,000 m from the sinkhole and downgradient with respect to groundwater flow

	Injectant water	Groundwater quality (1998/1999)		
	Mean	Min	Mean	Max
Electrical conductance (μS cm^{-1})	400	220	877	3,000
pH	7.75	6.5	7.3	7.9
T (°C)	17.8	14	15.4	17.7
N–NH$_4^+$ mg l^{-1}	Traces	0	0	Traces
N–NO$_2^-$ mg l^{-1}	0.03	0	0	0
N–NO$_3^-$ mg l^{-1}	8	0	6.8	18
Cl$^-$ mg l^{-1}	61	14	115	402
DOC mg l^{-1}	47	0	12	41
AOX μg l^{-1} as Cl$^-$	25.5	0	7	28

The analytical methods for chemical determination in this work are reported in specific manuals (*Standard Methods* 1995; CNR-IRSA 2003).

In order to reduce the number of water samples (i.e. lab analyses) for microbiological determination, selection of monitored wells was performed using a mathematical model (Masciopinto 1999). Assuming that (1) pathogens are transported with first-order adsorption and inactivation in a single fracture; (2) suspended particles (i.e. pathogens) migrate inside a fracture with the same velocity as the water; and (3) particle dispersion is negligible compared with the high interstitial velocity (pure advective transport), the Lagrangian approach suggests that pathogen transport is governed by the following set of ordinary partial differential Equations (Wood 1998):

$$\frac{dC(\mathbf{x},t)}{dt} + \frac{dC^*(\mathbf{x},t)}{dt} = -\lambda C(\mathbf{x},t) - \lambda^* C^*(\mathbf{x},t) \tag{1}$$

$$\frac{dC^*(\mathbf{x},t)}{dt} = r_1 C(\mathbf{x},t) - r_2 C^*(\mathbf{x},t) \tag{2}$$

with initial conditions (at $t = t_0$):

$$\mathbf{x}(t_0) = \mathbf{x}_0 \tag{3a}$$

$$C(\mathbf{x},t_0) = C_0 \tag{3b}$$

$$C^*(\mathbf{x},t_0) = 0 \tag{3c}$$

where C is the free pathogen count (PFU, or CFU, or MPN or IU) per unit of groundwater volume (l^3), $\mathbf{x}$ (l) is the particle pathway function (i.e. the function of all possible trajectories which a particle released at position $\mathbf{x}_0$ could follow to get to section $\mathbf{x}$), and t is time; λ (t^{-1}) and $\lambda*$ (t^{-1}) are the pathogen inactivation constants for suspended and adsorbed particles, respectively; r_1 (t^{-1}) and r_2 (t^{-1}) are the forward and reverse rate coefficients, respectively. For particle transport in porous and fractured media governed by both advection and dispersion the appropriate mathematical description has been presented by Sim & Chrysikopoulos (1995) and James & Chrysikopoulos (2000). Combining Equations (1), (2) and (3) yields a first-order differential equation that describes the metabolic decay of the pathogen particles suspended in the aqueous phase with accumulation (or elimination/inactivation) due to detachment (or attachment) from

(or to) the fracture walls:

$$\frac{dC(\mathbf{x},t)}{dt} = -\Omega C(\mathbf{x},t) \tag{4}$$

where Ω (t^{-1}) accounts for the pathogen inactivation constant. Under the initial conditions given by (3a), (3b) and (3c), Equation (4) can be integrated after separation of variables to yield

$$\ln \frac{C(\mathbf{x},t)}{C_0} = -\Omega t \tag{5}$$

and solving $C(\mathbf{x},t)$ leads to the desired expression:

$$C(\mathbf{x},t) = C_0 \exp[-\Omega t] \tag{6}$$

This inactivation constant Ω may be defined by Yates & Yates (1987) formula, as a function of groundwater temperature ((°C),

$$W = -0.66 + (0.054 \times T) \tag{7}$$

which produces $0.1\,d^{-1}$ at 14°C.

The groundwater flow problem in the Nardò fractured aquifer was solved under steady conditions after imposing suitable boundary conditions (Masciopinto *et al.* 1994). The mathematical model applied is based on a discrete fracture model (Nordqvist *et al.* 1996) made by a parallel set of horizontal fractures (Masciopinto 1999) using an anisotropic covariance matrix to reproduce the variable apertures of each fracture. Some adjustments to the model (Masciopinto 2005) were added to consider non-laminar flow resistances in fractures, owing to the presence of high velocity values. The piezometric heads in each grid node of domain were obtained by solving a system of equations imposing the continuity equation in each grid node. The code was successfully calibrated in a previous work (Masciopinto *et al.* 2000) by tracer (chlorophyll-A) tests on five wells drilled into the same fractured formation (*Calcare di Altamura*).

Collection and preparation of samples

The analytical methods utilised for microbiological determination (Barbuti *et al.* 1987; Aulicino 1989; Volterra &

Aulicino 1998) are also reported in specific manuals (EPA 1984; *Standard Methods* 1995; CNR-IRSA 2003).

Water samples of 20 l were pumped from wells and collected in sterile plastic containers. Each sample of 20 l was pre-filtered by a 10 μm polypropylene membrane (Gelman Sciences, Pall), at low virus adsorbent activity, in order to avoid successive filter obstructions. As virus particles can be retained on the filter, at the end of prefiltration the membrane was eluted (by shaking) using 3% beef extract (with NaOH to create a pH value of 9) for 10 min and then centrifuged at 1,500 × g for 30 min (Carducci *et al.* 1994). At the end the supernatant was buffered and added to a prefiltered sample of water (20 l).

Concentration procedure

The prefiltered sample was concentrated by means of two-step tangential flow ultrafiltration (Vivaflow 200, Vivascience Ltd, UK) in order to separate particles according to their molecular weight. The membranes are defined according to the nominal molecular weight limit (NMWL) (Lucena *et al.* 1991); that is, the cut-off limit of the molecular weight of the substances dissolved in the water sample which the membranes allow to pass through. The membranes polyethersulfone 10,000 Da (see for instance www.sartorius.com) were previously conditioned with beef extract (at pH 7) in order to minimise virus adsorption onto the filter membranes (Nupen *et al.* 1980). At the end of the first step of ultrafiltration, the concentrated sample (300 ml) was loaded with elution products of the membrane by using 3% beef extract (at pH 9). This sample was then re-concentrated with a Mini-ultrasette apparatus (Filtron Corp.) equipped with a similar membrane (Omega series, 10,000 NMWL; Filtron Corp.). The process was completed with a final eluting stage using 3% beef extract (pH 9), obtaining a concentrated sample of about 50 ml. The final sample (50 ml) was buffered and frozen at −70°C. The coliphage recovery efficiency in lab ultrafiltration was 92.4% (±3%), on average. The same technique was used in a previous work (Carducci *et al.* 1994) to collect enterovirus artificially added into 10 litres of seawater and the recovery efficiency ranged from 80 to 100%.

Somatic coliphage plaque assays

The double layer technique on 50 ml volume of lab sample (i.e. direct inoculation or concentrated water sample from 20 l) was performed by using 1.5% tryptic soy agar (TSA) as the bottom layer and soft TSA (0.7% of agar) as the top layer (EPA method 1601; 2001) on five large petri dishes (140 mm) at different dilution factors. The mutant strain of *E. coli* C ATCC#13706, which is nalidixic acid resistant, was utilised as host bacteria stock culture to detect somatic phi-X 174 stock coliphage (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) by means of the plaque method.

Clostridium spore and Pseudomonas aeruginosa enumeration

The water samples (100 ml) were heated before inoculation at 75°C for 15 min to eliminate vegetative cells and activate spore germination. After refreshing, the samples were inoculated by a selective medium SPS (Sulphite Polymixin Sulphadiazine Agar, Oxoid) using the tube inclusion technique. This method, as it is based on the selective medium SPS, allowed the recovery of 92 ± 5% *Clostridium perfringens* spores of the total *Clostridium* sulfite reducing spores present in wastewater samples according to the results reported by ISTISAN (2002). First the sterile SPS medium was melted and placed in a water-bath at 52°C to avoid medium solidification before use. Next, each tube was inoculated with 10 ml of the sample and was left to solidify at 20°C under anaerobic conditions by adding, on the solid forming surface, 4–5 ml of sterile Vaseline. The tubes were then incubated at 37°C for 24 h, after which the spores with a diameter over 3 mm were counted. Finally the tubes were incubated for a further 24 h and the spores were counted once more for confirmation.

The detection and enumeration of *P. aeruginosa* was carried out by membrane filtration method using a selective Cetrimide agar. The suspected colonies were confirmed by means of specific biochemical tests (ISTI-SAN 2000).

Virus assay

Two combined laboratory techniques were used here. The first, for qualitative presence/absence of enterovirus in the groundwater, was confirmed by a direct RT-nested PCR (reverse transcriptase polymerase chain reaction) carried out at Pisa (Italy) University Laboratory (Department of Experimental Pathology, BMIE), as described in Carducci *et al.* (2003) (Muscillo *et al.* 1999, 2001). The second, based on cell monolayer growth methods, was carried out in both Pisa and Bari (Hygiene, Internal and Preventive Medicine Department, DIMIMP) University Laboratories.

At Pisa Laboratory the PCR determination was performed on concentrated water samples preventively chloroform-treated to reduce bacteria and fungal contamination (Divizia *et al.* 1989) and, as required by the standard procedure (*Standard Methods* 1995, microbiological examination 9510 G), each sample was loaded by a pool of antibiotics at final dilution 1:20 (penicillin G 100,000 units ml^{-1}; streptomycin 120 mg ml^{-1}; Kanamycin 10 mg ml^{-1}; nystatin 3.2 mg ml^{-1}) and kept for 2 h at 37°C. The viruses were pelleted primarily by addition of PEG 6000 (10% weight/volume and NaCl 1.5%) and successively centrifuged (10,000 × g for 1 h at 4°C), then resuspended in 1,000 μl of Eagle's Minimum Essential Medium (EMEM, 2% fetal bovine serum) that was subjected to nucleic viral acid extraction. The water samples which were positive to PCR were also tested by cell method at Pisa, for quantitative estimations. The virus unit count was expressed as most probable number of infecting units (MPNIU) (Chang *et al.* 1958).

At Bari Laboratory cell determination was carried out for differential enterovirus analyses on three cell lines: BGM (continuous line derived from African Buffalo Green Monkey kidney cells) (Sattar & Westwood 1978; Dahling & Wright 1986) which are sensitive to poliovirus, echovirus, coxsackie virus B, reovirus and adenovirus; Hep-2 (human epidemoid larynx carcinoma) sensitive to enterovirus and adenovirus; and L20B (a genetically engineered mouse cell line) expressing the human poliovirus receptor (supplied by ISS Virological Laboratory in Rome). After the antibiotic treatment as described above, all the samples were centrifuged and seeded into cell culture monolayers grown in 25 cm^2 flasks and incubated for 90 min at 37°C and 5% CO_2.

Then the inoculum was removed and the flasks were incubated in a maintenance medium (EMEM, 2% fetal calf serum) at 37°C, 5% CO_2 for 7 days. During this period the flasks were checked daily with an inverted microscope, in order to estimate the cytopathic effect (CPE) from 1 + to 4 + (WHO 1993, 2001) by recording the percentage of cells affected by viral degeneration and monitoring possible effects due to cell toxicity or bacterial contamination. The lack of CPE in the second set of cultures was taken to indicate the absence, in the sample, of any virus detectable by the host. Wherever the CPE was confirmed, the centrifuged material was analysed by means of virological test identification. The virus typing procedure used was the microtitre neutralisation test (microtitre plate) by means of specific antisera (supplied by WHO) with virus log titration (i.e. the Kärber method (Hoskins 1975)). At Bari University Laboratory the viral load was estimated as the quantity of virus in a specific volume (i.e. 25 μl) that infected 50% of cell culture microplate wells. In other words, the quantity of viruses in a specified suspension volume (e.g. 25 μl) that will infect 50% of a number of cell culture microplate wells or tubes, is termed the cell culture infectious dose 50 [$CCID_{50}$].

Hypochlorination method

In this test 40 litres of prefiltered groundwater were concentrated by means of tangential flow and ultra-filtration. The concentrate (40 ml) was buffered and used to prepare groundwater samples at assigned coliphage counts of, respectively, 230, 350, 1,000 and 2,300 PFU 100 ml^{-1} and 10, 60, 100, 300 and 5,500 CFU 100 ml^{-1} of *Clostridium* spore counts. The water samples were used in order to determine the rate of viral indicator inactivation by adding sodium hypochlorite 0.269% (in weight) stock solution at different concentrations in order to have chlorine residuals of 2.5, 5 and 10 mg l^{-1} as HOCl or OCl- depending on the dose in the water samples, at assigned contact times. The maximum concentration of sodium hypochlorite used by local treatment plants for drinking water disinfection is 50 mg l^{-1} of 10% (in weight) stock solution (see for instance: www.oppo.it/home-calcoli.htm) whereas the Italian rule established a maximum concentration of 0.2 mg l^{-1} as total residual chlorine in the tap water. The recommended chlorine dosage in the groundwater is 2 mg l^{-1} as NaClO,

on average. In the laboratory tests, after 2, 5, 25, 35, 60, 100 and 300 minutes sodium thiosulphate (10%) was added in order to inactivate chlorine at different levels of concentration.

RESULTS

Simulations of groundwater flow and pathogen pathways

The model simulated the steady conditions that occurred during winter 2002, of frequent drought periods under a recharge flowrate of 140ls^{-1} (or 4.4 million m^3yr^{-1}) into the sinkhole. The estimated groundwater velocity was 50 m d^{-1} on average. The piezometric contour heads (in metres above sea level) (Figure 4) in the area around the injection site increased their initial values (i.e. without injection) by about 1.5 m. The groundwater discharge into the Ionian Sea increased from 102ls^{-1} to 208ls^{-1} with injection. The groundwater storage increased by 24% of the recharge flowrate ((140 – 106)/140). Subsequent results of the model were the identification of the area of subsoil which is dominated by the plume of wastewater injected in the sinkhole.

    The code determined the apparent pathogen pathways in fractures (see Figure 4) due to waste injection by using the particle tracking technique. As the pathogen particles preferred to follow the trajectories with higher discharge, simulation results presented in Figure 4 show the channelling effect (Moreno & Neretnieks 1993) on wastewater particle pathways. They did not take all possible radial flow directions around the injection site but principally followed two directions: SE-NW and NE-SW. Consequently the injection of wastewater only partially affected groundwater stored around the sinkhole. This result allowed four wells (#1, #2, #3 and #4 in Figure 4) out of the 16 total wells to be selected, located at progressive distances from the sinkhole and downgradient with respect to the pathogen migration direction SE-NW. One more lateral well (#5), at 600 m from the sinkhole and unaffected by the apparent wastewater plume (see Figure 4), was selected for background values of the groundwater quality. During simulation 2,000 particles were released into the inlet position (the sinkhole) and followed along their pathways. The elapsed time required to get to stationary particle counts at the observation wells were stored in the code as the term of filtration (see Table 2). When inactivation is negligible, and sorption/desorption and chemical inactivation are absent, the cumulative predicted counts in the observation wells are a function of the total number of collected particles. In order to take into account pathogen inactivation during simulation, the
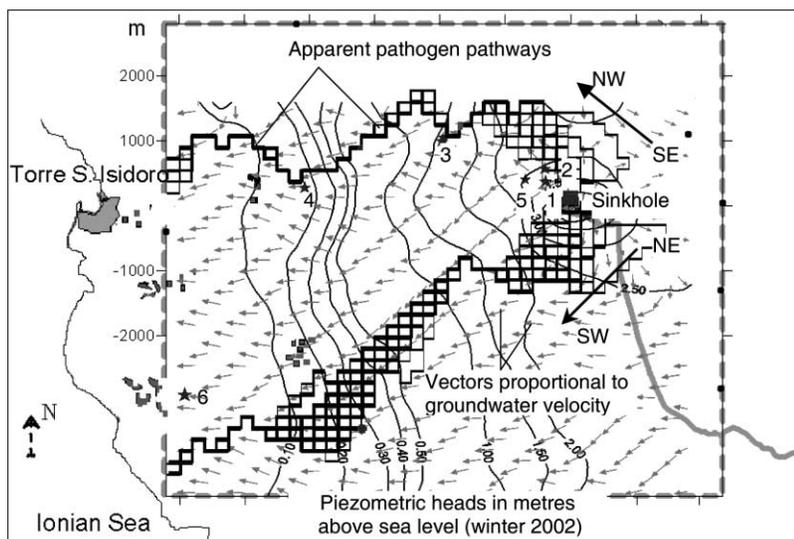

Figure 4 | Simulation results (Masciopinto & Carrieri, 2002): vectors of groundwater flow, piezometric heads and apparent pathogen pathways due to continuous injection of 140 l s^{-1} of 2^y municipal effluents into the sinkhole (recharge operation started in 1991) which allowed the selection of monitoring wells #1, #2, #3, #4 and #5 (the latter, unaffected by wastewaters, was used for background values) on the canalised plume direction SE-NW.

Table 2 | Pathogen indicators and viral infecting units in contaminated water samples of the Nardò aquifer (averages during winter 2002) in the monitored wells downgradient with respect to the groundwater flow and located at progressive distances from the sinkhole. Well #5 was used as background because it proved to be unaffected by wastewater injection into the sinkhole

	Sinkhole	Well #1 320 m	Well #2 500 m	Well #3 3,000 m	Well #4 5,000 m	Well #5 600 m
No. of samples	5	5	6	4	6	3
Term of raw water filtration in groundwater (days)*	0	3	10	65	250	–
Pathogen indicator:						
Bacterial count 22°C CFU ml^{-1}	170,000	80,000	20,000	150	100	262
Total coliforms MPN 100 ml^{-1}	410,000	408,000	26,000	3	50	50
Faecal coliforms MPN 100 ml^{-1}	400,000	400,000	1,200	3	4	8
E. coli MPN 100 ml^{-1}	25,000	800	150	0	0	0
Faecal streptococci MPN 100 ml^{-1}	3,400	3,500	400	0	2	0
Clostridium spores CFU 100 ml^{-1}	400	500	40	14	60	4
Somatic coliphages PFU 100 ml^{-1}	80,000	12,000	100	0	0	0
Viruses (IU l^{-1})**: RT-PCR	P$^+$	P	P	P	LP°	LP
Cell method:	2,860	4,600	2.9	2.9	2.9	2.9

*Time given by the code to achieve stationary count in each well without pathogen inactivation.
** Groundwater samples tested at Department of Experimental Pathology of Pisa (Italy) University.
P$^+$: positive.
LP°: low positive.

pathogen mass-particle quantity that was determined with the model each time was reduced according to the exponential decay Equation (6) (Yates & Jury 1995; Adelman *et al.* 1998). The inactivation constant Ω in this work was determined by computational code as the value which performed the best fit of the measured pathogen counts at the observation wells (i.e. stationary values in Table 2) and, consequently, it accounts for all the attenuation phenomena that may take place in reality during natural filtration in a fractured subsoil from the sinkhole to the observation wells.

Monitoring well results

In Table 2 the average counts of microbiological indicators in groundwater samples that resulted positive during winter 2002 (i.e. from November 2001 to May 2002) are reported. The Pisa lab detected a high number of virus infecting units

in well #1 and its injectant water. In the other wells (#2, #3 and #4) the IU were equal to the background value (i.e. 2.9 MPN l^{-1}). The PCR, in contrast, also gave positive tests for water derived from wells #2 and #3, whereas in well #4 the PCR gave results equal to the background (#5). At Bari University Laboratory (DIMIMP) in order to perform differential viral analyses, multiple cell lines were cultured as above and they were purchased and stored following two different protocols. This lab technique proved very time consuming and costly. Among the 18 analysed water samples only three were positive to enteric viruses. In particular, two positive samples were derived from the well 500 m from the injection (see well #2 in Figure 3); the third positive sample was derived from the well at 3,000 m (#3). In February/April 2002 in samples from well #2, echo 11 viruses at $10^{-5.5}$ CCID$_{50}$ 25 μl^{-1} and poliovirus (PV) 2-3 at 10^{-4} CCID$_{50}$ 25 μl^{-1} were detected. Indeed, in April 2002

in a sample derived from well #3, PV 3 at $\mathbf{10^{-5}}$ $CCID_{50}$ $25\,\mu l^{-1}$ were detected.

The groundwater pathogenic indicator count allowed us to estimate the distance required for natural pathogen inactivation in the Nardò fractures. The cell method detected a background count of IU (2.9 MPN l^{-1}) in well #2 after only 10 days of filtration (see Table 2), whereas the PCR continued to detect 'low positive' samples after 250 days of filtration into subsoil (i.e. 5,000 m from the sinkhole). This result agreed with positive PV 3 detection in well #3, at Bari University Laboratory.

Pathogen inactivation in the subsoil (see the Methodology section) was estimated by the transport model on the basis of the best fit between expected and measured values (i.e. stationary values in Table 2) at the observation wells. The expected infecting units of viruses, which gave us the best fit of detected values, were obtained by using the inactivation constant equal to $0.1\,d^{-1}$ in the model simulations. This inactivation rate agrees with the value defined by the Yates and Yates formula (see Equation (7)), when groundwater temperature is considered constant and equal to 14°C.

The $0.1\,d^{-1}$ is also within the range of values suggested by Yates & Jury (1995) for virus inactivation in groundwater and confirmed the resistance of viral particles in water (Keswick *et al.* 1985; Jiang *et al.* 2001).

On the basis of the model results, the inactivation constant of the faecal coliform ($0.08\,d^{-1}$) was close to the above-mentioned values for virus inactivation. The inactivation constants for streptococci, *E. coli* and somatic coliphages were also found to be close to $0.1\,d^{-1}$. The *Clostridium* spore counts also confirmed that a long time is required for inactivation in fractures (over 65 d). *P. aeruginosa* was not detected in the lab samples on any occasion. Only one water sample deriving from well #3 (3,000 m from the sinkhole) was contaminated by *P. aeruginosa* at a low count (68 CFU $100\,ml^{-1}$) and in the absence of other faecal indicators.

The experimental results reported above show that the setback distance in the studied aquifer is over 3,000 m and far exceeds the 1,036 m ($= 3,400$ ft) suggested by Abbaszadegan (2001) for similar geological formations used for drinking water supply in the United States. The setback distance also exceeds both the mean distance ($= 141$ m or 462 ft) reported by EPA (1997) for community groundwater systems

and the value ($= 200$ m or 656 ft) imposed by Italian law (D. Lgs. 258 2000).

A correlation between somatic coliphages and virus particles (infecting units) was attempted by a regression analysis of the results in Table 2. If a log-log transformation is applied, the following linear equation results:

$$\log (\mathrm{IU/L}) = 0.081 \times 2 \log PFU/100\,\mathrm{ml} - 0.1 \qquad (8)$$

while by considering *Clostridium* spores-viruses count we obtain:

$$\log (\mathrm{IU/L}) = 2.88 \times \log \mathrm{CFU}/100\,\mathrm{ml} - 9.4 \qquad (9)$$

Equation (9) was very similar to that determined by Payment & Franco (1993) (i.e. number of enteric viruses $= 2.65 \times$ number of colonies of *C. perfringens* $- 7.68$) for the same correlation on unfiltered and filtered river water samples of 100 l. The correlation coefficient determined by these authors was equal to 0.65 (with high significance level ≤ 0.001; i.e. the probability that the slope regression line is 0 is very low showing a good linearity). The correlation coefficients in this paper for the above Equations (8)–(9) were, respectively, 0.92 (with a small significance level $= 0.028$, associated with the slope, i.e. the linearity is uncertain) for somatic coliphages and 0.97 for *C. perfringens* spores (with a good significance level $= 0.005$ associated with the slope). The above-mentioned correlations were also documented by a factorial analysis carried out with SPSS (1995) statistical software, which extracted only one factor composed by variables log(IU/L), log(PFU $100\,\mathrm{ml}^{-1}$) and log(CFU $100\,\mathrm{ml}^{-1}$) with very high correlation coefficients (i.e. 0.99, 0.98 and 0.96, respectively). Additionally, the factorial analysis carried out by considering all the pathogen indicators (see Table 2) in groundwater samples together with chemical groundwater constituents (see Table 1), water temperature and pH, extracted only two principal components which were representative of viruses and water quality information. The results of the latter analysis would suggest that Equation (9) is better than Equation (8) to predict virus IU in groundwater because of the higher affinity of *Clostridium* spores, with respect to somatic coliphages, in order to explain the IU variability in groundwater samples.

Hypochlorination tests

The results of the hypochlorination tests are reported in Figure 5 and in Table 3, in terms of chlorine contact time (min). Even though the inefficacy of using chlorine for wastewater disinfection treatment is documented in the literature (Tyrrell *et al.* 1995; Gehr *et al.* 2003; Jo *et al.* 2005), chlorination is still used by the local community as a disinfectant for drinking water distribution. Shin & Sobsey (2003) showed the efficacy of ozonation in 4 $\log_{10}$ virus removal from drinking water. In this paper the resistance of *Clostridium* spores and somatic coliphages to chlorination was useful in testing the effectiveness of this groundwater treatment process currently used in the Salento. Therefore well #2 (see Figures 3–4) positioned 500 m from the sinkhole and downgradient with respect to the groundwater flow, was selected for the hypochlorination test. The physical and chemical groundwater constituents detected in well #2 (Table 4) are commonly observed in other wells of the Salento region.

Hypochlorination reduced somatic coliphages and *Clostridium* spores in groundwaters but did not achieve complete inactivation in all tests (see Figure 5). The latter indicator, after an initial decay (80%), showed a residual count (about 30 CFU 100 ml^{-1}) which was very resistant to chlorine disinfection, even at a high concentration of sodium hypochlorite (10 mg l^{-1} as residual chlorine) and
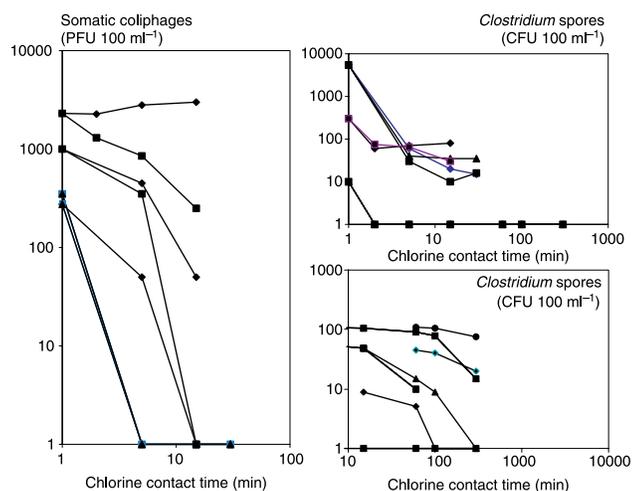

Figure 5 | Results of hypochlorination tests (somatic phages anti-*E. coli* (left) and *Clostridium* spores (right)) in contaminated groundwater derived from well #2 (500 m from injection), under different residual chlorine concentrations of, respectively, 2.5 mg l^{-1} (—◆—), 5 mg l^{-1} (—■—) and 10 mg l^{-1} (—▲—).

120 min of contact time. In addition, these residual counts did not correlate with the initial *Clostridium* spores count for values above 10 CFU 100 ml^{-1}. Indeed a low count of *Clostridium* spores (2–10 ufc 100 ml^{-1}) was found in almost every well of the Nardò aquifer. In contrast, hypochlorination (at 2.5 mg l^{-1} as residual chlorine) performed a complete disinfection of groundwater samples where there was an initial *Clostridium* spores count ≤ 10 CFU 100 ml^{-1}. In order to confirm the chlorine disinfection inefficacy in removing viral indicators in contaminated sites, the tap water in a house supplied by well #6 (see Figures 3–4), positioned at 8,000 m from the sinkhole and close to a village (Torre Inseraglio) along the Ionian Sea coast, was also analysed. For this test two samples of 40 l of tap water resulted positive for the presence of residual levels of *Clostridium* spores and somatic coliphages (see Table 4). Due to the relatively great distance of well #6 from the sinkhole (8,000 m), the existence of other groundwater pollution sources (i.e. wastewater spreading or injections) can be assumed. Here, as shown by electrical conductance values in Table 4, the water derived from well #6, after a reverse osmosis conventional treatment, was mixed again (in a ratio of about 50%) with hypochlorinated natural water thus reaching good salt concentrations in the finished water but also pathogen contamination from the untreated water.

Moreover, it has been established (Rebhun *et al.* 1997) that humic and fulvic acids, which may be included in the dissolved organic carbon (DOC) in groundwater (see Table 4), cause the formation of disinfection by-products such as trihalomethanes (THM), haloacetic acids and total dissolved organic halogens (DOX), which are carcinogenic (Aggazzotti *et al.* 2004; Vinceti *et al.* 2004). Following Rebhun *et al.* (1997), in secondary nitrified effluents a 1.2 mg dose of chlorine (or 0.34 of weight ratio Cl/C) yielded an average DOX/DOC of 49 μg Cl per mg C, and, THM formation ranged from 13 to 56%, on average.

HEPATITIS A RISK FACTOR DUE TO DRINKING WELLS

A realistic dynamic model to assess microbial health risks (Eisenberg *et al.* 2004) should take into account all risk

Table 3 | Log_{10} inactivation of contaminated groundwater after given contact time during hypochlorite treatment with different (residual) concentrations of chlorine

	Initial count	Residual chlorine mg l⁻¹	Mean of log_{10} count reduction after:							
			2'	%	5'	%	15'	%	30'	%
Somatic coliphages PFU 100 ml⁻¹	2,300	2.5	1.5	1.3	0.0	0.0	0.0	0.0	0.0	0.0
		5	3.0	43.5	3.2	63.0	3.3	89.1		
	1,000	2.5			2.7	55.0	3.0	95.0		
		5			2.8	65.0	3.0	100	–	
	350	2.5			2.5	100				
	275	2.5			2.4	81.8	2.4	100	–	
		5			2.4	100				
Clostridium spores CFU 100 ml⁻¹	5,500	2.5			3.7	98.9	3.7	99.6	3.7	99.7
		5			3.7	99.5	3.7	99.5	3.7	99.7
		10			3.7	99.3	3.7	99.4	3.7	99.4
	300	2.5	2.4	80.0	2.4	76.7	2.3	73.3		
		5	2.4	75.0	2.4	78.3	2.4	90.0		
	70	2.5			1.3	28.6			1.6	62.9
			60'	%	100'	%	300'	%		
Clostridium spores CFU 100 ml⁻¹	60	2.5	1.2	25			1.6	66.7		
		5	1.7	83.3						
		10	1.6	75			1.8	100	–	
	10	2.5	0.7	50	1	100	–			
		5	1	100	–					

factors associated with primary and secondary routes of HAV transmission, such as shellfish consumption, travel to high–medium endemic areas, contaminated food and water, and intravenous drug use. In this paper the risk factor estimation focused on the fraction of HAV cases in the Salento, which are predominantly waterborne. On the basis of the results of chlorination tests, a dose of $2\,mg\,l^{-1}$ as sodium hypochlorite in the water can fail to disinfect water when it is withdrawn from a well at a distance of 500 m from wastewater injection. For this setback distance it can be assumed that chlorine cannot perform complete groundwater disinfection (see Figure 5) and virus particles can be found in groundwater. The expected viral concentration in contaminated water, correspondent to $30–40\,CFU\,100\,ml^{-1}$ of residual count of *Clostridium* spores, can be determined by Equation (9), which gives $3.1\,IU\,l^{-1}$. Though the viral indicator occurrence in tap water is not unusual (Payment 1997; WaterTech Online

Table 4 | Water constituents and cultural indicator counts in the contaminated well #2 of the Nardò aquifer (see Figure 3) before and after hypochlorination, and tap water derived from well #6 after partial (50%) reverse osmosis and final hypochlorination

Groundwater constituents	Well #2	Well #6	Tap water from well #6 after treatment	Drinking water requirement (D. Lgs. 31, 2001)
pH	7.6	7.6	7.3	6.5–9.5
Electrical conductance $\mu S\ cm^{-1}$	551	3700+	1400	2500
COD (chemical oxygen demand) mg l^{-1} O_2	14	10	5	5
DOC mg l^{-1}	6.3	0	0	0.5
$N-NH_4^+$ mg l^{-1}	0	0	0	–
$N\text{-}NO_x$ mg l^{-1}	4	13.5	6	–
NO_3^- mg l^{-1}	3.8	13.5	6	50
NO_2^- mg l^{-1}	0.2	0	0	0.50
AOX $\mu g\ l^{-1}$ as Cl^-			< 1	< 30

Microbiological indicators:	Well #2			
	Before	After hypochlorination		
Total count 22°C CFU ml^{-1}	14,400	0*	5	100
Total count 37°C CFU ml^{-1}	4,800	32	30	20
Total coliforms MPN 100 ml^{-1}	6,300	0	0	0
Faecal coliforms MPN 100 ml^{-1}	700	0	0	–
E. coli MPN 100 ml^{-1}	0	0	0	0
Faecal streptococci MPN 100 ml^{-1}	34	0	0	0
Clostridium spores CFU 100 ml^{-1}	56	–	0	0**
Clostridium spores on concentrated 40 l sample CFU 100 ml^{-1}	5,500	15	35	–
Somatic coliphages PFU ml^{-1}	0	0	0	0**
Somatic coliphages on concentrated 40 l sample PFU ml^{-1}	3,120	0	65	–

+The values above the limits required by Italian regulation are underlined.

*Groundwater sample filtered in the lab.

** This determination is not required by Italian regulation in the absence of wastewater injections.

2002), 3.1 IU l^{-1} is several orders of magnitude over the EPA limit for drinking water. EPA-GWR (2000) assumes the water source to be contaminated when the viral pathogens are at a concentration of 3.56×10^{-3} virus l^{-1}. As the 3.1 IU l^{-1} count is about 1,000 times above the EPA limit, it may produce contamination in the Salento community consistent with the observed rates of illness presented in the introductory section of this work. On the basis of a daily intake for community water supply of $0.7\,l\,d^{-1}$ equal to the 50th percentile (see EPA-GWR

2000: Appendix A-5), with an exposure of 350 days per year, the individual annual probability of HAV infection can be estimated by means of the exponential model (Rose & Gerba 1991; Gerba & Rose 1993; Crockett *et al.* 1996; Crabtree *et al.* 1997; Lopez-Pila & Szewzyk 2000, 2001; Haas 2001):

$$P_{\text{Daily}} = 1 - \exp\left(-\frac{N}{\beta}\right) \rightarrow P_{\text{Annual}} = 1 - \left(1 - P_{\text{Daily}}\right)^{350} \quad (10)$$

where N is the daily IU of HAV ingested with water. In this work N was assumed equal to 10% of the daily intake (i.e. $3.1 \times 0.7 \times 0.1 = 0.22$ HAV l^{-1}) and β ($= 1822.9$ HAV $\times l^{-1}$) is the inverse constant probability of initiation of infection by a single HAV per 1 l (i.e. 1,000 g) of water drunk, derived from the Haas & Eisenberg (2001) (Eisenberg *et al.* 2004) risk assessment study. The estimation of the number of cases of HAV in the Ionian region was obtained by using the NRC (1983) method:

$$\sum_{\text{HAV}} = Pop \times P_{Annual} \times M_p \times (1 + M_S)$$
$$\times (1 + M_D) \times I_{cw} \times (1 - I_m)(1 - U) \quad (11)$$

where:

Pop	population exposed (subdivided by age group in the Ionian zone) in 2001
M_p	morbidity factor for primary route of illness as a result of infection
M_s	morbidity factor for secondary route of illness as a result of infection
M_D	morbidity factor for additional cases due to treatment failure in disinfected system and private distribution system sources
I_{cw}	percentage of wells contaminated in the community distribution system
I_m	percentage of population immunity
U	frequency of HAV underreporting cases in the database of notified infectious disease (in order to fit the notified cases)

The primary morbidity coefficient (M_p) was assumed variable by age group of the Ionian zone population (i.e. log-normal distribution with modal value 0.14), on the basis of de Almeida *et al.* (2002) estimations. The secondary and

additional morbidity factors were considered constant (i.e. $M_S = M_D = 0.1$) whereas the fraction ($I_{Cw} = 5 \pm 2\%$) of contaminated supplied water was assumed by local specific conditions in the community distribution system. Though the sensitivity of reporting (i.e. the frequency of underreporting) of HAV data in Puglia was estimated at 20% by Lopalco *et al.* (2001), this value was updated to 25% to consider asymptomatic diseases (Armstrong & Bell 2002), whereas the natural immunity to HAV was considered variable by age group and ranging from 15 to 50% (Canada Communicable Disease Report, Health Canada 1995; Germinario *et al.* 2000). Moreover, the vaccine immunity was considered equal to 100% for the population by age group < 4 years and 80% for age groups from 10 to 19 years, which have been considered target groups for vaccination programmes started in 1997 in order to prevent hepatitis A in Italy.

Equation (11) was implemented in a Microsoft Excel worksheet by considering the annual HAV probability of 4.1%, as defined by Equation (10) and the 2001 population data of the Ionian zone of Salento. The total HAV cases estimated in the region are reported in Figure 6 (histogram) together with the HAV cases ($= 50$) notified in 2001 by the regional database of infectious disease, subdivided by age group of the Ionian population. A good agreement between the observed and expected trend of HAV cases can be noted and the number of total cases ($= 19$) estimated by Equation (11) was enough (37.5%) to confirm that tap water might be
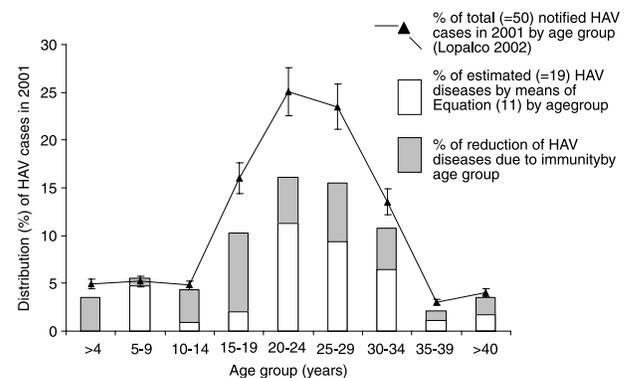

Figure 6 │ Distribution (%) of hepatitis A infection by age group in the Ionian zone of the Salento (Southern Italy): comparison between notified cases (—▲—) reported by database of infectious diseases in 2001 (Lopalco, 2002) and estimated illness (histogram) given by considering tap water as a primary route of HAV exposure.

considered as one of the primary routes of HAV exposure for the Salento population.

DISCUSSION

The experimental measurements and mathematical model predictions suggest that the minimum inactivation distance for pathogens in wastewater injected in the Nardò fractured aquifer is variable with groundwater flow direction around the sinkhole. The distance of 500 m can usually be considered acceptable but, along two preferential directions (SE-NW and NE-SW) around the injection site, it must be increased to 3,000 m. This distance also allows the removal of DOC, ammonia and other faecal contaminants as a result of biodegradation phenomena (Carrieri & Masciopinto 2003). The setback distance, as suggested by Yates & Yates (1987), is a function of groundwater temperature and was higher than both the 200 m (= 656 ft) fixed by Italian regulation (D. Lgs. 152 1999) and the mean distance for the average community groundwater system reported by EPA (1997). Moreover the setback value at the Nardò aquifer far exceeds the 1,036 m (= 3,400 ft) suggested by Abbaszadegan (2001) for similar geological formations used for drinking water in the US, only in the two above-mentioned directions.

The cell method implemented at Bari University, isolated PV 2-3 and Echo 11 viruses in the contaminated groundwater derived from a well 500 m from injections and PV 3 in one of five samples taken from a well 3,000 m away. The well 500 m from the injection site remained contaminated by *Clostridium* spores even after hypochlorination treatment at a high dosage. The water sampled 5,000 m from water injection resulted negative to virus presence (by PCR and cell methods) and showed indicator counts equal to background values. Though the PCR test compared with cell methods showed more immediate results (Divizia *et al.* 1989; Abbaszadegan *et al.* 1998; Panà & Divizia 1999; Abbaszadegan 2001), the cell method was necessary to detect infectivity of the sampled groundwater. The former method, even in qualitative form, found positive water samples at 3,000 m from injection. In contrast, according to a constant inactivation of 0.1 d^{-1} for viral indicators (i.e. *Clostridium* spores and somatic coliphages), the cell method did not find viral infecting units above the background

value (2.9 IU l^{-1}) in sampled water collected 3,000 m from the injection, even if one sample resulted positive to PV 3. The correlation between the selected viral indicators and IU detected in groundwater collected at progressive distances from injection, gave us very high correlation coefficients (0.93 and 0.99, respectively) whereas the factor analysis suggested a better affinity of virus IU with *Clostridium* spores, with respect to somatic coliphages.

The laboratory results confirmed the inefficacy of hypochlorination as a method for producing safe drinking water (WHO 2004) when the pumping well is sited in the contaminated plume direction and 500 m from waste injection (well #2). The risk assessment carried out on the basis of an infective dose of ingested HAV equal to 10% of total expected virus IU detected in the same well (i.e. 3.1 IU l^{-1}), confirmed that groundwater can be considered as one of the primary routes of HAV infection in the Salento region as it could have caused 37.5% of the total HAV cases notified in 2001.

In contrast, hypochlorination performed complete water disinfection for the well located in the contaminated plume direction and 3,000 m away from injection, where pathogens together with organic compounds, ammonia and other faecal contaminants were reduced to minimum concentrations by the natural filtration in subsoil and biodegradation.

CONCLUSIONS

The experimental study carried out at the Nardò aquifer shows that untreated water injection may affect groundwater quality even at a notable distance (3,000 m) from the pollution source only in restricted canalised areas (i.e. the contaminated plume) along two preferential directions (SE-NW and NE-SW), around the injection site. Otherwise a distance of 500 m can be considered acceptable and also allows the removal of DOC, ammonia and faecal contaminants by biodegradation phenomena. The estimated inactivation time for virus and pathogenic cultural indicators was 65 d. The numerous cavities and fractures of the carbonate rock formation allow the pollutants released in the soil or subsoil to spread rapidly into the groundwater. This percolation makes the Salento aquifer highly vulnerable to anthropogenic pollution which should be uniformly

controlled. As a result, a public health risk might arise in the Salento due to polluted water distribution when and where the pumping wells located in the plume direction do not respect the above setback distance of 3,000 m from pollution sources, even if the water is chlorinated prior to distribution. Indeed, the hypochlorination test on the polluted water samples taken from a well 500 m from the waste injection, confirmed the incomplete water disinfection even at high chlorine dosages. The presence of *Clostridium* spores in tap water should be considered significant and warrants immediate investigation. Here an appropriate method of water treatment, which may include coagulation, sedimentation and filtration (or ultrafiltration), should be considered by the local community and privately owned wells, in order to integrate natural filtration and avoid waterborne disease outbreaks. The integration of wastewater treatments before injection into a sinkhole is also recommended. Indeed, the epidemiological risk factor estimated in this work confirmed the possibility that polluted groundwater affected disease incidence in the Salento peninsula during 2001 as one of the primary routes of HAV infection.

In contrast, hypochlorination (at $2.5 \, \text{mg} \, \text{l}^{-1}$ as residual chlorine) performed complete disinfection of groundwater samples where there was an initial *Clostridium* spores count ≤ 10 CFU $100 \, \text{ml}^{-1}$. Low concentrations of pathogen indicators together with the absence of DOC, ammonia and other faecal contaminants can also be achieved in wells downgradient with respect to the wastewater plume direction but beyond the above defined setback distance into the Salento aquifer. This distance (3,000 or 500 m depending on the direction), established by mathematical model simulations at the Nardò site according to a $0.1 \, \text{d}^{-1}$ pathogen inactivation constant, is the minimum requirement together with chlorination, necessary to achieve safe tap water production from drinking wells in the Salento region.

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