Outbreak of unusual Salmonella enterica serovar Typhimurium monophasic variant 1,4 [5],12:i:-, Italy, June 2013 to September 2014

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Introduction

Monophasic variant of Salmonella enterica subspecies enterica serovar Typhimurium (monophasic S. Typhimurium), with antigenic structure 1,4 [5],12:i:-, appears to be of increasing importance in Europe. In Italy, monophasic S. Typhimurium represented the third most frequent Salmonella serovar isolated from human cases between 2004 and 2008. From June 2013 to October 2014, a total of 206 human cases of salmonellosis were identified in Abruzzo region (Central Italy). Obtained clinical isolates characterised showed S. Typhimurium 1,4 [5],12:i:- with sole resistance to nalidixic acid, which had never been observed in Italy in monophasic S. Typhimurium, neither in humans nor in animals or foods. Epidemiological, microbiological and environmental investigations were conducted to try to identify the outbreak source. Cases were interviewed using a standardised questionnaire and microbiological tests were performed on human as well as environmental samples, including samples from fruit and vegetables, pigs, and surface water. Investigation results did not identify the final vehicle of human infection, although a link between the human cases and the contamination of irrigation water channels was suggested.

Monophasic variant of Salmonella enterica subspecies enterica serovar Typhimurium (monophasic S. Typhimurium), with antigenic structure 1,4 [5],12:i:-, is considered an emergent pathogen in many European countries [1]. It accounted for 4.6%, 7.2% and 8.6% of total cases of salmonellosis reported to the European Surveillance System (TESSy) in 2011, 2012 and 2013 respectively [2], and ranked third among the Salmonella serovars identified in humans in the European Union (EU) during this period.

Human infection is usually acquired through the consumption of contaminated food and several food-borne outbreaks caused by this serovar have been reported in Canada, Europe and the United States (US) [1-3]. Fresh beef and pork account for the major sources of infection, but dried sausages and frozen chicken pies have also been implicated in food-borne outbreaks, as well as vegetables and juices. The majority of monophasic S. Typhimurium isolates detected in live animals originates from pigs [3]. Human outbreaks potentially related to environmental sources, including water, have been also observed [4-8].

In Italy, the laboratory-based surveillance network for enteric pathogens EnterNet Italia [9] provides information on the microbiological characteristics of Salmonella spp. strains isolated from humans. The data are gathered through a network of regional reference laboratories which characterise the strains isolated from peripheral diagnostic laboratories. The EnterNet Italia network is coordinated by the National Reference Laboratory for Salmonella infection in humans of the Istituto Superiore di Sanità (ISS).

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According to EnterNet data, monophasic S. Typhimurium represented the third most frequent Salmonella serovar isolated from clinical samples in the country between 2004 and 2008 [10], ranking second in 2009 [11]. It accounted for 2.95 isolates per 100,000 population/year between 1980 and 2011, with particularly high isolation rates in children aged one to five years [12]. Strains characterised by resistance to ampicillin, streptomycin, sulfafurazole, and tetracycline (ASSuT) (with or without additional Resistances) represented 75% of all the monophasic S. Typhimurium isolates from either 2008 or 2009. Forty-eight percent of strains belonged to DT193 and 13% to U302. The most common pulsed field gel electrophoresis (PFGE) profiles were STMXB 00131 (47%) and STMXB 0079 (37%) [3].

In October 2013 a significant increase of human cases of salmonellosis in L’Aquila province (Abruzzo region, Central Italy) was reported to EnterNet Italia. In the period between June and October 2013 only, ca 30 salmonellosis cases, corresponding to 9.9 cases per 100,000 population [13], were observed in the L’Aquila province, clearly exceeding levels of previous recent years. For example, between 2005 and 2009, the average number of salmonellosis cases notified in the province was equal to nine per year, with a maximum value of 30 annual cases in 2005 [14]. The vast majority of cases observed in L’Aquila province during 2013 and 2014 was due to monophasic S. Typhimurium. Isolates from patients’ stool samples were indistinguishable by traditional typing methods, such as phage type, PFGE and multilocus-variable number tandem repeat Analysis (MLVA) and were solely resistant to nalidixic acid, which had never been observed in Italy in monophasic S. Typhimurium neither in humans nor in animals or foods [15]. According to TESSy, monophasic S. Typhimurium with this peculiar antimicrobial profile had also rarely been reported in the EU.

Here we present the results of the epidemiological, environmental and molecular investigations carried out in the Abruzzo region in 2013–2014. The aims of these investigations were to gather relevant information on exposures and to identify the potential sources of infection so as to allow adequate control measures.

**Methods**

**Case ascertainment and case definition**

Following the observation of the significant increase of human cases of salmonellosis in L’Aquila province (Abruzzo region, Central Italy), an alert was sent to all public health services of Abruzzo region in October 2013. Prospective and retrospective active finding of cases was conducted for the period from January 2013 to October 2014, involving hospitals in Abruzzo as well as microbiological laboratories in the whole Italy through the EnterNet Italia surveillance network.
The outbreak strain was defined as: ‘monophasic S. Typhimurium resistant to nalidixic acid, with a PFGE profile equal to the reference type XBAI.0027 and MLVA 3-14-11-NA-211 encoded by the molecular surveillance service (MSS) of the European Centre for Disease Prevention and Control (ECDC)’.

Definitions of possible, probable and confirmed cases were established on the basis of phenotypic and genotypic characteristics of the Salmonella isolates, and the existence of a possible epidemiological link with Abruzzo region (people living in Abruzzo or reporting to have travelled to Abruzzo in the seven day-period before the disease onset). A confirmed case was a patient epidemiologically linked to Abruzzo, who was laboratory confirmed with the outbreak strain from January 2013 onwards. A probable case was a patient epidemiologically linked to Abruzzo, who was laboratory confirmed with Salmonella group B from June 2013 onwards. A possible case was a patient with gastroenteritis epidemiologically linked to Abruzzo from June 2013 onwards, but who was not laboratory confirmed with Salmonella.

An international alert was also launched through the European Epidemic Intelligence Information System (EPIS) in April 2014.

Epidemiological investigations
From October 2013, cases were requested to respond to a standardised questionnaire. This was administered by a face to face interview of either the patients or, if they were younger than 18 years-old, their parents. Until the end of March 2014 the questionnaire was generic and included only information on demographic data and presence of clinical symptoms. Starting from April 2014, a more elaborate version was used to also collect information on the occurrence, duration and severity of clinical symptoms, healthcare seeking behaviours, and exposures possibly presenting a risk for Salmonella infection in the seven days before symptoms onset. These included exposure to possible animal reservoirs, contact with people with gastrointestinal illness, as

Figure 2
Geographical distribution of outbreak cases (n = 115) and of surface and sewage water samples (n = 21) with a monophasic Salmonella Typhimurium outbreak strain isolate, L'Aquila province, Italy, April–October 2014

*Although there were a total of 179 outbreak cases in the Abruzzo region, only 115 are depicted in this figure because for 64 cases the residential address was missing information.
well as travel, details on food and beverages consumed including their purchase places, and the consumption of meals out of home. Cases were also asked about the consumption of local handmade food products of animal and vegetable origins.

The implementation of a case–control study was originally considered but could not be realised due several difficulties which are further discussed.

Data from the questionnaires were entered into a database using Epi Info 7 (Centers for Disease Control and Prevention, Atlanta, United States (US)). Data management and analysis were performed using Stata 12 (Stata Corporation, Texas, US). Data derived from the 2011 demographic census performed by the Italian National Institute of Statistics (ISTAT) were used to calculate the attack rates [16]. The possible existence of a relationship between the age of cases and both the duration of clinical symptoms and disease severity was assessed by the calculation of Kendall's rank correlation coefficient.

Environmental investigation
Given the temporal and spatial extension of the outbreak, a possible implication of environmental sources was suspected. Accordingly, the regional health authorities established a microbiological monitoring plan in April 2014. This included sampling of all commercial pig herds of L’Aquila province (faecal, dust, drinking water and feed samples were taken in each herd), and the examination of ileocaecal lymph nodes taken from swine at slaughterhouses to assess the status of *Salmonella* infection in pigs bred and slaughtered in the province. In addition, locally produced fruit and vegetables, mainly fresh vegetables grown in open fields and intended to be consumed raw, were directly collected in a subset of producing farms. These farms were selected on the basis of the results of surface water sampling, which was performed as further described. In each cultivated field at least eight samples of fruit and vegetables, with the minimum weight of 2 kg and chosen to be representative of the whole cultivated surface, were taken. Overall, 23 pig herds, 11 swine slaughterhouses and 17 fruit and vegetable-producing farms in L’Aquila province were investigated.

The monitoring plan also concerned surface water. Channels used for irrigation (including at farm sites) were sampled as well as surface water from three main catchment basins of the Tevere, Liri-Garigliano and Aterno rivers and their tributaries. Nine sewage water
treatment plants were additionally subjected to microbiological controls on fluids entering and leaving the plants. The sampling programme was performed until the end of October 2014.

Daily data on rainfall covering a period from September 2013 to June 2014 [17] were obtained from the Centre of Excellence for the Remote Sensing and Forecast of Severe Weather (CETEMPS) of L’Aquila University and a preliminary analysis was conducted to assess the possible association between rainfalls and the occurrence of salmonellosis cases, using the Pearson’s correlation coefficient. The correlation was evaluated through a cross-correlation approach by using two different time lags: the time shift between rain and cases detection and a time window used to average the quantities of rain along a period of time before the occurrence of cases. Data were aggregated using five days interval since this was considered the best temporal aggregation to summarise and analyse data on rainfall and on the occurrence of salmonellosis case. Only cases occurring in the L’Aquila municipality and in the neighbouring municipalities were included in the analysis, since available data on rainfall referred to that area. The analysis was performed using the R free package [18].

Characterisation of strains
Biochemical identification of isolates was conducted using the automated Vitek 2 system (Biomerieux, Marcy l’Etoile, France). The isolates were serotyped with commercial antisera (Statens Serum Institut, Copenhagen, Denmark) according to the Kauffmann–White scheme by slide agglutination. After having identified the somatic antigen and the phase-1 flagellar antigen, if the second one was negative, the phase inversion method was used to allow the expression of the second flagellar phase. Strains were assigned to *Salmonella* serovar Typhimurium monophasic variant 1,4,[5],12:i:- on the basis of a multiplex polymerase chain reaction (PCR) [19]. Antibiotic susceptibility of isolates was evaluated by disk diffusion method [20]. Phage typing was performed using *S.* Typhimurium phages according to the method described by Anderson et al. [21]. Strains were typed by PFGE and MLVA according to the ECDC Laboratory standard operating procedures [22,23].

The genomes of 20 respective strains matching the outbreak strain definition and isolated from human (n = 14) and environment samples (n = 6; five from sewage plants and one from surface water) were fully sequenced. They were chosen on the basis of geographical location, timeline isolation and biochemical properties. Moreover most of the strains chosen to be
fully sequenced were isolated from cases belonging to the same families, whereby cases belonging to a family lived in the same household. Three additional isolates, two unrelated outbreak monophasic S. Typhimurium isolates (differing from outbreak strain for antimicrobial resistance patterns) and a further strain isolated five years prior, were also sequenced and added as controls and outgroup respectively. Whole genome sequencing was performed by a Personal Genome Machine (PGM) Ion torrent platform. After quality trimming, retained reads were in the 40 to 350 nt length range, exhibiting an average phred score of 30 and providing an estimated coverage spanning from 18x to 45x for the selected isolates. Trimmed reads were submitted to the Sequence Read Archive (SRA) repository [24] under the Bioproject accession number SRP049581 and were used to build a single nucleotide polymorphism (SNP) reference-based matrix using the US Food and Drug Administration (FDA) SNP-pipeline programme [25]. The phylogenetic tree was built using the Tamura–Nei model in molecular evolutionary genetics analysis (MEGA)6 [26]. Confidence of branch points was estimated by Fast bootstrap analysis with 500 replicates.

**Results**

**Outbreak description**

A total of 206 cases (98 confirmed, 101 probable, 7 possible) were identified in Italy between June 2013 and September 2014 (Figure 1). No cases possibly linked to this outbreak were reported from the 14 countries (including the US) reacting to the EPIS alert.

Of the 206 cases, the place of residence was unknown for 19. Among those with available information, only eight cases were detected outside the Abruzzo region. In this region with a total of 179 cases, most (n = 146) were reported from the province of L’Aquila. The attack rate was 14 cases per 100,000 inhabitants in Abruzzo region (95% confidence interval (CI): 12–16), and 49 cases per 100,000 inhabitants in L’Aquila province (95% CI: 41–58). Information on patient’s age was collected for 192 cases and the median value was 4 years (range: 0–91 years). The age of cases significantly differed (Fisher’s test p < 0.01) from that of the Abruzzo population, taken as ‘control’ population, and the greatest number of cases (n = 98; 51%) occurred in patients aged between one and four years (Pearson’s chi-squared: 1,248; p < 0.01). No significant difference was observed by sex (53% males and 47% females).

Data on clinical signs were complete for 90 patients and the mean duration of illness was seven days (range: 1–30 days). Diarrhoea was the most important symptom (89/90; 99%), followed by abdominal pain (57/85; 67%), weakness (22/39; 56%), bloody diarrhoea (19/46; 41%), vomiting (32/86; 37%), fever defined as >37.5°C (28/92; 30%), nausea (13/77; 17%), and diffuse pain (4/35; 11%). Among symptomatic cases, 46 (51%) were hospitalised. The median age of hospitalised patients was four years (range: 1–91 years). No significant correlation was observed between the age of cases and the duration of the illness (Kendall’s coefficient = 0.03) or its severity (Kendall’s coefficient = 0.05). A man aged >70 years died following the S. Typhimurium infection.

**Epidemiological investigation**

Of a total 112 cases approached for interviews, 106 took part in the questionnaires, corresponding to a participation rate of 95%. Of these, 51 cases answered to the first generic questionnaire (48%) and 55 to the following more elaborate version (51%). Not all questions on clinical signs, travel history, animal or ill person

<table>
<thead>
<tr>
<th>Sampling place</th>
<th>Sample type</th>
<th>Number</th>
<th>Samples positive for the outbreak strain of monophasic Salmonella Typhimurium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pig herds</td>
<td>Dust</td>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Faeces</td>
<td>41</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Feed</td>
<td>34</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Drinking water</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>Farms</td>
<td>Bulb vegetables</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Root vegetables</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Leaf vegetables</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fruits</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Irrigation water</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Slaughterhouses</td>
<td>Swine ileocaecal lymph nodes</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Sewage treatment plants</td>
<td>Effluent water</td>
<td>111</td>
<td>9</td>
</tr>
<tr>
<td>Rivers and water channels</td>
<td>Surface water</td>
<td>191</td>
<td>11</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td><strong>498</strong></td>
<td><strong>21</strong></td>
</tr>
</tbody>
</table>
exposure, and on the food and beverage consumption were properly answered by all interviewed cases, thus the number of respondents for each question must be taken into account.

During the week before disease onset, eight of 49 respondents (16%) reported to have travelled. These eight cases resided in L’Aquila and had travelled outside the Abruzzo region (however, no cases travelled to the same place) and 24 of 95 (25%) respondents reported contacts with an individual presenting gastrointestinal illness. Contacts with pets were reported by 31 of 90 cases (34%).

In the week prior to the onset of the disease, 38 of 49 cases had consumed food outside their home: in school canteens (20/41), in restaurants (10/49), in bars (13/44), in fast foods (7/45), or in other places (8/23). As far as school canteens are concerned, the investigation was extended to verify possible common food suppliers and no common places or epidemiological links were identified.

The investigation on food eating habits did not reveal the consumption of any particular food considered at risk for Salmonella infection, apart from eggs and vegetables. Most of the cases reported drinking tap or bottled water, respectively 41/66 (62%) and 56/76 (74%).

Environmental investigation
All samples taken among the pig herds (n = 123), the fruit and vegetables produced by farms (n = 37), and in the slaughterhouses (n = 32) from April to October 2014 tested negative for Salmonella (Table). Monophasic S. Typhimurium isolates, fitting the definition of outbreak strain, were detected in 11 of 191 samples of surface water, in one of four samples of irrigation water taken on farms, and in nine of 111 samples of sewage treatment plants taken in several geographical locations of L’Aquila province (Table).

The positive sample of irrigation water was taken from irrigation pipelines with water from the Vera river, which is tributary of the Aterno river, and from which surface water samples also tested positive. The samples of fruit and/or vegetables taken from the fields irrigated with the contaminated water were negative for Salmonella. Monophasic S. Typhimurium outbreak strain was detected in five sewage treatment plants localised on the Aterno river and in one sewage treatment plant localised on the Raio stream flowing into Aterno river (Figure 2). The geographical location of 21 water samples testing positive for monophasic S. Typhimurium with the same characteristics as the outbreak strain is shown in Figure 2.

In addition, three further surface water and five sewage samples resulted positive for monophasic S. Typhimurium, but showing different phenotypic characteristics than the outbreak strain, while another 50 samples (34 of surface waters and 16 of sewage) were positive for other Salmonella serovars. The geographical locations of 302 sewage and surface water samples, which were microbiologically analysed as well as their testing results are reported in Figure 3.

The analyses performed to explore the possible correlation between rainfalls and the occurrence of salmonellosis cases gave a significant value of the Pearson’s coefficient (0.47) when an increase of the average value of rainfall over a 10 day period was considered and for a time shift between rain and cases detection of 20 days.

Characterisation of strains
Phylogenetic relationships among the selected isolates were investigated using the SNP matrix obtained from the whole genome sequencing. The single maximum parsimonious (MP) and maximum likelihood (ML) analyses resulted in a single tree with similar topology (Figure 4). The two strains not matching the definition of outbreak strain, clustered as a separate clade with high bootstrap support, and one of them differed from the antigenic profile (SAMN03162144), while the other exhibited a completely different antimicrobial resistance profile (SAMN03162146). In contrast, outbreak-related isolates were grouped in a well-separated clade from the other strains. However, genetic relationships among isolates (both from patients and water samples) inside the outbreak clade were supported by very low bootstrap values (range: 13–80%). Subclades with higher bootstrap values include both water and clinical-related strains.

Discussion
In the outbreak described here, cases were solely identified in Abruzzo region and occurred continuously and uninterruptedly from June 2013 to September 2014. The outbreak strain was repeatedly isolated in sewage treatment plants and surface water, including water used for irrigation in the region. A cross-correlation analysis between rain episodes and human cases moreover showed that an increased level of rainfalls (averaged over a 10 days period) preceded the onset of salmonellosis cases by ca 20 days. Whole genome sequencing phylogenetic analysis, albeit not robust, also indicated some relationships between clinical and water strains. To gain more information on whether outbreak strains could be of clonal origin, the phylogenetic distances among the outbreak isolates would need to be carefully evaluated taking into account the relative long period of time during which the human cases occurred and the subsequent higher probability of Salmonella population differentiation and heterogeneity.

Taken together, the results are in line with the hypothesis of a waterborne mechanism for the outbreak, and might suggest a possible relationship between heavy precipitations and the presence of Salmonella in surface waters, maybe via the flooding of sewage treatment plants, subsequently leading to contamination of irrigation waters. That floods, as result of intensive
precipitation events, bring pathogens from sewage water to surface water is a known phenomenon: heavy
rainfalls in a relatively short time can cause sewer overflows to surface water and/or soil, thus increasing
the risk of contaminating irrigation waters [27]. Here,
the contamination of surface and irrigation waters by
the outbreak strain could also have been due to prob-
lems with the treatment plants or to persisting damage
inflicted to water pipelines of the sanitary sewer sys-
tem of the city of L'Aquila and surrounding villages by
the devastating earthquake in 2009.

In the environmental investigation, the apparent absence of the outbreak strain in pigs reared in the
Abruzzo region and the negative microbiological
results in fruits and vegetables, may suggest the exist-
ence of yet undetected infection reservoirs, such as
wild animals. Nevertheless, given the time elapsed
between the possible occurrence of an environmen-
tal contamination and the collection of samples, the
results cannot definitely rule out pigs or vegetables as
sources of infection. Fruits and vegetables may be gen-
erally colonised by a wide variety of microorganisms and
Salmonella is usually associated to fresh produce
[28]. The occurrence of Salmonella in whole and fresh-
cut leafy greens has been reported [1] and outbreaks
due to S. Typhimurium, which may survive for extended
time periods in manure and manure-amended soils
[29], have been associated with the consumption of
fresh lettuce [30]. Rainfalls have also been proven to
play a role in Salmonella dispersal and contamination of
tomato plants in the field, especially during con-
centrated and relatively intense rain events and when
plastic mulch was used [6].

Aside from contamination via animal manure for fertili-
zation purposes, it is also well known that vegetables
can become contaminated with pathogens by irrigation
water [31]. In the US this was confirmed in repeated
incidents: a multistate outbreak of Salmonella Newport
infection associated with eating tomatoes in 2002 and
2005 [5], an E. coli O157:H7 outbreak associated with
shredded lettuce in 2006 [32] and the outbreak caused
by Salmonella Saintpaul, which was found in irrigation
water and in Serrano peppers [33].

A ban of using surface water to irrigate produce and
other crop due to the presence of Salmonella spp. in
some areas of L'Aquila province was already in force
since September 2013, before the peak of the out-
break. Unfortunately the ban was just applied to a
limited area of L'Aquila province. Only starting from
June 2014 did the local health authorities ban the use
of surface water for crop irrigation on a larger area
[34-36]. Sanitization interventions were subsequently
performed in water cleaning plants and no more
human cases were observed after September 2014.
The role of banning the use of surface waters for irri-
gation in the cessation of the outbreak can neverthe-
less not be ascertained and the possibility of multiple
transmission routes from the environment to humans,
involving food-vehicles, cannot be excluded.

The observed higher incidence in patients aged
between one and four years-old is not surprising in
Salmonella infections, given the increased vulnerabil-
ity of children and the elderly to this infection. On
the other hand, this finding could suggest the implication
of food vehicles normally consumed also by children or
the contamination of drinking water.

A case–control study would have added more signifi-
cant information on the possible source of this out-
break. Although originally considered and planned,
this study proved impossible. Indeed, the uncertain-
ties on the times of first exposures due to interventions
occurring late in the outbreak progress made it difficult
to find suitable controls. In addition, the realisation of
such a study was hampered by the extended geograph-
ical area involved.

During the period of the outbreak there was also an
increase of cases of Salmonella infections due to
Salmonella strains different from the outbreak strain.
Since laboratory diagnosis concerned solely Salmonella
spp. no other pathogen was investigated. The charac-
terization of all pathogens responsible for cases of gas-
troenteritis might have added more information on the
possible source of this outbreak and possibly further
supported the hypothesis of a waterborne mechanism.

The delays between generating hypotheses on the
contamination routes and implementing the sampling
programme and interviews of cases could also have
affected the identification of a food vehicle. This con-
firms once again the importance of having an effec-
tive epidemiological surveillance system in place, to
early identify all possible suspected clusters of infec-
tion and to quickly mobilise the human and diagnostic
resources for a rapid identification of sources and vehi-
cles of infection.

**Outbreak investigation group**

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Conflict of interest

None declared.

Authors’ contributions

FC: coordination of outbreak investigation, designed the questionnaire, data analysis, drafted the article. FB: designed the questionnaire, fulfilled the databases, data analysis, drafted the article. PC: coordination of outbreak investigation, drafted the article. EDG, LS, AMD, SO, NBB, CM, SS: laboratory typing on human and environmental samples. GS: coordination of outbreak investigation, drafted the article. MO: phylogenetic tree building, drafted the article. SI: Contributed data into the databases. IM: sequencing data analysis. LC: laboratory work on human samples. FDP, MG, MDL, GV: performed the environmental investigation. MG, CT: done the case interviews. All the authors: reviewed and approved the article.

References


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