Assessing natural mineral water microbiology quality in the absence of cultivable pathogen bacteria

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ABSTRACT

Italian Directives recommend the good quality of natural mineral waters but literature data assert a potential risk from microorganisms colonizing wellsprings and mineral water bottling plants. We evaluated the presence of microorganisms in spring waters (SW) and bottled mineral waters (BMW) samples. Routine microbiological indicators, additional microorganisms like *Legionella* spp., Nontuberculous mycobacteria (NTM), and amoebae (FLA) were assessed in 24 SW and 10 BMW samples performing cultural and molecular methods. In 33 out of 34 samples no cultivable bacteria $\geq$10 CFU/L was found. Cultivable FLA were detected in 50% of water samples. qPCR showed the presence of Legionella qPCR units in 24% of samples (from 1.1×10^2 to 5.8×10^2 qPCR units/L) and NTM qPCR units in 18% of samples (from 1×10^2 to 1×10^5 qPCR units/L). *Vermamoeba vermiformis* and *Acanthamoeba polyphaga* were recovered respectively in 70% of BMW samples (counts from 1.3×10^3 to 1.2×10^5 qPCR units/L) and 42% of SW samples (from 1.1×10^3 to 1.3×10^4 qPCR units/L). *Vahlkampfia* spp. was detected in 42% of SW and 70% of BMW samples (from 1.2×10^3 to 1.2×10^5 qPCR units/L). Considering the presence of FLA, we underline the importance of a wider microbiological risk assessment in natural mineral waters despite the absence of cultivable bacteria.

Key words | free living amoebae, legionella, natural mineral water, nontuberculous mycobacteria, qPCR, waterborne pathogens

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INTRODUCTION

European Regulation (EC) No 54/2009 (European Parliament 2009) defines the general principles and requirements of natural mineral water safety. Natural mineral water in its state at source may not be the subject of any addition of chemical products, as disinfectants, and may not be the subject of any treatment such as the separation of unstable elements, as iron and sulphur compounds, in order to not alter the original water composition and property in ions constituents.

During the last decades an increase of bottled mineral water (BMW) consumption was observed in Italy and over 200 liters are consumed yearly by every person on average (Altamore 2004). In the country there are about 140 companies bottling over 260 mineral water brands and the large production and consumption of natural mineral water is due to the high number of Italian springs and the good organoleptic quality of bottled water. Therefore, more than 30% of Italian people prefer to drink mineral water, despite being more expensive in comparison to domestic tap water (Istituto Nazionale di Statistica 2012). In Italy, the collection and distribution of natural mineral water is regulated by a Legislative Decree and Ministerial Decree (Legislative Decree 176/2011; Ministerial Decree of 10 February 2015) which establishes the evaluation criteria for geological, chemical, microbiological and pharmacological characteristics of natural mineral waters. Regarding the

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microbiological parameters spring water (SW) and BMW must be bacteriologically pure with a microbial community (environmental microorganisms) free from changes during the production cycle phases, from production to distribution and sale. Routine microbiological analysis performed in SW and BMW aims at ensuring the absence of *Escherichia coli* and further coliform bacteria, fecal streptococci, *Pseudomonas aeruginosa, Staphylococcus aureus,* sulfate-reducing bacteria, and further parasites or pathogenic microorganisms in cases of suspected contaminations (Legislative Decree 176/2011; Ministerial Decree of 10 February 2015).

Literature data (Bates *et al.* 2000; Kobayashi *et al.* 2014) suggest a widespread presence of other microbial hazards in SW and BMW such as non-tuberculous mycobacteria (NTM), *Legionella* spp., fungi, free living amoebae (FLA), etc. NTM and *Legionella* spp. are widely present in soil, freshwater, hot springs and replicates in a large range of temperatures (20–45 °C). These bacteria can adapt and resist to stressful environmental conditions because of their ability to enter in a viable but non-culturable state, showing a low metabolic activity despite their cell integrity (Oliver 2005). Under favorable conditions waterborne bacteria can recover their vegetative state and regain pathogenic potential within FLA, which are considered as natural primary hosts (Declerck *et al.* 2007; Chang *et al.* 2009). The World Health Organization classified several NTM species and *Legionella pneumophila* as emerging pathogens transmitted by water that may cause severe pneumonia and health-care associated pathologies, including skin and soft tissues infections, mostly in immunocompromised people (Pedley *et al.* 2004; World Health Organization 2011; Delafont *et al.* 2014). Gastrointestinal symptoms may be caused by water contaminated by FLA and in less common cases by *Legionella pneumophila* sg 1 (ISO11731 1998) and some NTM species as *Mycobacterium avium* (Pedley *et al.* 2004), which are capable of survival and growth in phagocytic FLA as *Acanthamoeba* spp. and *Vermamoeba* spp. with consequent increasing of virulence and resistance to environmental stress (Cirillo *et al.* 1997; Miltner & Bermudez 2000). Therefore, pathogens like *Giardia* spp., *Cryptosporidium* spp. and *Entamoeba* spp., are recognized as important waterborne disease pathogens and are frequently associated with severe gastrointestinal illness, while amoebiasis outbreaks have been reported, especially in South-American countries (Kumar *et al.* 2014; Faria *et al.* 2017). Moreover, skin and respiratory tract infections are linked to waterborne bacteria, fungi and virus present in bathing waters. In particular, old and immuno-suppressed people receiving bath thermal treatments with contaminated natural mineral water may be subjected to infection occurrences. *Pseudomonas aeruginosa* has proved responsible for cases of skin infections related to contact with spa water while *Legionella pneumophila* has been the cause of outbreaks of pneumonia when the water is nebulized in the form of aerosol for respiratory hydrotherapy (Leoni *et al.* 2015).

The aim of this research is to assess the microbiological quality of SW and BMW samples of different Italian brands, searching for microbial indicators and microorganisms not routinely searched according to current regulations.

METHODS

Setting

The study was performed during the period from April 2016 to November 2016 on 34 natural mineral water samples belonging to 11 different brands. Samples were subdivided for their productive state (SW and BMW). In detail, the research was performed on 24 SW and 10 BMW samples, which were classified according to their fixed residue at 180 °C (FR).

Therefore, 12 minimally-mineralized (FR $\leq$ 50 mg/L), 10 oligo-mineralized (FR = 51–1,499 mg/L) and 12 rich-mineralized (FR $\geq$ 1,500 mg/L) water samples were collected and analyzed for the routine microbiological test recommended by Legislative Decree 176/2001 and Ministerial Decree of 10 February 2015. Further microbiological tests as *Legionella* spp., NTM, and FLA were performed in the same SW and BMW samples.

SW samples were collected directly from the environmental springs, localized in Tuscany, Italy (Tuscan-Emilian Appennines, Apuan Alps, Val d'Orcia and Tuscan archipelago). BMW samples were collected at the storage zone, after the bottling procedure and before being transported to the points of sale. Temperatures of the storage zones were 10–15 °C. BMW samples were collected and analyzed 10 days after the day of production.

Table 1 shows details about the 11 water brands collected and analyzed at different steps of the production chain.

Routine microbiological and physical-chemical analysis

As suggested by Italian Regulations, 3 L each of SW and BMW samples were collected and mixed in a sterile container. The mixtures were analyzed for the detection of the total viable counts at 22 and 37 °C (ISO 6222 2001) in two 1 mL aliquots using Plate Count Agar (Oxoid Ltd, Basingstoke, Hampshire, UK) as medium. Coliform bacteria, *E. coli* (ISO 9308-2 2012) and fecal streptococci (ISO 7899-2 2000) were enumerated filtering two 250 mL aliquots using Colilert 250 Test (Idexx, US) and Slanetz Bartley Agar (Biolife, Italy), respectively. *Pseudomonas aeruginosa* (ISO 16266 2006) and *Staphylococcus aureus* (Superior Institute of Health 2007) were searched by one 250 mL aliquot using CN Pseudomoas Agar (Biolife, Italy) and Mannitol Salt Agar (Biolife, Italy) Sulfate-reducing bacteria (Superior Institute of Health 2007) were enumerated filtering one 50 mL aliquot using Sulphite Polymyxin Sulfadiazine (SPS) Agar (Biolife, Italy). Filtrations were performed through a 0.45 μm membrane (Nalgene, USA), which were layered on the respective culture media before being incubated at the proper temperature and for the requested period. At the same time, some physical-chemical water parameters such as temperature, pH, and conductivity were measured (Legislative Decree 176/2011; Ministerial Decree of 10 February 2015). For BMW samples temperature values reported in bottle labels were considered.

Table 1 | Information about the correspondence between the number and type of spring water (SW) and bottled mineral water (BMW) samples and their geographical position for each brand

Brands	Number of SW and BMW samples collected	Type of water	Geographical position
BRAND 1	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 2	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 3	2 SW; 2 BMW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 4	1 SW	Minimally-mineralized	Apuan Alps
BRAND 5	1 SW	Minimally-mineralized	Apuan Alps
BRAND 6	2 SW	Minimally-mineralized	Tuscan-Emilian Appennines
BRAND 7	4 SW; 4 BMW	Oligo-mineralized	Tuscan-Emilian Appennines
BRAND 8	2 SW	Oligo-mineralized	Tuscan-Emilian Appennines
BRAND 9	2 SW	Rich-mineralized	Tuscan archipelago
BRAND 10	2 SW	Rich-mineralized	Tuscan-Emilian Appennines
BRAND 11	4 SW; 4 BMW	Rich-mineralized	Val d'Orcia

Legionella spp. search

Legionella spp. isolation in SW and BMW samples was performed in accordance with standard procedures (ISO 11731 1998). One liter of water was filtrated through a 0.2 μm membrane (Millipore, Billerica, MA), which was subsequently immersed in 10 mL of the same water and sonicated for 5 minutes, allowing the detachment of cells from the membrane and their suspension in water. Suspension was subjected to a thermal inactivation treatment at 50 °C for 30 minutes with the aim to select *Legionella* spp., inactivating all microbial species not resistant to high temperature. Afterwards 0.1 mL of the suspension was seeded in triplicate on Legionella BMPA selective medium (Oxoid Ltd, Basingstoke, Hampshire, UK) and the plates were incubated at 37 °C for 7–10 days within jars with a modified atmosphere (2.5% CO_2). Suspected Legionella colonies grown on the medium were subjected to species and serogroup identification analysis using a multi-purpose latex agglutination test (Legionella Latex Test, Oxoid Ltd, Basingstoke, Hampshire, UK).

NTM search

SW and BMW samples of 1 L were centrifuged at 5.000 × g for 20 min. Pellets were suspended in 1 mL of sterile distilled water, and 0.1 mL samples were spread on the surface of Middlebrook 7H10 agar medium (BBL Microbiology Systems, Cockeysville, Md.) containing 0.5% (vol/vol) glycerol

and 10% (vol/vol) oleic acid–albumin enrichment. Plates were incubated at 37 °C and examined after 21 days of incubation. Following the incubation period the total number of colonies (from one to 15 per plate) was treated with an acid-fast staining (Falkinham *et al.* 2001).

From one to five acid-fast colonies per plate were identified by sequencing of the *hsp65* gene (439 bp). DNA was extracted using QIAamp DNA Mini Kit (Qiagen) and for each Polymerase Chain Reaction (PCR) 50 µL of mix were prepared with 31.25 µL of water; 5 µL of 10X PCR Buffer (15 mM MgCl2), 1 µL of dNTPs mix (10 µM); 1.25 µL of Tb11 (5′-ACCAACGATGGTGTGTCCAT) (20 mM); 1.25 µL of Tb12 (5′-CTTGTCGAACCGCATACCCT) (20 mM); 0.25 µL of HotStarTaq DNA Polymerase (5 U/µL); and 10 µL of extracted DNA (HotStarTaq DNA Polymerase, Qiagen, USA). The reaction was subjected to 45 cycles of amplification (1 min at 94 °C, 1 min at 60 °C, 1 min at 72 °C); followed by 10 min of extension at 72 °C (Telenti *et al.* 1993). Ten µL of the amplified PCR mixture was loaded to a 1% agarose gel with ethidium bromide. A 1.5 Kb ladder was used to compare amplified PCR product.

After an electrophoretic run, applied at 110 V for 30 minutes, *hsp65* gene amplification results were visualized in a UV transilluminator. Amplified *hsp65* gene was sequenced in outsourcing (GATC, Biotech, Germany) and sequence alignment was performed by BioEdit Version 7.0.0. Sequences identification was obtained by Basic Local Alignment Search Tool (BLAST) Database.

FLA search

To detect FLA cells, 1 L of SW and BMW samples was filtered through a 0.2 μm membrane (Millipore, Billerica, MA), which was suspended in 10 mL of Page's modified Neff's Ameoba Saline (PAS) (Page 1988). Three mL of suspension were centrifugated at 750 × g for 20 minutes and then 1 mL of sample was seeded on non-nutrient agar-*E. coli* ATCC 11229. After 2 hours, any excess liquid was gently pipetted off and the plates were closed in a polythene bag and incubated at 37 °C for 7 days. The cells were observed daily by inverted microscope with a 20× objective. Trophozoite plaques or outgrowths from deposit inocula were gently scraped from the plate and added to 1 mL of PAS (Health Protection Agency 2005). Differentiation of FLA strains was performed by Giemsa-Romanowsky staining (Vráblic *et al.* 1998) applied on 100 µL of suspension while the other 900 µL were analyzed by multiplex PCR assay to identified FLA strains. Briefly, DNA was extracted using a QIAamp DNA Mini Kit (Qiagen) and multiplex PCR on the *18S rRNA* gene was performed using two pairs of primers (Amo_1400_F5′ATGCCGACCARSGATYMGGAG3′/Amo_1540_R5′CAAGSTGCYMGGGGAGTCAT3′ and Vahl_560_F5′AGGTAGTGACAAGMYRTAGYGACT3′/Vahl_730_R5′GGGCGTTTTAACTACARCAGTATTA3′), which allowed the amplification of 130 bp fragment for Acanthamoeba DNA, a 50 bp fragment for Echinamoeba DNA and Hartmannella DNA, and a 150 bp fragment for Vahlkampfiidae DNA. The run was performed using the following protocol: initial denaturation step at 94 °C for 15 minutes, and then 35 cycles at 94 °C for 30 seconds, 62 °C for 1 minute, and 72 °C for 1 minute, followed by a final elongation step at 72 °C for 10 minutes (Le Calvez *et al.* 2012). Electrophoretic run and sequencing methods are described above.

qPCR tests

To perform the quantification of Legionella, NTM and FLA qPCR units, all SW and BMW samples were subjected to a qPCR, in accordance with the protocol of the SsoAdvanced SYBR Green Supermix (Bio-rad) using the CFX96 qPCR detection system (Bio-rad). DNA extraction (QIAamp DNA Mini Kit, Qiagen) was performed on 500 µL of concentrated water, which was prepared through filtration of 1 L of water as described for *Legionella* spp. search. To detect Legionella qPCR units the *mip* gene (558 bp) was amplified with one pair of primer (mip595R5′-CATATGCAAGACCTGAGGGAAC/mip58F5′-GCTGCAACCGATGCCAC). Briefly, 12.5 µl of Supermix were added to 5 µL DNA template in a 25 µL volume, with 0.3 µM of each primer. Reaction conditions were 98 °C for 2 minutes, followed by 40 cycles of 98 °C for 2 seconds, 55 °C for 20 seconds, and 72 °C for 20 seconds (Escmid Study Group for Legionella Infections 2012; Kim *et al.* 2015). NTM and FLA qPCR units were detected with the same protocols applied for qualitative PCR on *hsp65* and *18S rRNA* gene, respectively.

CFX96 qPCR detection system (Bio-rad) can detect and quantify the copy number of target genes present in 5 µL of

DNA sample and each samples and standard control were amplified in triplicate.

Results are expressed as qPCR units per litre (qPCR units/L). For *mip*, *hsp65* and *18S rRNA* genes the limit of detection (LOD) and quantification (LOQ) of the qPCR assay were 5 and 25 qPCR units/well, respectively. The presence of PCR inhibitors in extracted DNA was considered if there was no amplification of the internal control (standard gene). In case of inhibition, extracted DNA was diluted in sterile water (1:5 and 1:10) and then amplified again.

Standard curves were obtained by decimal dilutions of the plasmid, which was obtained by the cloning procedure of Legionella, NTM and FLA genes. Genes were amplified and cloned in a plasmid vector (pGEM-T Easy Vector System, Promega). Vector-insert ratio 1:3 was calculated by Biomath calculator program (Promega, Italy) and the insert was quantified by spectrophotometric reading at 260/280 and 260/230 nm. Bacterial transformations were obtained in *E. coli* JM HighEfficienty Competent Cells (Promega, Italy).

Statistical analysis

Correlation tests were performed and Pearson's coefficients were calculated with the aim of analyzing the correlations between physical-chemical parameters (temperature, pH, conductivity) and qPCR units belonging to *Legionella* spp., NTM, Amoebozoa and Vahlkampfiidae. These tests were independently applied for SW and BMW samples. Ninety-five per cent confidence levels were defined for the statistical tests. Therefore, we considered the following ranges of values: 0–0.3 (weak correlation); 0.3–0.7 (moderate correlation); 0.7–1 (strong correlation). The statistical analysis was carried out using the SPSS software package, version 17.0.1.

RESULTS

Routine microbiological and physical-chemical results

In all 34 natural mineral water samples, all microbiological parameters respected the limits suggested by Italian Regulations. Therefore, in SW samples we detected total viable counts at 22 °C lower than 20 CFU/mL (mean value 4.8 ± 1.3 CFU/ml) and total viable counts at 37 °C lower than 5 CFU/mL (mean value 1.2 ± 0.1 CFU/mL), respectively. Furthermore, in BMW samples total viable counts at 22 °C lower than 10^2 (mean value 14.9 ± 3.1 CFU/mL) and total viable counts at 37 °C lower than 20 CFU/mL (mean value 3.2 ± 0.7 CFU/mL) were always obtained. In accordance to the same Regulations, Coliform bacteria, *E. coli, Pseudomonas aeruginosa, Staphylococcus aureus* and sulfate-reducing bacteria were not isolated in natural mineral water samples. Physical-chemical results showed temperature values ranging from 10.7 to 42 °C. A mean pH value of 6 ± 0.24 was observed in water samples, while conductivity values ranged from 43 to 3,765 μS/cm. No statistically significant difference of physical-chemical results was observed between SW and BMW samples (Table 2).

Legionella spp. results

No viable and cultivable Legionella cells were isolated in SW and BMW samples. Legionella qPCR units were detected in two out of 24 (8%) and in seven out of 10 (70%) SW and BMW samples, respectively. Overall, qPCR showed the presence of *mip* gene in nine out of 34 (24%) samples, with a mean count of $2.9 \times 10^2 \pm 1.7 \times 10^2$ qPCR

Table 2 | Mean temperature, pH and conductivity values obtained in minimally-mineralized, oligo-mineralized and rich-mineralized spring water (SW) and bottled mineral water (BMW) samples

	Mean temperature (°C)	Mean pH	Mean conductivity (μS/cm)
Minimally-mineralized SW	11.30 ± 0.70	6.29 ± 0.10	76.70 ± 3.20
Oligo-mineralized SW	12.90 ± 0.13	5.80 ± 0.01	1104.00 ± 0.84
Rich-mineralized SW	35.20 ± 10.10	5.90 ± 0.05	3316.00 ± 476.00
Minimally-mineralized BMW	12.00 ± 0.70	6.00 ± 1.00	46.50 ± 3.00
Oligo-mineralized BMW	12.60 ± 0.14	5.80 ± 0.09	1110.00 ± 1.37
Rich-mineralized BMW	28.30 ± 11.70	6.10 ± 0.06	3195.00 ± 551.00

units/L. The highest percentage of positive *mip* gene amplification and the highest Legionella qPCR units/L counts were recovered in rich-oligo-mineralized bottled water samples. The amplification efficiency was mean SLOPE = −3.155 (±0.17); mean E = 91.2% (±0.82%). Figures 1 and 2 show Legionella qPCR units, conductivity and temperature values detected in 34 water samples.

NTM results

Cultivable NTM cells were not detected in natural mineral water, with the exception of 83 CFU/L of *Mycobacterium gilvum* isolated in one rich-mineralized SW sample (similarity value of 97.9%) having a temperature of 42 °C, pH 5.8 and conductivity of 3,740 μS/cm. These physical-chemical values represent the optimal environmental conditions for the NTM growth and colonization in wellsprings. Moreover, qPCR results showed the presence of NTM qPCR units in six out of 34 (18%) samples, with a mean count of $5.7 \times 10^3 \pm 4.1 \times 10^3$ qPCR units/L. In detail, the *hsp65* gene was amplified in three out of 24 (13%) SW samples and in three out of 10 (30%) BMW samples.

The highest percentage of positive *hsp65* gene amplification was detected in rich-oligo-mineralized bottled water samples while the highest NTM qPCR units/L counts were recovered in the rich-mineralized spring positive to cultivable *Mycobacterium gilvum*. The amplification efficiency was mean SLOPE = −3.123 (±0.14); mean E = 90.6% (±0.74%). Figures 1 and 2 show NTM qPCR units, conductivity and temperature values detected in 34 water samples.

FLA results

The cultivable method allowed the detection of viable FLA in 17 out of 34 (50%) of water samples analyzed, and showed shapes resembling trophozoites and cyst cells by inverted microscope with a 20× objective. Shapes were confirmed by Giemsa-Romanovsky staining. Identification of FLA was completed detecting the presence of Amoebozoa DNA *(Acanthamoeba polyphaga* and *Vermamoeba vermiformis)* and Vahlkampfiidae DNA *(Vahlkampfia inornata)* (similarity values from 97.2 to 98.1%).

qPCR results showed the amplification and sequencing of *18S rRNA* gene (50 bp) related to *Vermamoeba vermiformis* in seven out of 10 (70%) BMW samples with counts ranging from 1.3×10^3 to 1.2×10^5 (mean count of 3.9×10^4) qPCR units/L. Among these BMWs, two out of seven (29%) were minimally-mineralized water, further two out of seven (29%) were oligo-mineralized water and three out of seven (42%) were rich-mineralized waters. An

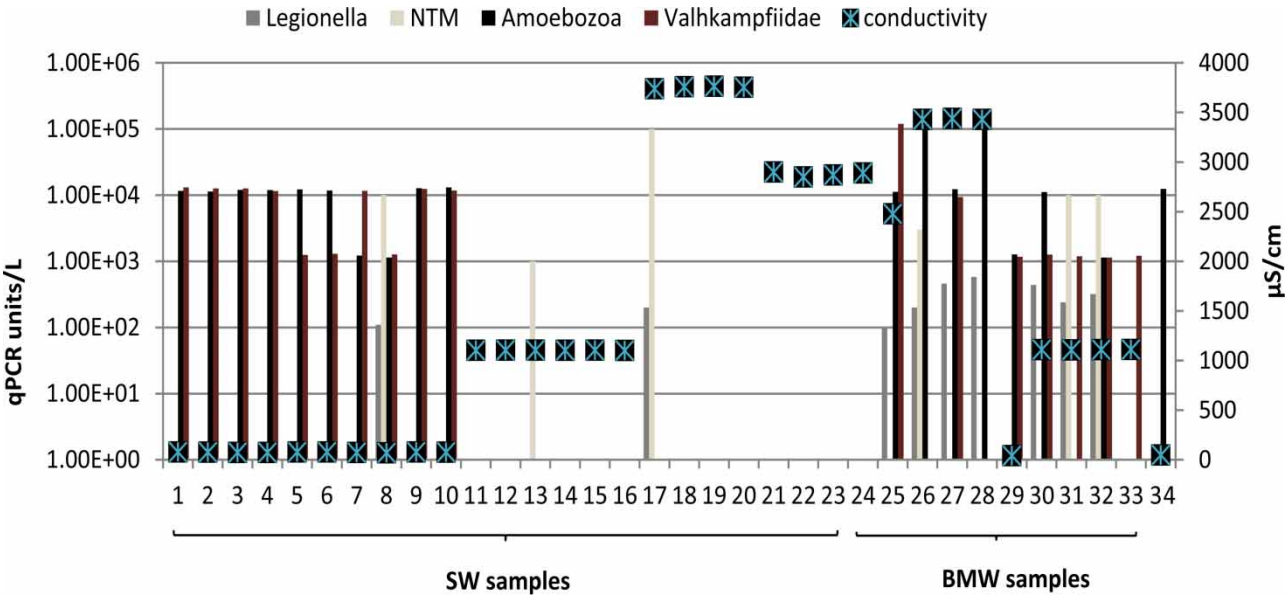

Figure 1 | Legionella, NTM, FLA qPCR units (qPCR/L) and conductivity values detected in 34 water samples.

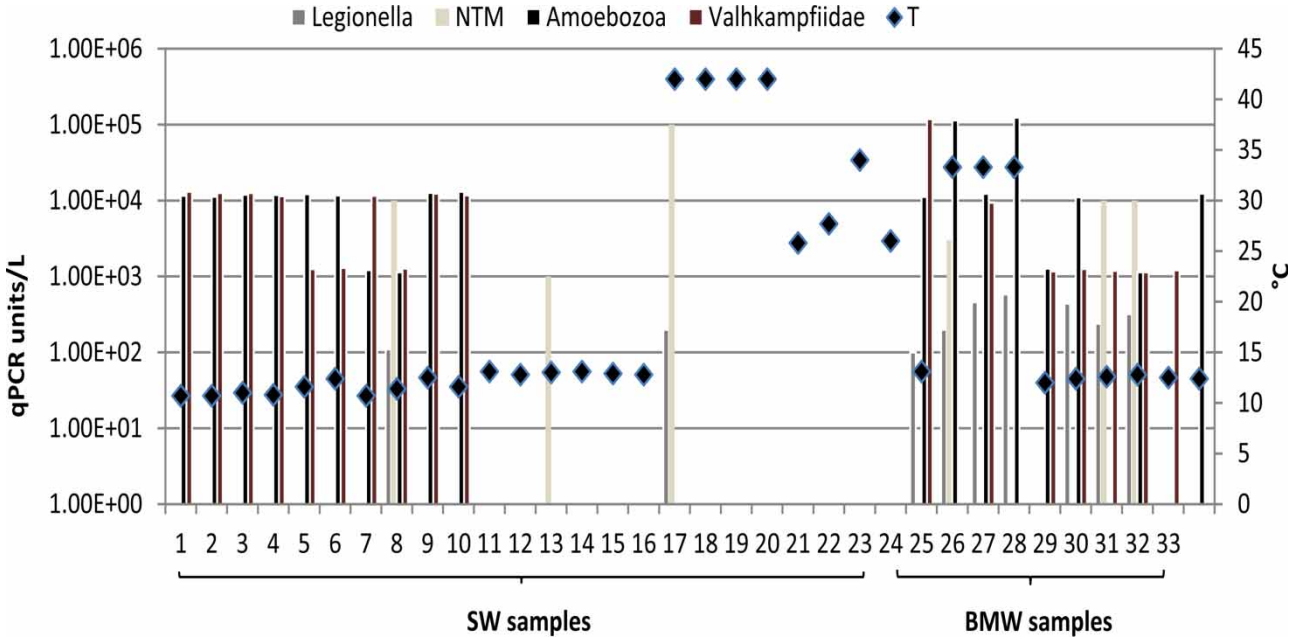

Figure 2 | Legionella, NTM, FLA qPCR units (qPCR units/L) and temperature values (T) detected in 34 water samples.

amplification and sequencing of *18S rRNA* gene (130 bp) belonging to *Acanthamoeba polyphaga* was recovered in 10 out of 24 (42%) SW samples with counts ranging from 1.1×10^3 to 1.3×10^4 (mean count of 9.9×10^3) qPCR units/L. All these springs were minimally-mineralized waters.

Furthermore, an amplification of *18S rRNA* gene (150 bp) belonging to the genus *Vahlkampfia inornata* was shown in 10 out of 24 (42%) SW and in seven out of 10 (70%) BMW samples, with a mean count of $1.3 \times 10^4 \pm 2.9 \times 10^3$ qPCR units/L.

The amplification efficiency was mean SLOPE = −3.261 (±0.21); mean E = 91.4% (±0.36%). Figures 1 and 2 show Amoebozoa and Vahlkampfiidae qPCR units, conductivity and temperature values detected in 34 water samples.

Statistical results

In SW samples statistical results showed moderate correlations between the physical-chemical parameters (conductivity; temperature) and the FLA qPCR units (r values from −0.788 to −0.221; p values from 0.003 to 0.027). In SW samples a strong correlation was detected between the Amoeboza and Vahlkampfiidae qPCR units (r values from 0.633 to 0.832; p values from 0.0008 to 0.013) and between the NTM and Legionella qPCR units (r values from 0.788 to 0.965; p values from 0.0009 to 0.012).

In BMW samples we detected strong correlations between the physical-chemical parameters (conductivity; temperature) and the Legionella qPCR units (r values from 0.588 to 0.844; p values from 0.009 to 0.02).

Moreover, a further strong correlation was detected for Legionella and Amoebozoa qPCR units (r values from 0.998 to 1; p values from 0.0009 to 0.0011), proving the strong relationship between these microorganisms in the environment.

The statistical correlations between the pH values and microbial qPCR units were not detected in SW and BMW samples (r values from 0.08 to 0.15; p values from 0.54 to 0.82).

DISCUSSION

Quality assessment of natural mineral water is needed to ensure safe water consumption. The Directives cited above represent the only legislative tool which requires the assessment and the management of water risk (European

Parliament 2009; Legislative Decree 176/2011; Ministerial Decree of 10 February 2015). These documents mention the possible microbiological hazards present in environmental habitats such as springs, soils, etc., ensuring a tight control of bottled waters. Microbiological parameters (total viable counts at 22 and 37 °C, *E. coli*, coliform bacteria, fecal streptococci, *Pseudomonas aeruginosa, Staphylococcus aureus* and sulfate-reducing bacteria) are considered for routine tests. On the other hand, the Italian Directives do not require the microbiological control of further microbiological hazards as Legionella, NTM, and other environmental opportunistic human and animal pathogens, which may colonize springs and bottling plants (Cabral & Fernández 2002; Bahrami *et al.* 2013). The growth of these microorganisms could be due to the presence of biological reservoirs and FLA, which are considered to be natural hosts of waterborne bacteria. These pathogens can benefit from symbiosis for replication, spread (amoebae are vectors of *Legionella* spp. and NTM), protection, virulence and resuscitation of viable non-culturable cells (Oliver 2005; Declerk *et al.* 2007; Chang *et al.* 2009).

In this study we evaluated the possible presence of viable cells of and qPCR units belonging to *Legionella* spp., NTM, and FLA in SW and BMW samples. The lack of cultivable bacteria isolation in almost all samples may be due to the environmental physical-chemical conditions, which limit the widespread colonization of bacteria in springs and bottling plants. In particular, the absence of cultivable Legionella cells could be due to the nutritional requirements of the bacteria, which need specific growing conditions (Superior Institute of Health 2007). As an exception, 83 CFU/L of *Mycobacterium gilvum* were detected in only one warm rich-mineralized SW sample. *Mycobacterium gilvum* does not represent a hazard because it is a saprophyte detected with a low concentration (Lavania *et al.* 2014). Cultivable FLA (*Acanthamoeba polyphaga, Vermamoeba vermiformis* and *Vahlkampfia inornata),* were detected in 50% of the water samples. Molecular tests, performed with PCR and qPCR, suggest the presence of high qPCR units belonging to *Legionella* spp., *Mycobacterium* spp., Amoebozoa and Vahlkampfidiiae genus.

Bacteria qPCR units, shown in Figures 1 and 2, are often associated with the presence of protozoan *18S rRNA* gene, mostly in BMW samples. Moreover, the percentage of samples positive to FLA qPCR units is higher compared to the percentage of samples positive to Legionella and NTM genes. Moreover, a strong correlation was detected between Legionella and Amoebozoa qPCR units. These data assert the possible role of FLA as reservoirs of waterborne bacteria, which may increase their resistance to environmental conditions, as described in further studies performed on drinking water and other environments (Chang *et al.* 2009; Delafont *et al.* 2014). Another issue confirmed by this study concerns the correlations between the physical-chemical parameters and the presence of microbial qPCR units. In accordance with further studies (Lindström *et al.* 2005; Lee *et al.* 2016), the temperature, pH and conductivity parameters are important environmental factors influencing the bacterial community in wellsprings, mostly for *Mycobacterium* spp. and *Legionella* spp. In fact, we detected strong correlations between the physical-chemical parameters (conductivity; temperature) and the Legionella qPCR units. As described elsewhere (Fields 1996; Steinert *et al.* 1997), the presence of minerals in waters represent an important growing factor for the microbial colonization.

CONCLUSIONS

In conclusion, the absence of disinfection procedures for natural mineral water highlights the importance of microbiological control plans for SWs and BMWs in accordance with international food hygiene regulations. Our study suggests the lack of viable and cultivable waterborne bacteria despite the presence of cultivable FLA, which may be a bacteria reservoir for protection and resuscitation activity. For this reason, we highlight the need for additional routine microbiological tests aimed at ensuring water safety, mostly for high risk people such as those who are immuno-suppressed. Therefore, to avoid the occurrence of biological risk factors a wider assessment of microbial indicators presence is recommended.

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AUTHORS CONTRIBUTIONS

Angelo Baggiani conceived and designed the experiments. Michele Totaro, Beatrice Casini and Paola Valentini performed the experiments and wrote the paper. Pier Luigi Lopalco and Mario Miccoli analyzed the data.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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