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The microbiota as a candidate biomarker for SPA pools and SPA thermal spring stability after seismic events

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ABSTRACT

Worldwide, the location of thermal springs overlaps seismic areas, and the higher occurrence of earthquakes may impact on water stability and safety. The hydrogeological perturbations pose environmental and public health risks that can be monitored by well-established chemical, physical and biological parameters. Specific health concerns involve the exposure of the population to the medical or wellness uses of SPA thermal waters, e.g. in respiratory or hydropinic treatments as well as during rehabilitative or recreational activities in pools. Since SPA waters are characterized by their own microbiota, we analysed by 16S amplicon sequencing the dynamics of water microbial communities after the August 2017 Ischia island earthquake. For the first time, we report the impact of a seismic event on a thermal spring water, whose microbiota was deeply characterized before and immediately after the natural disaster. The biodiversity stability of the water underwent a dramatic disturbance following the earthquake, as summarized by a Shannon index moving from 1.300 during May 2016-July 2017, up to 1.600 during the first 20–70 h after the event and slightly slowing down to 1.500 after 30 days and to 1.400 after 6 months. Microbiota analysis showed a sudden reduction of the relative abundance of autochthone thermophilic species within the first 20 h and a parallel increase of other thermophilic species as well as of ectopic bacteria from soil, sediments, sea, freshwater and wastewaters. Cultivable mesophilic bacteria were observed only in the first 20 h sample (7×10^3/L), even if the presence of faecal contamination traces was detected by Real Time PCR also up to 70 h after the disaster. OTUs analysis of putative metabolic functions showed several changes between pre and post event, such as in the distribution of Sulphur metabolizing and Carbon fixation species. The restoration of the original pattern followed a slow trend, requiring over six months. The observed results confirm the impact of the earthquake on the microbiota structure of the underground thermal spring water, suggesting further perspectives for monitoring water stability and safety issues by a metagenomic approach.

1. Introduction

Hydrological response to earthquakes is related to the nature of the seismic event and involves changes in both underground spring waters and different superficial waters (Montgomery and Manga, 2003; Muir-Wood and King, 1993). The action of crustal deformations and ground shaking on water resources was deeply studied and several models proposed to explain and predict the possible effects on aquifers or aquitards (Wang and Manga, 2015; Zhang et al., 2019). Water management after seismic events represents a public health priority and waterborne infections are a key component of earthquake-related injuries among survivors (Zhao et al., 2018; Tang et al., 2017; Bagcchi, 2015). After a natural disaster, indeed, waters may often not be safe due to chemical, radioactive or biological hazards (CDC; Sekine and Roskosky, 2018; Klise et al., 2017). However, in addition to the urgent need for detecting health risks, a major issue concerns the disruption of the original stability of the water and its restoration during time. Traditional surveillance methods are based on a well-established set of chemical, physical and microbiological markers, that are basically the same used for monitoring quality and safety in thermal spring waters (Directive, 2009). Information on changes in the composition of underground waters after a seismic event is relevant for public health, but also for environmental and hydrogeological studies (Wang and Manga, 2015; Grasby et al., 2019). Moreover, since thermal waters often are used worldwide for SPA wellness, medical, rehabilitative and recreational purposes, stability markers are desirable to allow appropriate

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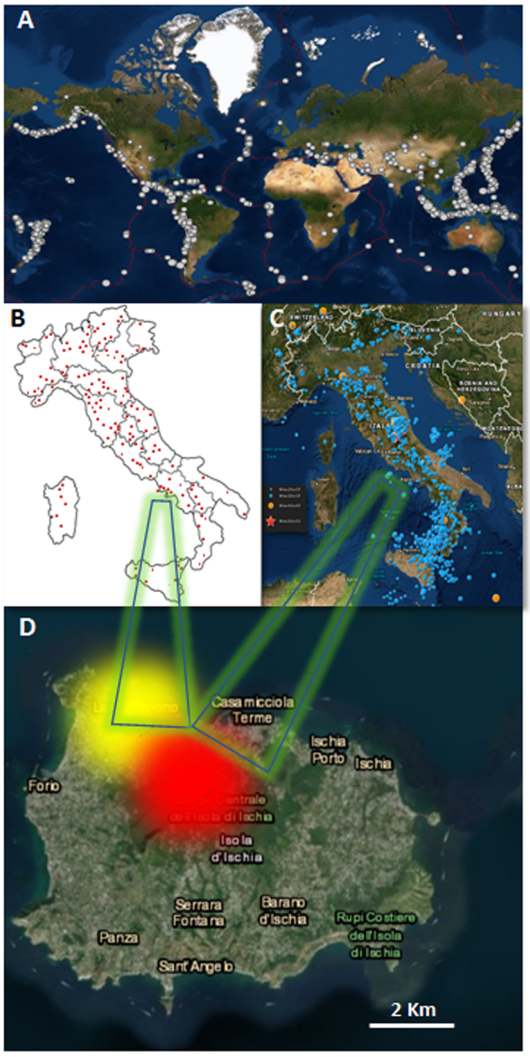

Fig. 1. Thermal springs distribution overlaps seismic areas. (A) Worldwide distribution of earthquakes (> 3.5 Richter magnitude) during August 2017 (modified from USGS, 2019, (USGS). (B) Map of the Italian thermal spring waters that are accessible to the population for wellness or recreational purposes (Federterme). (C) Map of the earthquakes occurred in Italy during year 2017, including the Ischia earthquake of August 21st, 2017 h 18.57 (UTC) of 3.9 Richter Magnitude (Pignone et al., 2017; Centre for Research on the Epidemiology of Disasters (CRED), 2018). (D) Enlargement of Ischia island with the indication of the epicentre area (Red) and the sampling area (Yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

management and prevent risks in the exposed populations (Valeriani et al., 2018d; Mavridou et al., 2018).

For hydrogeological reasons, the geographical distribution of seismic or volcanic areas tends to overlap thermal springs distribution, posing these water sources at an increased frequency of earthquakes and in closer proximity to their epicentres (Fig. 1) (Montgomery and Manga, 2003; Wang and Manga, 2015; Grasby et al., 2019; Pignone et al., 2017). Worldwide, about 10^5–10^6 earthquakes are reported annually, whose 10^3–10^4 overcome a magnitude of 3 on Richter scale (INVG). During billions of years the composition of each thermal water reached a hydrogeological equilibrium as shown by monitoring physico-chemical parameters (Gagliano et al., 2016; Song et al., 2013). This spring stability is also characterized by an own biological component, mainly consisting in thermophilic and other extremophilic microbial species that were recently described by massive sequencing methods (Valeriani et al., 2018c, 2018a; Scherer et al., 2017; Valeriani et al., 2016).

Hereby, we report the changes observed in the water microbiota of an underground spring located in the proximity of the epicentre of the August 2017 Casamicciola earthquake (Magnitude 3.9 Mw), occurring in Ischia, a quiescent volcanic island located in Italy, in the Gulf of Naples (Fig. 1) (Iovine et al., 2017; Piochi et al., 2019). These hot springs were known since ancient times and this Mediterranean Island (originally called Pithacusae) was included in the first century AD Plinio's description of the interactions between earthquakes and water sources (Pliny the Elder). Still today thermal waters are deeply studied and extensively used for tourism, health or wellness purposes, so that Ischia hydrogeology and seismology is very well characterized (Iovine et al., 2017; Piochi et al., 2019; Paoletti et al., 2013).

To our knowledge, this is the first study that describes the microbiota dynamics in a thermal spring water after an earthquake. Recently, other reports considered the analysis of the environmental microbiome in drinking waters after seismic events, e.g. during the 2010 Haiti and 2015 Nepal earthquakes (Roy et al., 2018; Uprety et al., 2017). However, the target of these studies were superficial waters sampled after the event to the aim of evaluating possible risks for waterborne diseases. These studies support the suitability of a microbiota analysis by massive sequencing to assess presence of contaminants and to evaluate the stability and safety of a water source. In particular, the Haiti study was based on a wide metagenomic approach involving different microorganisms including bacteria, fungi, protists, viruses and showing its effectiveness in detecting different pathogenic species as well as in identifying virulence and antibiotic resistance genes (Roy et al., 2018). The Nepal study was based on a 16S sequencing approach applied to water samples collected directly from the faucets; it contains information on the microbial community before the earthquake, providing additional data on microbial stability before the seismic event and on the restoration as a function of time and sanitation practices (Uprety et al., 2017). Several other studies considered the application of metagenomic analysis to water sources of different kinds, supporting the reliability of the general approach (Ghai et al., 2011; Saleem et al., 2018; Chu et al., 2018; Paduano et al., 2017; Meyer-Dombard et al., 2005). Here, we focused on the impact of a medium magnitude earthquake on the water microbiota of an underground volcanic thermal spring, mainly characterized by an extremophilic and autochthone microbial community.

2. Materials and methods

2.1. Site description of the spring and seismic event

The water from a sulphurous thermal spring located underground within a radius of 1 km from the epicentre of the 2017 Casamicciola earthquake (Ischia, Italy) was monitored in the days immediately after the seismic event and compared with preceding and following samples (Fig. 1). Ischia is a volcanic island located in a seismic area along the Neapolis gulf, whose hot springs were known since ancient times and still today widely used for tourism, health and wellness purposes (Pliny the Elder; Paoletti et al., 2013). Its hydrogeology and seismology are well characterized (Iovine et al., 2017; Piochi et al., 2019; Pliny the Elder; Paoletti et al., 2013). The Casamicciola earthquake occurred on August 21st 2017 at time 18:57:51 (UTC), with an epicentre at the following geographic coordinates (lat, lon) 40.74, 13.9 at 2 km depth and with a Mw 3.9 magnitude (Pignone et al., 2017).

2.2. Sampling protocol

Within a period ranging from May 2016 up to January 2018, for each sampling time at least 2 L of water were collected from a well in the direct proximity of the spring using sterile glass bottles and standard procedures. Samples were kept at room temperature and processed within 24 h. A total of 8 sampling times were considered in this study. Before the earthquake event 3 samples were collected: (i)

15 months before on May 2017 (Sample Pre1), (ii) 2 months before on June 2017 (Pre2), (iii) 1 month before (Pre3). Soon after the earthquake other samples were collected immediately after 20 h, 48 h, and at 70 h, respectively named Post1, Post2, Post3, and then after 30 days (Post30d) and six months (Post6m).

2.3. Microbiological analysis

The microbiological analysis was performed using three different culture-based approaches: (i) classical culture methods: briefly, the detection and count of intestinal *E. coli* and Enterococcus spp., in 100 ml of water, were performed in agreement with the membrane filtration method using nitrocellulose filters of 0.45 μm pore size (ISO, 2000a, 2000b). Total bacterial count (22° and 37 °C) was done following validated protocols (ISO, 1999); (ii) rapid bacterial enzyme detection technologies (ISO, 2012; ISO, 1998): for *E. coli* and Enterococcus spp. were conducted using the IDEXX Quantitray/2000 system following the manufacturer protocol (IDEXX Laboratories, Westbrook, ME); (iii) detection and isolation of thermophilic bacteria were performed through agar plate with medium D modified (Castenholz, 1969) and Medium APL/iron-reducer (Spanevello and Patel, 2004). Water samples (1 Liter) were filtered with a 0.47 μm sterile nitrocellulose membrane (Whatman-GE Healthcare, USA) and incubated on D or APL medium plates mainly for 18 h at 54 °C (also other temperature and times were considered: 24–48 h, 55–70 °C). Selected strains of similar morphology were isolated, DNA extracted and characterized by Sanger sequencing according to previously reported protocols applied to thermal spring waters (Valeriani et al., 2018a).

2.4. DNA extraction protocol

Water samples (2–5 L) were concentrated by membrane filtration (0.4 μm polycarbonate membrane, Minerva Biolabs, Germany). DNA extraction was performed following a modified manufacture protocol as previously described (Valeriani et al., 2018c, 2018a). Briefly, the filter membrane was transferred (turned upside down) onto an incubation dish filled with 2 ml of Lysis buffer (Minerva BioLabs, Germany) and then incubated at 37 °C for 30 min. Subsequently, the lysis solution was transferred into an incubation tube with 0.1 mg of glass beads (Sigma Aldrich, USA) and incubated at 56 °C for 15 min after 1-minute vortex. DNA purification was carried out using Aqua screen Fast Extract Kit according to the manufacturer's protocol (Minerva BioLabs, Germany).

2.5. Analysis of mfDNA by multiplex real-time PCR and data interpretation

The analysis of *mf*DNA was performed, following a protocol previously validated for detection of human microflora traces as previously described (Valeriani et al., 2018b, 2014, 2016), briefly: amplifications were combined in 3 multiplex reactions (Microsan-F Kit; MDD, Viterbo, Italy): mix F1, for the identification of human biological traces using *Staphylococcus aureus/Enterococcus spp* probes; mix F2 for not-specific human fecal traces using *Bacteroides fragilis/Bacteroides vulgatus* probes; mix F3 for specific human fecal traces using *B. vulgatus/Escherichia coli* (probes were labeled FAM/JOE/ROX, with the BHQ-1 quencher) (Valeriani et al., 2018b). For each mix, samples were tested in triplicate. Reactions were performed in a volume of 25 μl, of which 12.5 μl JumpStart Taq ReadyMix for Quantitative PCR (Sigma Aldrich, St. Louis, MO), containing primers (900 nM), and probe (250 nM) (Valeriani et al., 2018b). The amplifications were performed using Bio-Rad CFX96 (Bio-Rad, Hercules, CA) programmed for 10 min at 95 °C and 40 cycles of 15 s at 95 °C and 1 min at 60 °C. For each sample 5–10 μl template reaction was amplified (Spanevello and Patel, 2004). The PCR output was expressed as cycle threshold (C_T), a measure of the quantity of detected DNA. Positive samples were considered those where C_T data analysis provided at least 1 positive indicator ($C_T \leq 33$). Conversely, a microbial indicator was considered not detectable (ND) when over the $C_T \geq 33$ threshold.

2.6. Bacterial community 16S amplicon sequencing profiles

16S rRNA paired-end sequencing was performed according with "16S Metagenomic Sequencing Library Preparation" protocol (Part# 15044223 rev. A; Illumina, USA). Briefly, the primers containing Illumina adapter and linker sequence and targeting the V1-V2 regions of bacterial 16S rRNA genes were used (Valeriani et al., 2018c, 2018a; Kittelmann et al., 2013; Mucci et al., 2019). Three libraries with unique tags were generated for each sample as technical replicates. Each amplification reaction had a total volume of 25 μl containing 12.5 μl of KAPA HiFi HotStart ReadyMix (Roche, Pleasanton, CA), 5 μl of each primer (1 μM), and 2 μl template DNA. Reactions were carried out on a Techne®TC-PLUS thermalcycler (VWR International, LLC, Radnor, USA). Following amplification, 5 μl of PCR product from each reaction was used for agarose gel (1%) electrophoresis to confirm amplification. The final concentration of cleaned DNA amplicon was determined using the Qubit PicoGreen dsDNA BR assay kit (Invitrogen, Grand Island, NY, USA) and validated on Bioanalyzer DNA 1000 chip (Agilent, USA). Libraries were prepared using the MiSeq Reagent Kit Preparation Guide (Illumina, San Diego, CA, USA). Raw sequence data were processed using an in-house pipeline which was built on the Galaxy platform and incorporated various software tools to evaluate the quality of the raw sequence data (e.g. FastQC, http://www.bioinformatics. babraham.ac.uk/projects/fastqc/). All data sets were rigorously screened to remove low quality reads (short reads $<$ 200 nt, zero-ambiguous sequences). Demultiplexing was performed to remove PhiX sequences and sort sequences; moreover, to minimize sequencing errors and ensure sequence quality, the reads were trimmed based on the sequence quality score using Btrim (Kong, 2011). OTUs (Operational Taxonomic Units) were clustered at a 97% similarity level and final OTUs were generated based on the clustering results and taxonomic annotation of individual OTUs was based on representative sequences using RDP's 16S Classifier 2.5. Observed OTUs were defined as observed species. The sequence reads were analysed, also, in the cloud environment BaseSpace through the 16S Metagenomics app (version 1.0.1; Illumina®): the taxonomic database used was the Illumina-curated version (RefSeq RDP 16S v3 May 2018 reference taxonomy database using data from https://benjjneb.github.io/dada2/training.html; (Wang et al., 2007). The raw sequencing data have been submitted to NCBI Sequence Read Archive (http://www.ncbi.nlm.nih.gov/sra/) with the project accession number of PRJNA564198.

2.7. Data analysis

Relative abundances of community members were determined with rarefied data and summarized at each taxonomic level. Alpha and Beta diversity were calculated using EstimateS software at a level of 97% sequence similarity. Regarding Alpha diversity, microbial richness and biodiversity were computed through Shannon Index (SI) and other indexes (Equitability index, Chao index, Inverse Simpson Index; data not shown) at species level (Colwell et al., 2012; Magurran, 2013). A diversity index is a mathematical measure of species diversity in a community and the Shannon diversity is one of most commonly used to provide more information than simply species richness, as well as to take into account also the relative abundances of the different species. We applied different indexes (data not shown) obtaining very similar trends and reported results in the Shannon Index form. Principal Coordinates Analysis (PCA) was performed using METAGENassist platform (Arndt et al., 2012), in order to investigate the dissimilarity between groups. To assess sequencing depth, alpha rarefaction plots were done in 'mothur' (v 1.31.1) and R (version 3.1.3) using packages 'ggplot2′ and 'vegan' (R Core team 2013). All statistical analyses were performed with software package IBM SPSS statistics, version 22. The putative functional profiles based on the 16S community composition were investigated by automated taxonomic-to-phenotypic mapping using a METAGENassist platform and NCBI microbial taxonomy (Arndt et al., 2012). Based on taxonomic assignment, pathogens were calculated considering those OTUs with a $>$ 97% sequence similarity

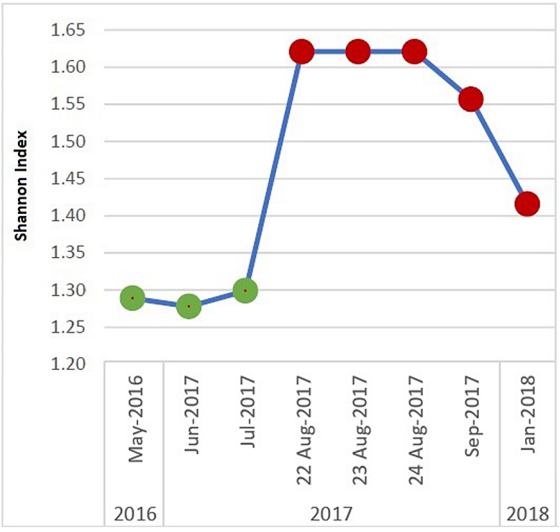

Fig. 2. Perturbation in microbiota biodiversity after earthquake. The trend of alfa diversity was summarized by the Shannon index, showing a sudden change in the microbiota of the spring water before (green) and after (red) the 21st of August 2017 earthquake.

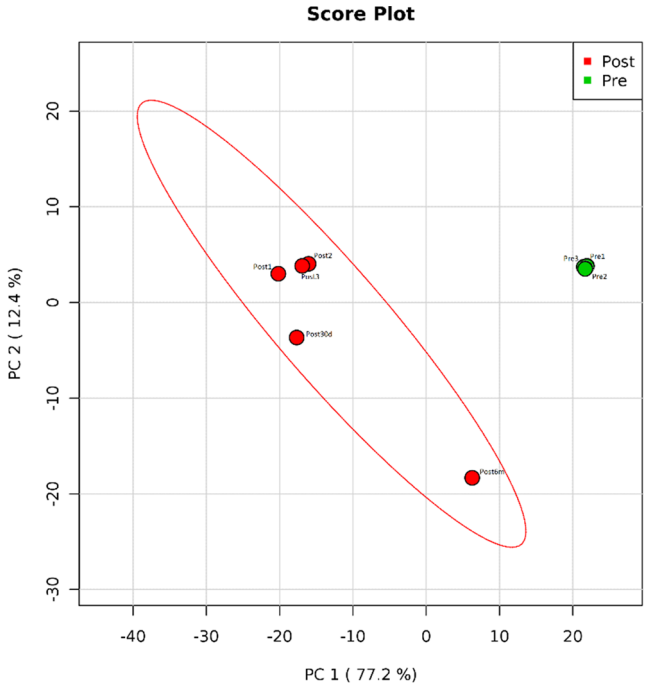

Fig. 3. Weighted PCoA plot representing the bacterial community structure. Before (green) and after (red) earthquake samples show a different distribution along the score plot. The close overlapping of samples collected before the event supports the high stability and consistency of the natural spring microbiota up to over 1 year before, while the vector-based analysis shows the disruption of the equilibrium and the instability of the post-event microbiota (red circle area). Beta diversity analysis is presented as weighted UniFrac distance matrix.

respect to known metabolism and habitat phenotype. The result is presented as a heatmap. Agglomerative hierarchical clustering was performed by treating everyone as a separate cluster and then proceeds to combine them until all samples belong to a single cluster (the data are analysed for metabolism and habitat phenotype, 53).

3. Result and discussion

3.1. Earthquake impact on thermal spring biodiversity

The microbiota of a thermal spring water located in the area affected by the Casamicciola 2017 earthquake, showed a sudden perturbation of its structure that was consistently maintained in the following days, as summarized by Shannon index (SI) analysis of alfa diversity (Fig. 2). The SI value -as well as other indexes, e.g. Inverse Simpson or Chao Index- represented a putative marker for measuring biodiversity changes after the hydrogeological perturbation. It was already described and applied for other ecological contexts (Valeriani et al., 2018a; Magurran, 2013). The Shannon's index accounts for both abundance and evenness of the species, allowing to monitor the dynamics of water biodiversity after the seismic event.

In contrast to the elevated stability of the microbiota during the previous 15 months (SI: 1.289 ± 0.01), a novel microbiota pattern appeared during the hours following the 21st of August 2017 earthquake, as shown in independent samples collected at 20, 48 and 70 h after the event (SI: 1.620 ± 0,001). If stability and individuality in the microbial signature of natural thermal SPA waters were already established and associated to different environmental factors, however to our knowledge this is the first report describing the impact of an earthquake on the thermal spring microbiota and its extremophilic species (Valeriani et al., 2018c, 2018a; Meyer-Dombard et al., 2005; Fouke, 2011). Indeed, previous metagenomic studies on the impact of an earthquake on waters were performed after the Haiti 2010 and Nepal 2015 disasters, but they considered surface waters and their mixing, focusing on pathogen contaminations more than on disruption of an original biodiversity (Roy et al., 2018; Uprety et al., 2017). Hereby, we report the 16S amplicon sequencing analysis of a thermal water microbiota from an underground volcanic spring, focusing on the environmental microbiology itself and monitoring the disruption of this unique bacterial community. For well-known hydrogeological reasons, indeed, the location of thermal springs along seismic areas is not an exception but the rule, in Italy as well as worldwide (Fig. 1) (INVG). The effect of an earthquake on drinkable water supplies is a priority issue for public health, involving also the concern for stability of underground springs (Reeder and Turner, 2011; Ahmad et al., 2018). Moreover, since thermal SPA waters are used for wellness, medical, touristic, rehabilitative and recreational purposes, novel biomarkers are desirable to evaluate their stability after seismic events, allowing appropriate management to prevent health risks in the exposed populations. In addition to the available traditional standards, also metagenomics may represent a further promising strategy for surveillance of underground spring waters after seismic events.

3.2. Thermal spring microbiota stability and perturbation

Fig. 3 reports the Principal Component Analysis (PCA) score plot, showing a different distribution of data from samples before and after the earthquake, based on the bacterial community structure. Interestingly, it can be noticed the close overlapping of samples collected before the seismic event, showing the high stability and consistency of the natural microbiota naturally derived from the underground volcanic spring. Otherwise, the vector-based analysis of the beta diversity highlights the disruption of the equilibrium and the persistence of an instability of the microbial community after the earthquake. The whole of PCA data suggests an effective role of the microbiota as a candidate marker for monitoring underground spring waters after seismic events.

Moreover, the disruption and naturally occurring restoration process toward the original microbiota structure can be followed during time (Fig. 4). In particular, the distance between the different OTU patterns showed a trend in accordance with the sampling time. The clustering analysis highlights how the microbial community is disrupted within few hours, and how its restoration process requires several months, showing an own drift progression toward the original

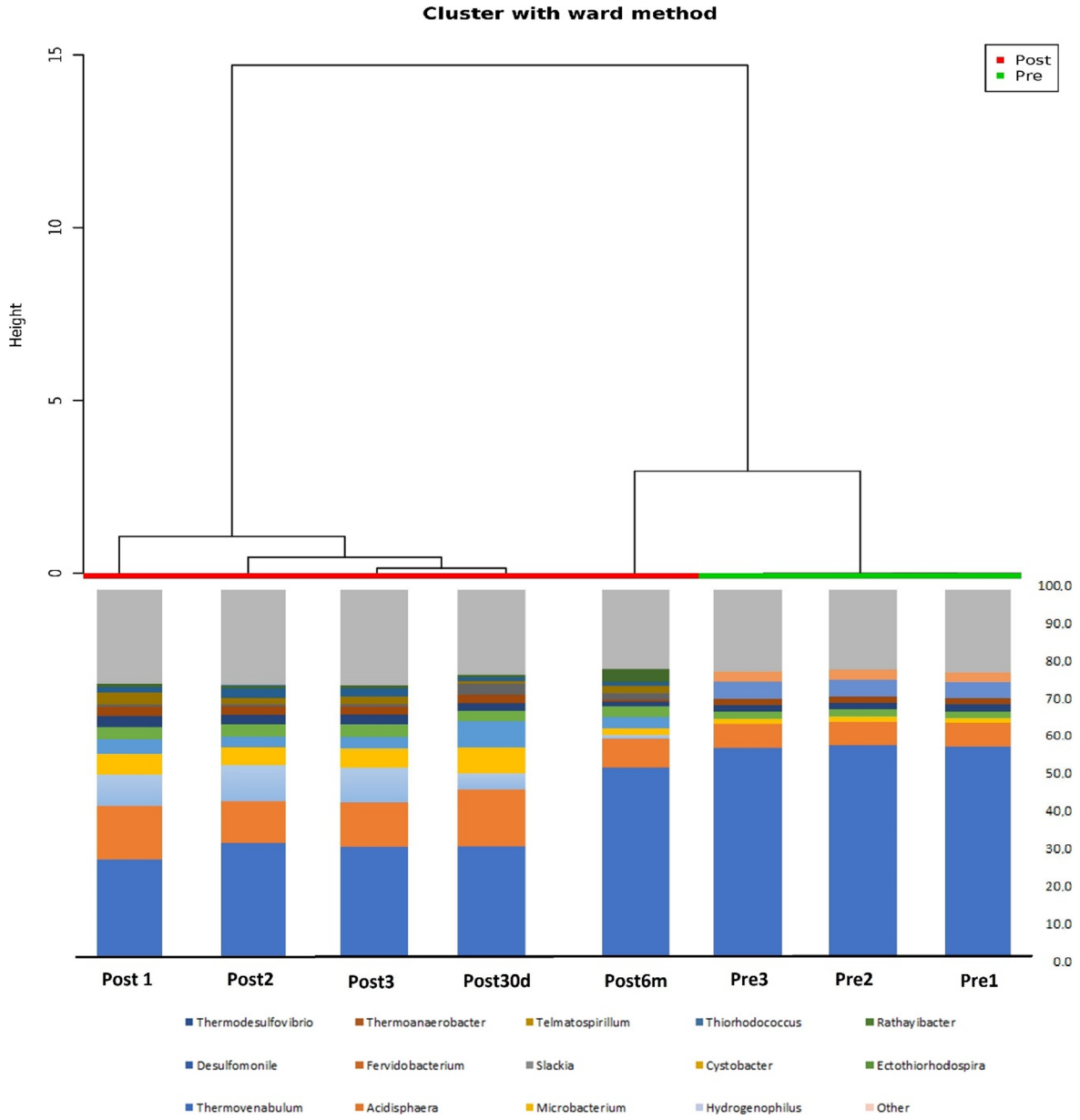

Fig. 4. Clustering dendrogram. The distance in the different OTU patterns and their relative abundance show the consistently stable structure before the earthquake (green line), the ordered distribution of post-event samples (red line) in accordance with the sampling time, but the independent aggregation of the 6-month sample (Post6m) in a dendrogram branch rooting in the pre-event group cluster. The clustering analysis highlights that the microbial community is disrupted within few hours, but that its restoration is a long process requiring several months and showing an own trend towards the original structure of the spring microbiota. Data are reported at genus-level and are representative of the biodiversity fluctuation rather than of the detection of a particular bacteria at species level. Average linkage (UPGMA) clustering dendrogram and the histogram with the percentage composition of sequences belonging to the indicated genus (cut off < 2% abundance).

microbiota structure. Interestingly, the water microbiota obtained 6 months after the earthquake represents an independent cluster in the dendrogram, characterized by being a post-event sample, but rooting with the pre-event group (Fig. 4). Bray–Curtis similarity analysis revealed that it shares a much lower similarity (< 70% threshold) with all the other post-event samples, that share a 99% of similarity within the first 70 h samples and 91% of similarity still 30 days after the earthquake. Together these data indicate that the spring water microbiota tends toward a natural restoration of its original ancient layout. This process seems to require month-long periods, in agreement with previous studies performed on superficial waters supplied from large river basins, after the 2010 Haiti earthquake or other seismic events (Grasby et al., 2019; Roy et al., 2018; Uprety et al., 2017). Therefore, it seems as if water microbiota structure retains an its own equilibrium and inertia, but additional studies are needed to understand the parameters involved in this phenomenon, such as dimension of the reservoir, presence of biofilm, hydrodynamics of the aquifer, hydrogeology changes after the earthquake. The disruption of the biodiversity pattern and its natural trend toward the original structure suggest the presence of an intrinsic biological component in both underground and surface basins. Surface waters, indeed, can be more easily contaminated by wastewaters and other surrounding environmental sources than the deeper and more protected volcanic springs, that are characterized by a lower availability of organic Carbon substrates, higher temperatures, absence of light, presence of salts, heavy metals or hydrogen sulphide (Valeriani et al., 2018d; Giampaoli et al., 2013; Gagliano et al., 2016; Song et al., 2013; Valeriani et al., 2018c).

The fluctuations observed in the microbiota biodiversity after the 2017 Casamicciola earthquake are due to modifications in quality and relative abundance mainly related to autochthon and extremophilic

genera. Two-way ANOVA analyses showed significant differences between samples collected before and after the earthquake ($p < 0.05$). The dominant genus *Thermodesulfovibrio* reduced from 56.7% to 29.3%, while *Thermoanaerobacter* from 6.6% to 12.4%; and several other genera increased in all post-event samples collected within 30 days after the seismic event: *Telmatospirillum* (7.9 ± 2.5%), *Thiorhodococcus* (5.3 ± 0.8%), *Desulfomonile* (3.3 ± 0.2), *Fervidobacterium* (2.5 ± 0.3%), *Slackia* (2.4 ± 0.2%), *Cystobacter* (0.8 ± 1.6%). Also, some new genera appeared overcoming the 0.1% threshold, such as *Rathayibacter* (4.4 ± 1.9%), *Bellilinea* (1.8 ± 0.14%), *Ectothiorhodospira* (1.6 ± 1.3%), *Thermovenabulum* (1.8 ± 0.7%), *Streptomyces* (1.3 ± 0.5%) and others disappeared after the earthquake perturbation such as *Microbacterium*, *Marinobacter*, *Bradiryzobium*, *Symbiobacterium*, *Hydrogenophylum*. The earthquake impact on this thermal water supports a major role for an inner reassemble of genera rather than a prevalent contamination by ectopic species from wastewaters of surface sources.

3.3. Putative functional profiles

The inferred microbial function and the agglomerative hierarchical clustering were calculated for metabolism and habitat phenotypes (Fig. 5). A clear modification in the putative functional profiles was observed after the earthquake. However, these data were not obtained from a systematic metagenomic analysis of prokaryotic genes or from transcriptomic analysis but were inferred from the 16S rDNA amplicon sequencing and the subsequent search for the related metabolic information available in the databases. For this reason, it would be overambitious to assign detailed metabolic differences to each specific pathway as shown in the report of Fig. 5; this result should be better considered as a whole, showing the novel metabolic pattern and its correspondence with the biodiversity disruption. The prokaryotic patterns, indeed are reported at Genus level, so that any change should not be confused with the role of single bacteria or their metabolic role at species level. Nevertheless, the heat map of functional profiles contains consistent information on the potential use of bioinformatics in indicating habitat and metabolic changes of the microbial component after a seismic event. For example, several interesting highlights included changes in Carbon, Nitrate and Sulphur pathways, an increase of activities related to carbon fixation or atrazine and chloramphenicol degradation, suggesting possible soil-specific or soil-contaminated surface pollutions. After 6 months most of metabolic functions tend to return to the original pattern, but still several differences remain, such as those related to dinitrogen fixing, propionate metabolism, sugar fermentation and methanogen activity. The habitat phenotype reported in Fig. 5B, suggests the possibility of contaminations from soil and fresh waters as well as from sea; that's likening event being both epicentre and spring close to the coast along a small island (Fig. 1D).

3.4. Water safety issues

Even if we focused on inner changes within the specific thermal spring microbiota, we also considered water quality and sanitation conditions by assaying the traditional microbial indicators. This approach is essential for monitoring faecal contaminations and waterborne infectious risks as well as for comparing data acquired through 16S amplicon sequencing. For this reason, we used in parallel different culture-based methods, but also Real Time PCR amplifications of the whole microflora DNA (mfDNA) used as template. All methods confirmed the absence of *E. coli* and *Enterococcus* spp., excluding that wastewaters may have contaminated the aquifer, even if the underground spring is located within villages in an inhabited area. Total bacterial count was always negative unless for the presence of mesophilic bacteria ($7x10^3$ CFU/l) in sample Post2, collected after 48 h from the disaster. This observation is in agreement with Fig. 5B, suggesting for sample Post2 a possible higher contamination from soil bacteria rather than from wastewaters or other sources. Real Time PCR resulted negative for *Staphylococcus aureus*, and for the following indicators of human faecal contamination: *Enterococcus faecalis*, *Escherichia coli*, *Bacteroides fragilis* and *Bacteroides vulgatus*. Only one set of primers for *B. vulgatus* provided positive results in samples collected between 20 and 70 h. However, this primer set is not restricted to human species, but can cross-amplify faecal sources from other homeothermic animals or fishes (Lee and Lee, 2010; Jenkins et al., 2009). This information from classical culture-based microbiology is consistent with the possible underground contamination of the spring water due to the temporary shaking of the aquifer and is not in contrast with the 16S DNA detection of different environmental and thermophilic species, that cannot be isolated on aspecific media (e.g. TSA). Conversely, these culture-based results are in agreement with the 16S amplicon sequencing data. Indeed, the extremophile species present in the thermal water microbiota cannot grow under traditional culture conditions, having specific requirements, but can be isolated using appropriate culture media (Valeriani et al., 2018a, 2016). So, we tested the water samples also on dedicated media, confirming the presence and growth of viable thermophilic species in all samples. Moreover, randomly selected colonies

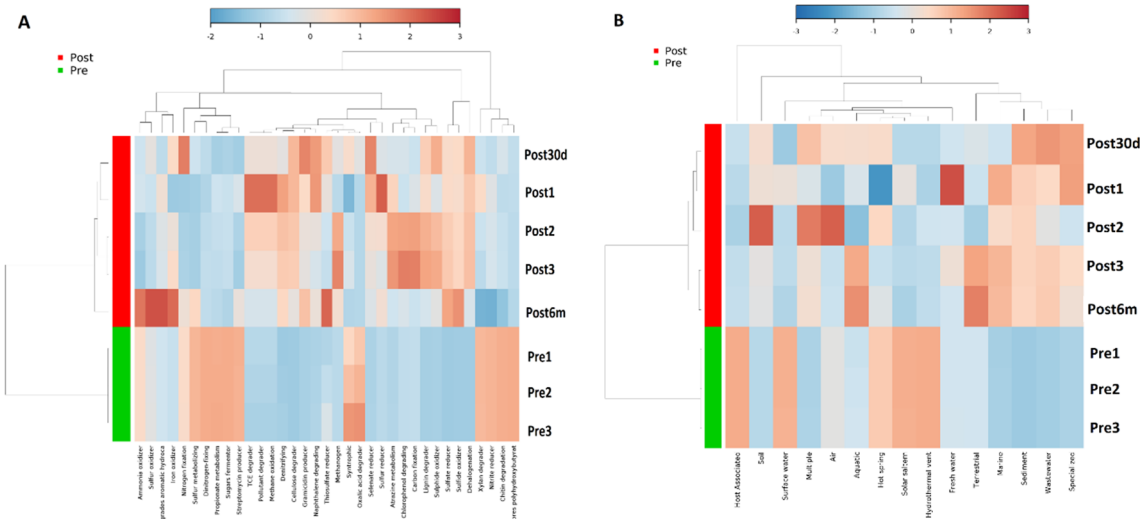

Fig. 5. Heat map of putative functional profiles. Agglomerative hierarchical clustering was performed, and data were analysed for metabolism (**A**) and habitat (**B**) phenotype. Bacterial genera were calculated considering those OTUs with a > 97% sequence similarity respect to known phenotype.

Table 1
Bacterial contamination after earthquake. Culturable bacteria were present only during the second day after the event and no traces of VBNC of anthropic origin were detected by Real Time PCR. Faecal traces of possible animal origin were observed by mfDNA analysis in the first 20–72 h after the earthquake. VBNC: viable but not culturable; CFU/L: Colony Forming Unit per Litre; *mf*DNA: microflora DNA; Indicators for human microflora were *Staphylococcus aureus, E. coli, Enterococcus spp* and for other fecal traces were *Bacteroides fragilis*, *Bacteroides vulgatus*. Real Time PCR positive samples were considered those where C_T data analysis provided ($C_T \leq 33$), conversely, a microbial indicator was considered not detectable (ND) when over the $C_T \geq 33$ threshold.

		Samples							
	Indicator	Pre1	Pre2	Pre3	Post1	Post2	Post3	Post30d	Post6m
Bacterial culture test	***Total bacterial count 22 °C (CFU/L)***	0	0	0	0	0	0	0	0
	Total bacterial count 37 °C (CFU/L)	0	0	0	0	$7x10^3$	0	0	0
	Enterococcus spp (CFU/L)	0	0	0	0	0	0	0	0
	E. coli (CFU/L)	0	0	0	0	0	0	0	0
mfDNA test	***Human microflora traces***	***ND***	***ND***	***ND***	***ND***	***ND***	***ND***	***ND***	***ND***
	Aspecific fecal traces	***ND***	***ND***	***ND***	***Positive***	***Positive***	***Positive***	***ND***	***ND***

with different morphology were typed by rDNA sequencing, showing a correspondence with the OTUs detected by 16S amplicon analysis, such as: *Anoxybacillus, Bacillus, Thermomonas*. Therefore, the absence of colonies on aspecific culture media excluded the presence of classical environmental or anthropic mesophilic microorganisms in the spring after the earthquake, even if several thermophilic species are clearly present and could be isolated on dedicated media. In accordance with a previous study on drinkable water safety after the Nepal 2015 earthquake (Uprety et al., 2017), and in consideration of a possible antibacterial activity of the sulphurous thermal water (Valeriani et al., 2018d; Fouke, 2011), we used also Real Time PCR to detect viable but not countable cells (VBNC) or dead bacteria, further confirming the absence of anthropic contaminations after the seismic event (Table 1).

3.5. Limitations of the study

This study describes the dynamics of water microbiota after the Ischia earthquake in a natural SPA spring water, reporting a slow restoration of the process over time. Even if the general approach is straightforward and data quite consistent, hereby we want to underline several limitations present in this study and mainly due to methodological issues. First, although 16S rRNA gene-based sequencing is an established approach to characterize bacterial genera, the information related to viruses and eukaryotes including fungi and protozoa genera is not included. Second, 16S rRNA gene-based amplicon sequencing does not provide direct information related to functional genes, involved in the overall nutrient and biogeochemical cycling or virulence-associated factors. Third, although this study aims to assess the degree of perturbation as a function of time, sampling immediately after the earthquake and 6 months after the earthquake may not be enough to comprehensively characterize all important genera because restoration properties may likely differ among specific groups of bacteria. Finally, a whole genome approach may have provided additional information on the hydrogeological ecology of the event, even if at higher costs. However, the 16S sequencing analysis revealed effective in defining a spring microbial signature and in monitoring its variability, even if with restrictions in the possibility to further consider functional genes and detailed metabolic patterns in different microbial species.

3.6. Perspectives for surveillance

After a major earthquake, water safety represents a principal concern for public health (Ivers and Ryan, 2006). Hygiene management implies hard decisions on forbidding or allowing access to superficial or underground waters for human purposes (Watson et al., 2007; Rasheed et al., 2009). Availability of additional information for tracing water stability may provide a further hint to support decisions based on a risk-benefit ratio. Being often far from urbanized areas, underground spring waters are considered more protected regarding mixing events or contaminations with wastewaters or external pollutants but may imply novel or unpredicted pollutants including radioactive or chemical hazards (Watson et al., 2007; Somboonna et al., 2014; Hiraoka et al., 2016; Bell et al., 2018). The high variability in the presence of traces of different elements or salts is part of the properties of several mineral spring waters and in particular those used for thermal SPA treatments and pools (Valeriani et al., 2018d; Giampaoli et al., 2013; Gagliano et al., 2016; Song et al., 2013; Valeriani et al., 2018c). Even if traditional parameters used for chemical, physical and microbiological test represent the gold standard for assessing water safety and avoiding health risks, however, changes in the autochthone microbiota component may represent a valuable information to detect differences in the hydrogeology of the aquifer after a seismic event and allow an advanced monitoring of water stability during time.

4. Conclusions

Stability of water supplies after a major earthquake represents a current hydrogeological issue and a public health concern. We had the extraordinary opportunity to characterize the microbiota of an underground spring located in the proximity of the epicentre of the 2017 Ischia earthquake, before and after the seismic event. The autochthone microbiota showed an own stability, that was suddenly disrupted in the hours after the natural disaster and persisted in the following months, although no potability indicators were modified. Biodiversity indexes obtained by metagenomics can represent valuable markers for surveillance of spring waters. The water microbiota is influenced by the spring hydrogeology, providing a promising tool for monitoring spring stability, tracing contaminants and supporting public health decisions after seismic events.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

F.V. performed data curation, formal analysis, investigation, writing - review & editing; G.G. performed the samples processing, methodology application, resources, software, validation, visualization; V.R.S. performed and designed the study and conceptualization, funding acquisition, project administration, supervision, and writing - original draft. All authors edited and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Availability of data and material

NCBI Sequence Read Archive project accession number PRJNA564198.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.105595.

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