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Geochemistry and microbial diversity of cave waters in the gypsum karst aquifers of Emilia Romagna region, Italy

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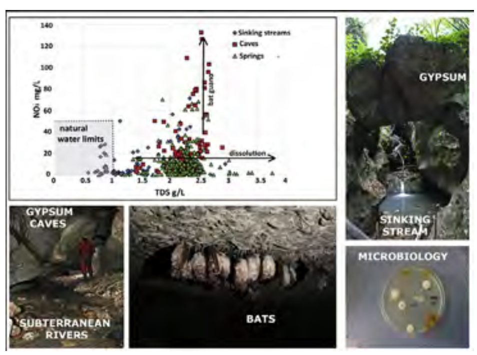
HIGHLIGHTS

- A five year geochemical and microbiological monitoring campaign has been carried out.
- Water samples in 57 points have been taken in gypsum caves and karst areas.
- Major and some minor elements have been analyzed over the seasons.
- Microbiological diversity has been assesses by molecular biology techniques.
- Fluctuations of microorganisms are correlated to season and to biological activity of bats.

GRAPHICAL ABSTRACT



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ABSTRACT

Fifty-seven control points of waters (sinking streams, rivers in caves, and resurgences) hosted in gypsum karst areas in Emilia Romagna region (N-Italy) were sampled in the framework of a Project LIFE + 08NAT/IT/000369 "Gypsum" in the period 2010–2014. The microbiology and chemistry of these waters have been analyzed to evaluate the impact of human activities or natural factors, in the gypsum karst systems. Waters have been analyzed for major chemistry (Ca, Mg, Na, K, SO_4, HCO_3, Cl, NO_3) and some minor constituents (F, Br, NH_4 and PO_4), measuring pH, electric conductivity (EC), total dissolved solids (TDS) and temperature (T) in situ. The same samples have been analyzed with traditional microbiology techniques focused on total microbial count and on fecal microbiota, as index of human and/or animal contamination, and molecular biology techniques (sequencing of 16S rRNA segment and PCR-DGGE), focused on the characterization of microbial populations in the different sampling sites and determination of their variations and/or changes during the five years of the project. As expected, waters tend to be increasingly mineralized from sinking streams to resurgences, with only local and temporarily high contents in nitrates and ammonium, often related to the presence of bat colonies. PCR-DGGE revealed ecological changes, in terms of microbial populations present in the bulk water samples, in different sampling sites within the same cave. Although the impact of fecal microorganisms only rarely exceeded 2 log UFC/ml, the results evidenced fluctuations of these microorganisms mainly correlated to the season and to the biological activity of bats.

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1. Introduction

Karst aquifer systems are becoming increasingly important as groundwater resources, covering about 25% of the world's demand (Ford and Williams, 2007) and are used extensively in many Mediterranean countries (Bakalowicz, 2015). They can deliver large amounts of drinkable water, often having their recharge areas confined to scarcely populated high mountain areas. Where urbanization is taking place upon karst, special care should be taken in preventing or minimizing pollution by human activities (Gutiérrez et al., 2014; Dodgen et al., 2017) putting in place correct policies and legal rules (Ravbar and Šebela, 2015).

Most studies on water quality in karst, focused on domestic groundwater resources, have been concentrated on carbonate areas, especially those where important springs are connected to aqueducts and deliver water to important cities (Stevanović et al., 2007; Jiang et al., 2009; Bicalho et al., 2012; Jeannin et al., 2016). Gypsum karst areas have been much less studied from a chemical and qualitative point of view. This is mainly because these waters are not suitable for domestic use, and have thus less interest of being studied and analyzed.

Gypsum is widely present in the Earth's crust, and is exposed less at the surface respect to carbonate rocks because of its lower resistance to weathering in most climates. In fact, gypsum is easily dissolved (Klimchouk, 1996) by both surface and hypogene undersaturated waters due to its high solubility (Ford and Williams, 2007). This has caused the formation of extensive cave systems in a wide variety of structural and climatic settings, and gypsum karsts have been studied in a great number of countries, including the circum-Mediterranean, Ukraine and other Central Asiatic countries, Poland, Germany, UK, Iran, North-America, Argentina and many others (Klimchouk et al., 1996; Calaforra, 1999; Klimchouk, 2005). The biggest gypsum caves are deep-seated and artesian in origin, caused by the upward movement of undersaturated waters into a soluble gypsum bed confined in between non-soluble aquifers and aquitards (Klimchouk et al., 1996). This type of intrastratal karst systems normally has no surface springs, and accessibility to the phreatic waters is occasionally possible mainly due to quarrying activities (Andrejchuk and Klimchouk, 2001; Vigna et al., 2010a). Waters flowing in these systems are normally close to saturation in gypsum (Klimchouk and Aksem, 2005; Vigna et al., 2010b).

Of completely different behavior are the so-called epigenic gypsum cave systems, related to surficial water flows that, through surface streams or seeping waters reaching the soluble gypsum formation, start to create caves and voids in less than a century. These karst systems are typically composed of sinkholes and sinking streams, through-caves, and springs or resurgences. Caves are often accessible and generally contain flowing waters. These systems have been widely studied from a morphological and speleogenetic point of view, and because of their fast evolution, can cause typical hazards such as sinkholes (Gutiérrez et al., 2014, 2016).

Water analyses in these epigenic gypsum karsts have been carried out in many countries, such as in Turkey (Günay, 2002; Kaçaroğlu et al., 2001), and in Iran (Raeisi et al., 2013), in which good examples of river quality deterioration have been described (Aghdam et al., 2012).

Several gypsum karsts have been studied from a geochemical point of view also in Spain, and in particular in the Betic Cordillera (Calaforra et al., 2002), in Salinas-Fuente Camacho area close to Granada (Calaforra and Pulido-Bosch, 1993), in Malaga (Benavente Herrera and Carrasco Cantos, 1986; Calaforra and Pulido-Bosch, 1999) and in Sorbas basin (Pulido-Bosch and Calaforra, 1993).

Gypsum outcrops are widely present also in Italy, especially in Sicily, Calabria, Piedmont, Marche, and Emilia Romagna regions (Madonia and Forti, 2003; De Waele et al., 2017). Caves in Emilia Romagna are almost exclusively formed in gypsum rocks, which cover only 1% of the entire region. These systems, often of the through-flow type (with a river sinking upstream and crossing the gypsum ridge flowing out at a downstream spring), are among the longest epigenic gypsum caves in the world, with developments of over 10 km (Lucci and Rossi, 2011). Some of these (i.e. Re Tiberio cave system) are still-active multi-level caves, formed by a climate-driven speleogenesis in an uplifting mountain belt (the northern Apennines) over at least the last 130 ka (Columbu et al., 2015).

A) SIC IT4030009 Triassic Gypsum (Alta Val di Secchia, Reggio Emilia)
B) SIC IT 4030017 Ca' del Vento-Ca' del Lupo-Borzano (Reggio Emilia)
C) SIC IT 4050001 Gypsum areas of Bologna (Bologna)
D) SIC IT 4050027 Gypsum of Mts. Rocca, Capra and Tizzano (Zola Pedrosa, BO)
E) SIC IT 4070011 Romagnola Gypsum Vein (Faenza/Imola)
F) SIC IT 4090001 Onferno (Rimini)

Fig. 1. Protected evaporite areas studied during the LIFE + 08NAT/IT/000369 "Gypsum" project. Area A is composed of Triassic gypsum, while areas B to F are all Messinian evaporites.

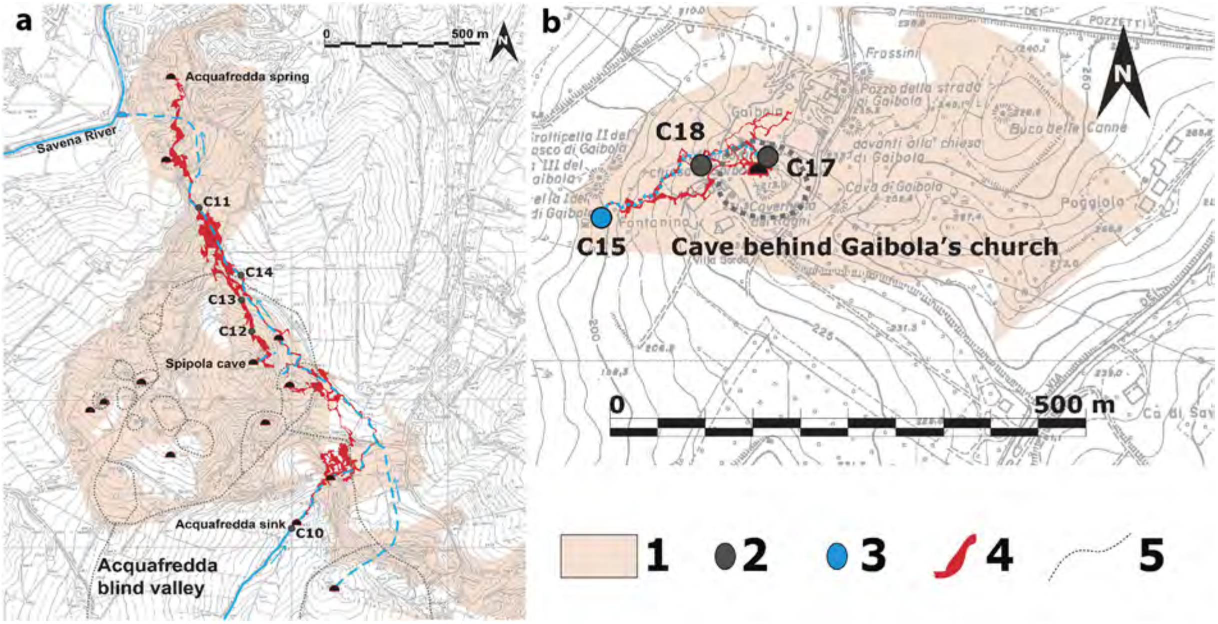

Fig. 2. The most extensive epigenic gypsum cave systems of Emilia Romagna region, located in area C in Fig. 1. a. Acquafredda–Spipola system close to Bologna b. Gaibola cave system close to Bologna. Legend: 1. Messinian gypsum outcrops; 2. Sinking and in-cave water sampling points and their ID; 3. Spring water samples; 4. Cave systems; 5. Doline contours. The blue lines with the arrows show perennial external water flows, dashed blue lines illustrate underground water flow. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The hydrogeology of these gypsum karsts has been studied from a rather general point of view starting in the 70s (Casali, 1972), and especially during the 80s (Forti et al., 1985, 1989; Forti and Francavilla, 1990), with dye tests, geostructural and speleological studies, and some water analysis. These studies concentrated on the most important caves, such as Acquafredda-Spipola system near Bologna, Rio Stella-Basino south of Faenza, and on the brackish Poiano springs and the nearby Tanone cave. Some episodes of pollution with nitrates and ammonium have been documented in the Acquafredda-Spipola cave (Forti et al., 1985) and at Tanone cave spring (Forti and Francavilla, 1990), while nitrates due to agricultural activities have been registered at Rio Stella sinking stream (Forti et al., 1989). In the late 90s the quality of the waters in the Messinian gypsum area of Borzano-Albinea (close to Reggio Emilia) has been studied, showing a limited pollution in nitrates in Tana della Mussina di Montericco, and a heavier pollution in nitrates in Tana di Mussina di Borzano. This pollution is attributed both to natural and anthropogenic causes (Forti and Chiesi, 2001). Poiano springs, the biggest of Emilia Romagna region, have been studied in detail since the mid 80s (Forti et al., 1988), and especially in 2006–2008, in the framework of the project called "Trias" (Chiesi and Forti,

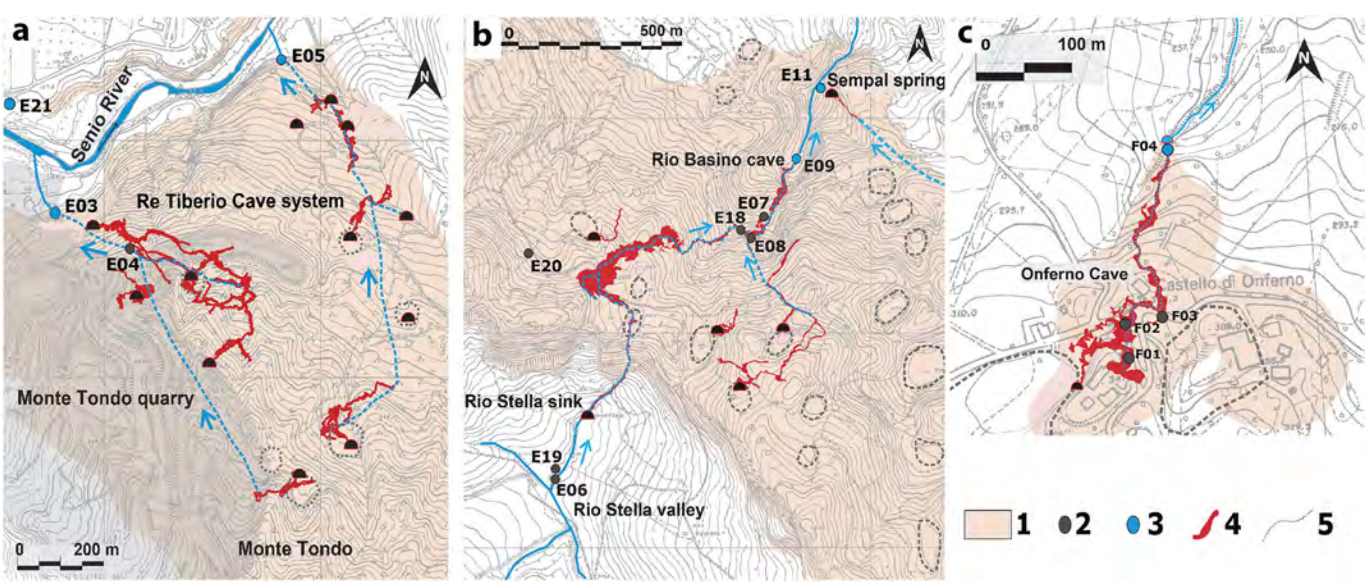

Fig. 3. The most extensive epigenic gypsum cave systems of Romagnola Gypsum Vein (area E in Fig. 1) and Rimini province (area F in Fig. 1). a. Re Tiberio cave system in the Romagnola Gypsum Vein along the Senio River, b. Rio Stella-Basino in the Romagnola Gypsum Vein, c. Onferno cave system in Rimini province. Legend: 1. Messinian gypsum outcrops; 2. In-cave water sampling points and their ID; 3. Spring water samples; 4. Cave systems; 5. Doline contours. The blue lines with the arrows show perennial external water flows. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Protected gypsum karst areas and number of investigated sampling sites. See Fig. 1 for the position of the areas.

Protected area	Number of sites	Sinks	Caves	Springs
SIC IT4030009 Triassic gypsum	8	0	0	8
SIC IT4030017 Cà del Vento–Cà del Lupo–Borzano	4	0	0	4
SIC IT4050001 Gypsum areas of Bologna	16	4	7	5
SIC IT4050027 Gypsum of Mts Rocca, Capra and Tizzano	2	1	0	1
SIC IT4070011 Romagnola Gypsum Vein	23	5	4	14
SIC IT4090001 Onferno	4	0	3	1
Total	57	10	14	33

2009). These springs, having around 6 g/L in NaCl, and a saturation in calcium sulfate, are of course undrinkable. The detailed monitoring of the springs has demonstrated the variable salt amount to derive from dissolution of salty lenses in the sedimentary sequence in a slowly uplifting area characterized by halokinetic movements (Chiesi et al., 2010).

From May 2010 until October 2014 a detailed sampling campaign has been carried out on 57 water points (sinking streams, caves, and springs) in the main gypsum karst areas (Fig. 1) located in several Natura 2000 sites of Emilia Romagna region. A total of 560 water samples have been analyzed for their major components (Na^+, K^+, Ca^{2+}, Mg^{2+}, HCO_3^-, SO_4^{2-}, Cl^-, and NO_3^-) and some minor constituents (F^-, Br^-, NH_4^+, and PO_4^{3-}) and several have also been analyzed from a microbiological standpoint. This is the first real systematic quality assessment of waters in gypsum areas in Italy. This study, carried out in the framework of the European Project "LIFE + 08NAT/IT/000369 Gypsum" (De Waele et al., 2013), had the aim of assessing the quality of the natural environment in these protected areas, and deciding proper interventions for the requalification of compromised sites.

2. Study sites

2.1. Geological outline

From a geological point of view two different gypsum formations crop out in Emilia Romagna region. In the Upper Secchia Valley (A in Fig. 1), Triassic microcrystalline gypsum crops out as intensely tectonized units together with anhydrites and cavernous limestones. These rocks belong to the Burano Formation (Norian), and are allochthonous units displaced during the compressive phases of the Apennine formation. After their deposition in a shallow marine environment, they underwent deep burial, thermometamorphism, and exhumation with rehydration and dissolution (Lugli, 2001). Cretaceous-Paleocene Ligurian units mainly composed of fine sediments mostly surround them.

All other protected areas (B–F in Fig. 1) in which waters have been sampled are located in Messinian, mostly macrocrystalline gypsum units, belonging to the Gessoso-Solfifera Formation (Primary Lower Gypsum Units; 5.96–5.60 Ma) (Lugli et al., 2010). Most of these tectonic thrust sheets have been overthrusted by Ligurian Units to the South, mainly composed of fine-grained sediments and are overlain by Late Messinian-Pliocene deepwater fine sediments to the North. Their general alignment along the northern Apennine flank is controlled by high angle faults, with a general NW-SE direction, and the same Apennine tectonics has caused their fragmentation in isolated parts of thrusted sheets.

Table 2
Legal surface water limits for some constituents based on the CEC 1993. A01 refers only to Poiano springs, A to Triassic Gypsum areas excluded Poiano springs, B to Cà del Vento, Cà del Lupo, Borzano, C to Gypsum areas of Bologna, D to Gypsum of Mts. Rocca, Capra and Tizzano, E to Romagnola Gypsum Vein, F to Onferno. For each element (Cl^-, SO_4^{2-}, Na^+, F^-, NH_4^+, NO_3^-) the descriptive statistics including minimum (min), maximum (max), standard deviation (st.dev) and median (med) are reported.

	Cl^- (250 mg/l)	SO_4^{2-} (250 mg/l)	Na^+ (150 mg/l)	NO_3^- (50 mg/l)	F^- (1.5 mg/l)	NH_4^+ (0.5 mg/l)	Tot. samples
A01							
Min	3217.36	1839.97	2105.88	1.259	0.015	0.01	11
Max	4039.24	2131.61	2783.67	44.66	0.06	0.11	
St.dev	233.1	89.91	200.62	16.13	0.018	0.03	
Med	3611.48	1973.83	2219.35	16.54	0.015	0.07	
A							
Min	2.945	625.754	1.5713	0.021	0.012	0.01	47
Max	45.91	1586.12	92.64	13.45	1.485	0.12	
St.dev	11.33	208.84	19.86	3.5	0.33	0.03	
Med	19.31	1301.95	18.815	2.185	0.205	0.05	
B							
Min	6.96	1142.92	12.33	0.36	0.036	0.01	33
Max	51.599	1632.63	36.38	13.51	1.459	0.23	
St.dev	8.2	89.74	6.37	4.428	0.29	0.053	
Med	17.8	1425.61	22.77	3.1	0.33	0.05	
C							
Min	9.355	243.85	3.71	0.015	0.012	0.01	159
Max	230.27	1628.12	390.5	50.31	3.012	0.33	
St.dev	54.26	266.3	40.79	10.297	0.57	0.059	
Med	39.87	1326.9	29.64	12.02	0.418	0.04	
D							
Min	15.47	393.41	18.60	2.28	0.19	0.01	18
Max	99.11	1397.83	49.51	31.24	1.60	0.11	
St.dev	19.2	339.2	7.51	8.56	0.37	0.035	
Med	22.15	836.14	24.72	7.72	0.46	0.04	
E							
Min	4.55	205.43	4.22	0.01	0.02	0.01	252
Max	403.49	2020.76	385.69	71.43	13.88	5.00	
St.dev	61.57	357.59	45.98	12.09	1.47	0.6	
Med	19.44	1361.49	16.77	7.08	0.97	0.04	
F							
Min	29.63	852.05	6.00	4.10	0.04	0.01	40
Max	124.53	1556.94	84.08	132.98	7.56	0.26	
St.dev	19.9	183.03	16.69	34.24	1.38	0.055	
Med	62.19	1350.23	51.95	56.24	0.62	0.05	

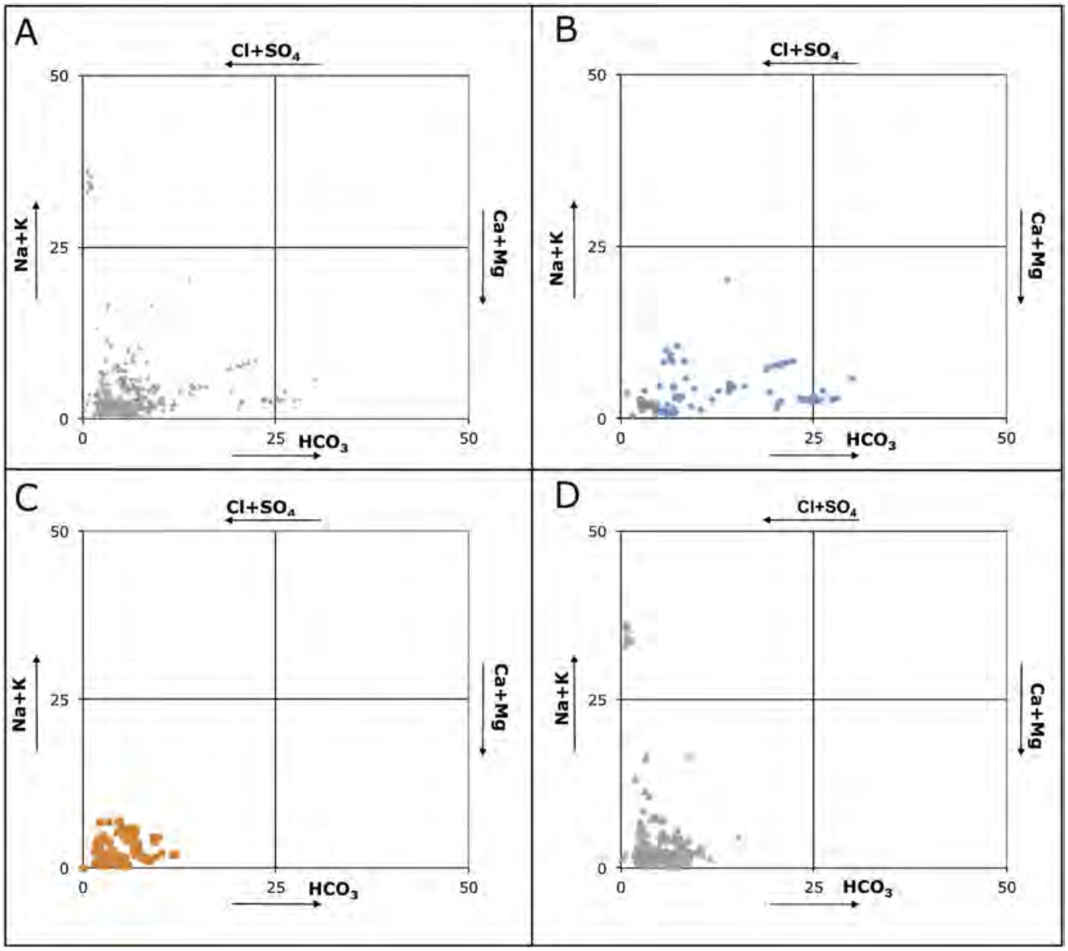

Fig. 4. Classification of waters in the Ludwig-Langelier diagram: A. All samples; B. Sinking streams; C. Caves; D. Springs.

2.2. The Emilia Romagna gypsum aquifer systems

All the studied evaporite aquifer systems are composed of well-karstified units in which surface water enters the soluble rocks along sinking streams or dolines, flows underground along cave passages and ends its subterranean flow at springs. In many cases only the springs have been sampled, being the sinking points of difficult access or often dry during most seasons. This is the case for all samples in areas A and B, and in some samples of areas C and E (e.g. C09, E02, E12, E16, E21, E22, E23, E24) (see Supplementary Table 1). In the gypsum outcrops near Bologna (areas C and D in Fig. 1), in the Romagnola Gypsum Vein (area E in Fig. 1) and at Onferno (area F in Fig. 1), on the other hand, samples could be taken at their upstream and downstream ends of the gypsum outcrops (i.e. sinking streams and springs respectively), and often also along their flow paths inside the caves. In these areas, in fact, cave waters can generally be followed from their sinking points down to their springs crossing important and accessible cave systems (Figs. 2 and 3). All gypsum aquifers of the region are of the dominant drainage type, with most of the water running along vadose conduits and passages, and enlarged fractures. This means that springs generally respond quickly to infiltration events, with rapid increases in flow rate, and changes in water chemistry and temperature.

2.3. Sample sites

Fifty-seven points of water have been sampled throughout the gypsum areas of Emilia Romagna region. All sites are located in protected areas according to Natura 2000 (Sites of Community Interest of the Directive Habitat of EC) or in regional parks or natural reserves (Fig. 1). The number of investigated points for each of these protected areas is reported in Table 1. A total of 10 sinking streams, 14 in-cave sites, and 33 springs have been sampled.

Samples have been taken more or less every three months, four times in a year, for a total of 17 sampling campaigns. Some areas have not been sampled in certain periods because of the lack of water (dry seasons) or unfavorable conditions. Landslides or snow/ice sometimes made some sampling points unreachable for a period of time.

Supplementary Table 1 reports all sampling sites divided per protected areas.

3. Material and methods

3.1. Sampling and chemical analysis

Three samples were collected at each site: 250 ml and 50 ml (sterile container) of normal water and 100 ml of water filtered with a 0.45 μm sterile filter (sterile cellulose acetate, Minisart©) and acidified with 1 ml of concentrated 65% HNO_3.

At each sampling site pH, temperature and electric conductivity were measured in situ with a previously calibrated Hanna Combo portable sensor (accuracy 0.5 °C, 0.05 pH, and 2% EC/TDS). The sterile 50 ml bottle was used for microbiological analyses.

Fundamental metals (Na^+, K^+, Mg^{2+} and Ca^{2+}) were analyzed with an Atomic Absorption Spectrophotometer Thermo S (AAS). Major and minor anions (SO_4^{2+}, Cl^-, F^-, Br^-, PO_4^{3-}, and NO_3^-) were analyzed with Ionic Chromatography (Metrohm 881bIC Pro), ammonium (NH_4^+) by Colorimetry (portable spectrophotometer Hack DR 2010), alcalinity (HCO_3^-) through a titration with HCl and methylorange as indicator.

3.2. Microbiological analyses

The samples (collected using the sterile 50 ml container) were stored in the dark at 4 °C until the analysis. To determine the total microbial concentration water was plated on R2A medium (Dewettinck et al., 2001; Wu et al., 2006) and the plates were incubated for 10 days at 20 °C. Moreover, total and fecal coliforms were counted on VRBA medium (Oxoid, Milano, Italy), incubating the plates at 37 °C or at 45 °C, respectively. From each plate different colonies, corresponding to different morphological types, were randomly isolated. Genomic DNA was extracted from the pure cultures by Insta Gene Matrix kit (Bio—Rad Laboratories Milano, Italy) (Patrignani et al., 2015). The isolates were identified sequencing the 16S rRNA region (Boubendir et al., 2016). Moreover, after the microbial counts, the plates were used for bulk formation as previously described (Ercolini et al., 2001). Bulk cell suspensions (1 ml) from the countable plates for each medium were used for the DNA extraction as described below and subsequently used for DGGE analysis.

3.3. Total DNA isolation

The total microbial concentration of the water samples analyzed rarely exceeded 4 log UFC/ml. For this, DNA templates for DGGE experiments were harvested from the viable-count plate (2 log UFC/ml). All the colonies were collected by saline solution suspension and centrifugation (4000 rpm for 5 min). The pellet obtained was used for DNA isolation by Insta Gene Matrix kit (Bio-Rad Laboratories Milano, Italy).

3.4. DGGE analysis

16SrDNA fragments were amplified with primers GC-clamp-EUBf933 and EUBr1387 specific for universally conserved bacterial 16SrDNA sequences. The sequences of the two primers are EUBf933: 5′-GCACAAGCGGTGGAGCATGTGG-3′, a 40-bp GC clamp was attached to the 5′ end of this primer; and EUBr1387: 5′-GCCCGGGAACGTATTCACCG-3′. PCR mixture containing 2.5 U of Taq, 20 pmol of each primer, 5 μl of a 200 μmol/l of each deoxyribonucleoside triphosphate, 5 μl of 10 × PCR buffer, was made up to 45 μl with DNA-free water (Wu et al., 2006). All the PCR reagents were provided by Takara (Takara, Otsu, Shiga, Japan). PCR was performed in agreement with Wu et al. (2006). PCRs were performed with T3000 Thermocycler (Biometra, Göttingen, Germany).

PCR products were loaded onto a 6.5% (wt/vol) polyacrylamide gel in 1 × TAE. The 6.5% (wt/vol) polyacrylamide gel (acrylamide/bisacrylamide ratio, 37.5:1) was made with denaturing gradients ranging from 40%–60% for 16SrDNA fragments. Denaturant (100%) contained 7 mol/L urea and 40% formamide. The electrophoresis was run at 60 °C for 18 h at 80 V. After electrophoresis, the gels were stained with ethidium bromide rinsed in distilled water, and photographed under UV illumination (Theunissen et al., 2005). The analysis of the DGGE gel combined with the use of the program Fingerprinting II (Bio-Rad) allowed the characterization of the samples in relation to the genetic patterns whose differences have led to the formation of clusters with Pearson correlation.

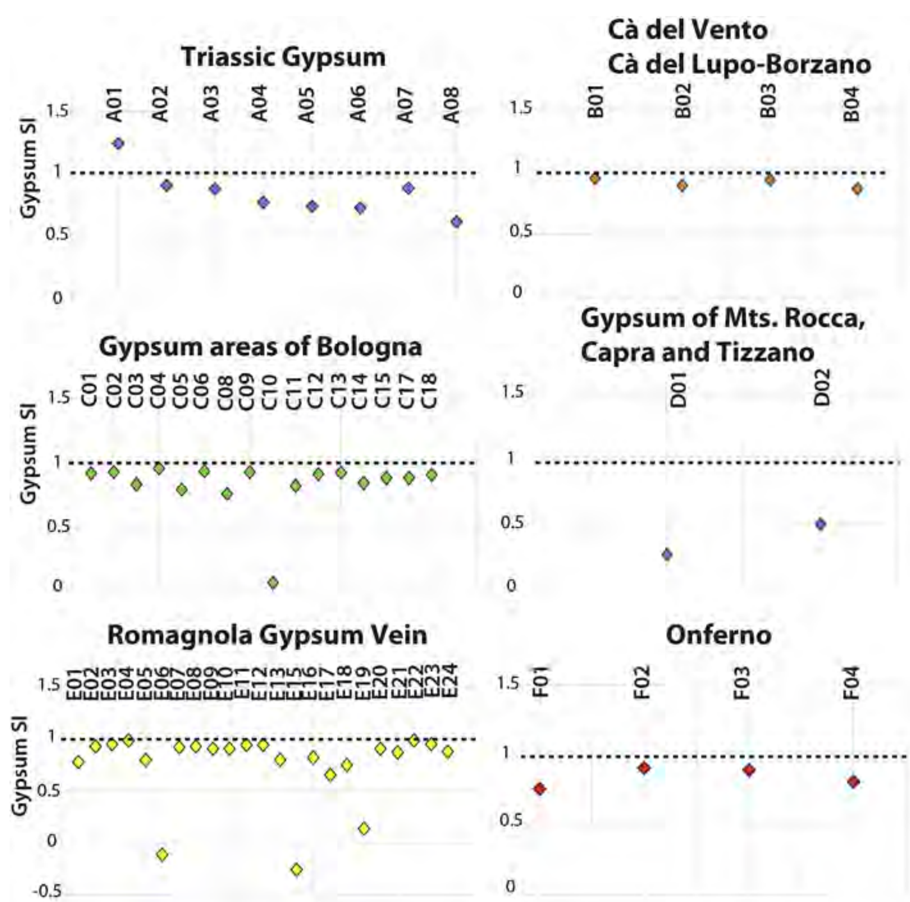

Fig. 5. Gypsum Saturation Index is reported as the mean value for each sample. Only A01 presents a SI > 1 (supersaturated). For the other sites the SI is close to 1 (equilibrium condition, i.e. saturation with respect to gypsum) or < 1 (undersaturated).

4. Results and discussion

4.1. Geochemistry

Kallis and Butler (2001) give a concise and critical summary on EU environmental and water policy from 1973 to 2000. They distinguished three main periods of environmental directives: the first from 1973 to 1986 was known as "public health protection" and "harmonization of environmental rules to avoid market distortion", the second from 1987 to 1992 was influenced by the Maastricht Treaty mainly dominated by "pollution control" and "environmental protection" ideas, whereas the third period from 1993 to 2000 saw the birth of the Water Framework Directive (WFD) characterized by "sustainable development", "integrated management", and "subsidiarity". Important standards for quality of water were pointed out with the Council of the European Communities (CEC) (1993) (Kallis and Butler, 2001) addressing the problems of wastewater and nitrate pollution and the safeguard of general "ecological quality" of all waters. The major and minor elements obtained from geochemical analyses, in all studied karst areas, are reported in Supplementary Tables 2, 3, 4, 5, 6, and 7.

Table 2 shows the maximum allowed values for Cl^-, SO_4^{2-}, Na^+, NO_3^-, F^-, NH_4^+, on the basis of the CEC 1993 standards. For each natural park karst area (A, B, C, D, E, F) it is possible to observe minimum (min), maximum (max), standard deviation (st.dev.) and median (med.) values.

Phosphates only rarely exceed the legal limit of 5 mg/l. High PO_4^{3-} values have been observed one time in A01, A02, B01, and E02 (see Supplementary Tables 2, 3, 6). Except for A01 (Poiano springs), the most abundant ion is SO_4^{2-}, as can be expected for waters in contact with gypsum. As a matter of fact, most waters are of the calcic-sulfate type, but some sinking streams classify as calcic-bicarbonic, and only Poiano (A01) falls in the field of the sodium-chloride waters according to the Ludwig-Langelier diagram (Fig. 4).

We observed a hydrochemistry homogenization of water flows from sinking streams to springs, commonly observed in karst systems from all over the world and in different kinds of lithologies (e.g. carbonates, evaporites, quartzites, etc.). Sinking stream waters, related to surface environments (river, rainfall, etc.) are usually undersaturated with respect to groundwaters, which flow in contact with soluble rocks over longer distances. Differently from carbonate rocks mainly ruled by the presence of P_{CO2} (Jeannin et al., 2016), gypsum dissolution is influenced by different mechanisms, well-explained in Klimchouk (1996) such as: temperature, pressure, grainsizes, sulfate reduction by microbes (that removes ions from the solution enabling water to dissolve more sulfate), and mainly by diffusion processes and flow velocity over the dissolving external surface. As demonstrated by James and Lupton (1978) gypsum flow time (distance) necessary to reach saturation is very short, while anhydrite requires longer travel distances. Such behavior is also shown in Fig. 4 where it is possible to see how in-cave and spring samples are very similar. Fig. 5 (mean Gypsum Saturation Index) illustrates how only sinking streams are highly undersaturated, whereas, generally, in-cave and spring waters are close to saturation in gypsum. Except for Poiano (A1), the other sites present values close to 1 (equilibrium condition), all being almost saturated with respect to gypsum, or <1 (undersaturated condition) especially for the sinking points, indicating a high potential dissolution of evaporites.

The concentration of sulfates is practically always above the limits set by the law (only 3 samples C03, E06, E15, all taken in sinking streams, are slightly below the limit of 250 mg/l).

Nitrates have exceeded the limit of 50 mg/l one time in C17, E01, E11, E21, two times in E10, four times in F01 and F03, seven times in F02, and F04. Fluorides are over limits one time in C06, C09, C18, D02, E04, E06, E09, E10, E22, E23, F02, F04, two times in C13, C15, E02, E05, E11, E15, E18, F1, three times in C14, E12, E13, E20, F3, four times in E07, E08, five times in E21 and seven times in E03. In Fig. 6 the distribution of sampling points respect to NO_3^-, F^-, and TDS is shown. Here we decided to use the WHO (World Health Organization) limit for TDS (1000 mg/l), because the European one is not established in CEC 1993.

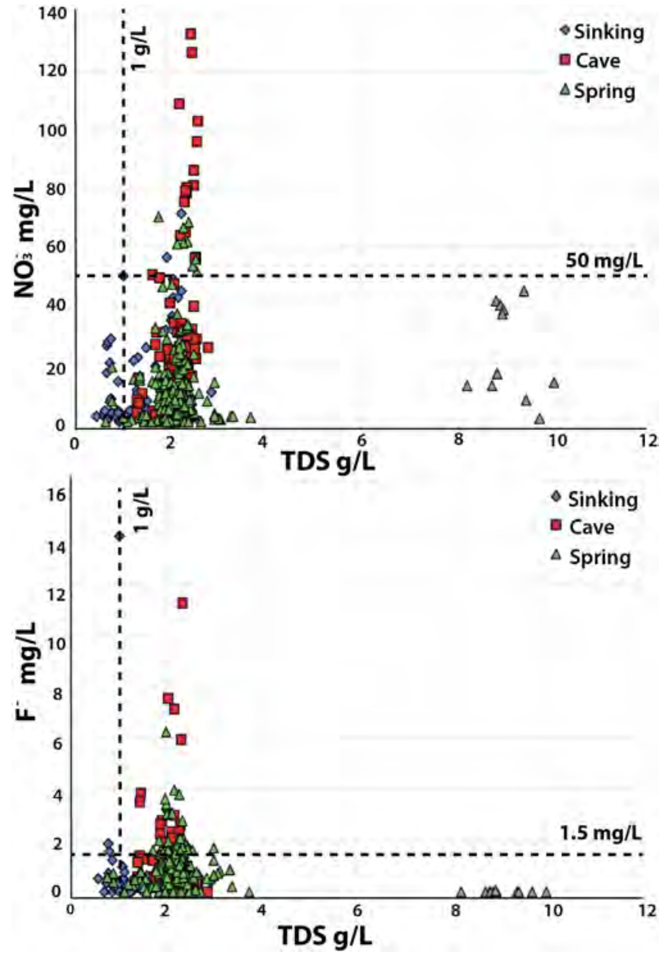

Fig. 6. Variation in nitrates (above) and fluorides (below) respect to TDS in all samples, and indication of natural surface water quality limits. The legal limit for nitrates is 50 mg/l, the recommended limit for fluorides is 1.5 mg/l, and the WHO limit of TDS is 1 g/l.

High fluoride contents are most probably related to the dissolution of Messinian Gypsum, and are particularly evident in the Romagnola Gypsum Vein area (E), and to a less extent in the Bologna area (C) and Onferno (F), keeping low values in both Reggio Emilia gypsum areas (Triassic gypsum of area A, and Messinian evaporites of area B).

The chemical variability of the waters is shown in Fig. 7, regarding four through-cave systems in which both sinking waters and spring waters could be sampled throughout the years. In the bigger systems, such as Acquafredda-Spipola (C10–C14) and Rio Stella-Basino (E06–E09), in which the stream has flow rates varying between 5 and 500 l/s (Forti et al., 1989), sinking waters are clearly undersaturated (with a lower TDS) and increase their TDS by over 150–200% during the pathway to the springs. In both the cave systems during rainy periods, such as the one occurred in autumn 2012, the high flow conditions (dilution processes) make the waters homogeneous from sinking point to spring. In contemporary in these flow conditions nitrates increase in the sinking points, indicating a provenance from the allogenic areas upstream the gypsum outcrops and out of the protected areas (parks and natural reserves) and probably interested by agricultural (Acquafredda-Spipola system) and by livestock wastes (Rio Stella-Basino system), whereas these concentrations are diluted crossing the gypsum karst area, normally lacking agricultural, cattle-raising and human activities (they are located in protected natural areas). Nitrate pollution is very evident in the Colombaia sinkhole (E10), where a small stream drains an area characterized by the presence of a couple of houses, some of the few

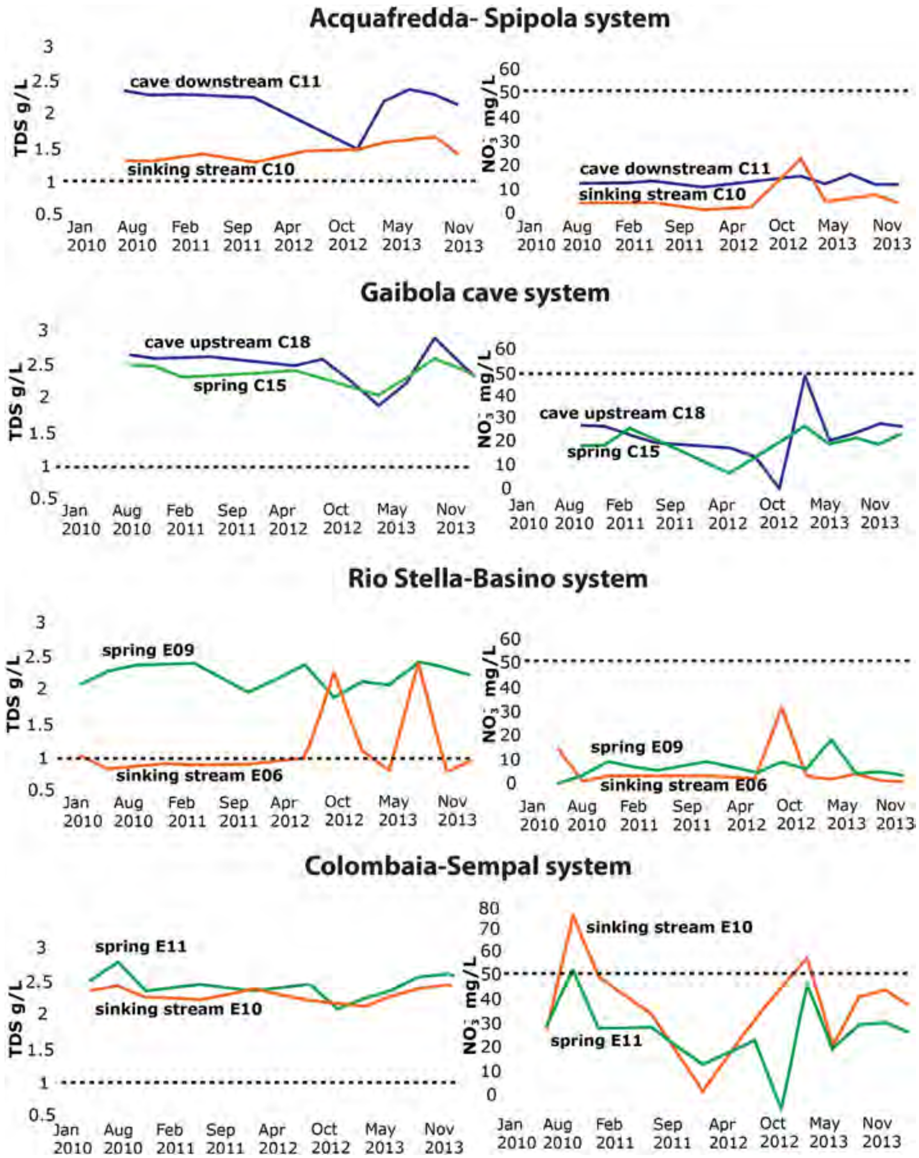

Fig. 7. Variation in TDS and nitrates in four cave systems from sinking point to spring. The orange line indicates sinking streams, the blue one in-cave samples and the green one refers to springs. Several karst systems are reported: Acquafredda-Spipola and Gaibola (SIC IT 4050001, C on the map in Fig. 1), Rio Stella-Basino and Colombaia-Sempal systems (SIC IT 4070011, E on the map in Fig. 1). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

settlements in the protected areas. These nitrate-rich waters however are slightly diluted throughout the transit from sink to the Sempal spring (E11) in particular during the driest seasons. Also in this Colombaia-Sempal system (E10–E11) the waters seem to be enriched in nitrates during the rainy periods, likely related to the mobilization of by-products of agricultural and human activities. A clear example of dilution process occurred is autumn 2010, when we observed 71.43 mg/l of NO_3^{2-} in the sinking stream (E10) and 51.58 mg/l in the spring (E11).

The TDS concentration of the waters from the Gaibola cave system (C15–C18) is clearly over the WHO limit (1 g/l), and the nitrate concentration slightly decreases moving from the cave stream to the spring. This pollution also appears to be related to human activity (drainage loss of sewage pipes from the small built-up area located above the cave system and small agricultural activities).

Maximum, minimum, and mean nitrate contents are shown in the boxplots of Fig. 8. A comparison between areas immediately shows the much higher nitrate values in the Onferno area, and the generally lower values for both Reggio Emilia areas (A and B), excluding the brackish Poiano springs (A01). In the other areas especially some monitoring points have frequently evidenced high values in nitrates (C08, C12, C14, E10, E11, F01, F02, F03, F04). Fig. 9 shows that total dissolved solids (TDS) are always above the WHO limit (1 g/l), mainly due to the fast dissolution of gypsum. The mean nitrate values for the monitoring period 2010–2014 exceed the legal limit of 50 mg/l in all Onferno samples (from F01 to F04) and in the samples of the system Colombaia-Sempal (from E10 to E11), and Befana (E01), while C17 from Gaibola cave system only one time exceeds this value reaching 50.31 mg/l, during the winter 2013. The abundance of nitrates could be related to leaking sewage systems of residential areas located close to or above the caves (C15, C17, C18 located under a residential area, E10–E11 with a sinkhole near to some houses with no sewage system), or to flood events able to mobilize nitrates coming from agricultural activities out of protected areas especially in the sinking stream (C10) of Acquafredda-Spipola system. Nevertheless this event can be considered not significant especially because the maximum NO_3^- value reaches 22 mg/l during the winter season 2012 (December 2012–February 2013).

The high nitrate contents at Onferno (F01-F04) must be explained in another way. Agricultural areas mainly interested by grapes surround

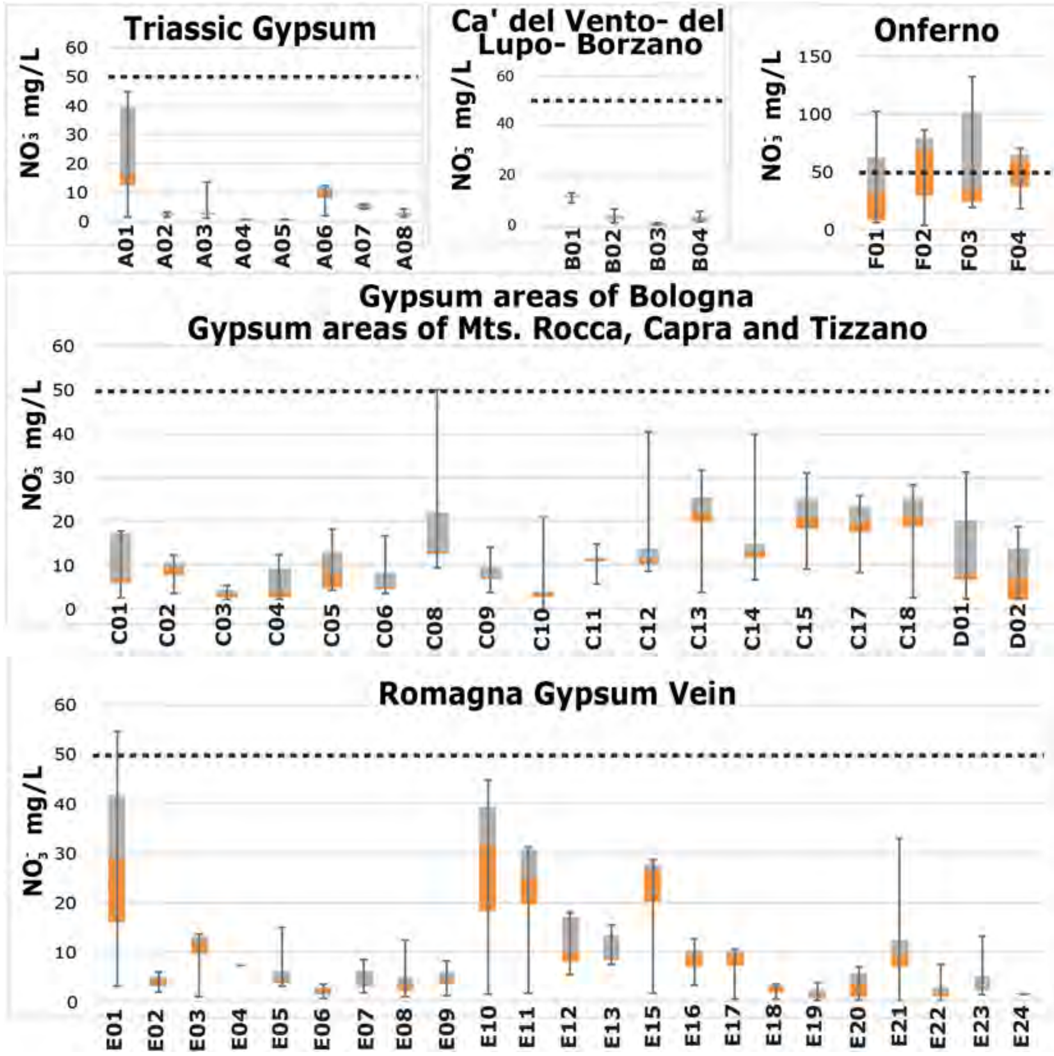

Fig. 8. Boxplots for nitrate contents in all sampled waters.

this small outcrop, but no cattle raising is present. Above the cave there is a settlement characterized by a small group of houses and a holiday farm. Nitrates are always high, suggesting these substances to be released into the cave stream over the entire year (Fig. 10). Agricultural evidences are not very clear. On the other hand, Onferno is famous for its bat colonies with six species of bats (*Miniopterus schreibersii*, *Myotis blythii*, *Myotis myotis*, *Rhinolophus euryale*, *Rhinolophus ferrumequinum*, *Rhinolophus hipposideros*), the first four use the cave for reproduction (spring-summer), while the other two use the cave to get through the winter (hibernation state) (Grazioli and Peron, 2016). These annual colonies are made out of approximately 3500 individuals, dwelling mainly in F01 and F02 sampling points and also scattered in other places of the cave. Their guano forms heaps close to the underground river, and is the source of nitrates. In the first period of monitoring the highest NO_3^- values are reported in the upstream sample, F01, (Fig.10) reaching values >100 mg/l. In the winter, when bat activity is reduced (the hibernation period, in which guano production is less), the infiltration and stream waters continue their leaching of the guano produced during the months of great bat activity, causing a constant high value of nitrates in the waters. Nevertheless, the "Pearl room" (F03), from May 2011 to February 2013 shows the highest nitrate contents. This sampling point represents a small lateral branch fed by seepage waters, probably enriched with sewage liquids from the houses above. The spring (F04) shows values that commonly are lower than F03, as a normal behavior due to dilution processes, and seem to be the sum of the other three components.

From an overall view on all samples, an evolution of the waters can easily be identified considering the concentration of nitrates and values of total dissolved solids (TDS) (Fig. 11). The sinking stream waters, with lower salinity and generally low nitrate values, increase their TDS rapidly flowing through the caves toward the springs, and some cave samples also increase their nitrate contents. This can be explained by the enrichment in nitrates due to the presence of bat colonies (and their guano) close to the flowing streams.

4.2. Microbiology

Microbiological analyses have been done in order to characterize and determine, from an ecological point of view, human and animal impact and to evaluate the evolution/change of microbial colonies with respect to the sampling site, moving from the sinking stream to the spring of several cave systems. In particular, the samples have been analyzed both with traditional microbiological techniques and with PCR-DGGE in the first year, while the microflora concentration by plate counting has been determined in the remaining part of this 5-year project.

Moreover, the relationship between microbiological and chemical results was assessed to understand the impact of water microflora on chemical parameters and vice versa. In particular, we focused on total coliform concentration: in fact this microbial group, frequently occurring in aquatic environments (including cave waters) as well as in soil,

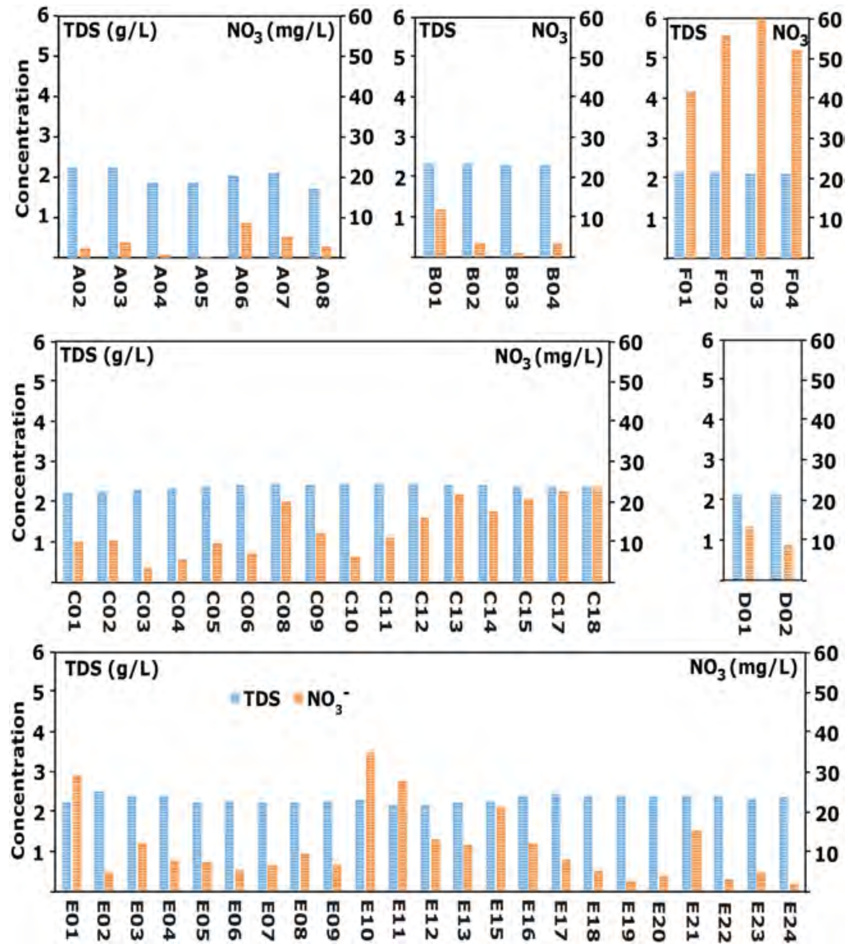

Fig. 9. Mean values in TDS and nitrates for all samples for the monitoring period 2010–2014 (excluding brackish spring Poiano A01).

is commonly used as bacterial indicator of hygienic quality of food and water (Seman et al., 2015).

Fig. 12 shows the PCR-DGGE profiles obtained for the year 2010–2011; two main groups (X and Y) (similarity 25%) were observed, and smaller clusters with increasing correlation degree have been identified. The first group (X) is subdivided in two clusters (X1 and X2, similarity 45%) that are strongly influenced by sampling season; inside these two clusters, the samples are grouped depending on the sampling

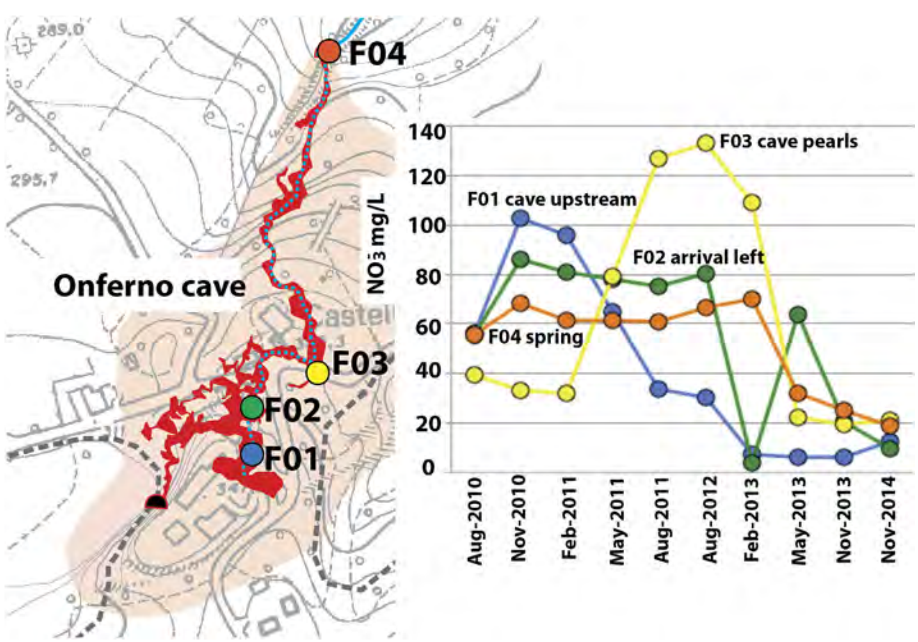

Fig. 10. Onferno cave system: survey of the cave on the left and location of samples. The graph on the right shows nitrate values during the whole project.

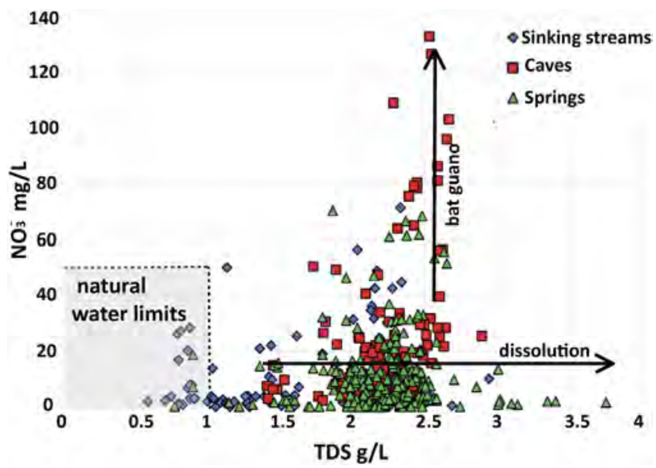

Fig. 11. Evolution of waters from the sinking point, across the caves toward the springs as shown by the comparison of TDS and nitrates in all analyzed waters (excluded A01 Poiano springs).

period. In particular, X1 includes almost all the samples collected in the fourth season of 2010 (December 2010–February 2011) and in the first season of 2011 (March–May). Otherwise, cluster X2 contains samples belonging to the third (September–November), fourth (December 2010–February 2011) season of 2010, first (March–May) and second (June–August) season of 2011. Moreover, within this cluster we created also additional sub-clusters considering geographical proximity. In fact, Onferno cave upstream (F01) showed a 98% similarity with Onferno cave spring (F04).

Surprisingly the sample collected inside the cave (F03, deriving from a lateral branch in Onferno where dripping waters are causing calcite cave pearls to form) clusterized in another small sub-cluster showing a different microflora in this specific niche. Such behavior, confirmed in two different seasons: III-2010 (September–November) and IV-2010 (December 2010–February 2011), demonstrates the different input of NO_3^- (probably due to sewage liquids) in this sampling point (F03). On the other hand, group Y is characterized by higher heterogeneity. Samples are not grouped depending on the season, with the exception of I-2011 (March–May). This group, Y, includes more than half of the samples deriving from Rio Stella-Basino (E06, E07, E18, E19 and E20) and Acquafredda-Spipola (C10–C14) systems.

Microbial biodiversity in samples deriving from the same location has also been observed; this would suggest that environmental niches typical of gypsum caves have a strong impact on cave microbiology.

Due to the complexity of the obtained microbial results, some interesting considerations can be focused on specific sites, characterized by high nitrate concentration to establish a potential correlation between nitrates and microbial growth. This relationship was previously demonstrated by Seman et al. (2015) in water samples collected from Slovak karst caves. The authors correlated nitrate and oxidability to the total coliform counts as index of anthropic activities. In particular, the brackish spring of Poiano (A01) showed, in some sampling during 2010 and 2011, a correlation between the total coliform concentration (>4 log UFC/ml) and high nitrate concentrations (38.5 mg/l). In the following two years (2012 and 2013) no monitoring has been done. Poiano waters were collected again starting from the end of 2013. In this second sampling period no total coliforms have been detected and, it results, therefore, difficult to confirm a potential correlation between nitrates and total coliforms. High nitrate pollution and total microflora are visible in the sample group E10–E11 (Colombaia cave and Sempal spring respectively), in Fig. 13, in which cell counts are often higher than 2 log UFC/ml (defined as limit contamination threshold). Interestingly, some strains collected from these sites have been identified as *Enterobacter amnigenus*, a microorganism previously isolated from water and

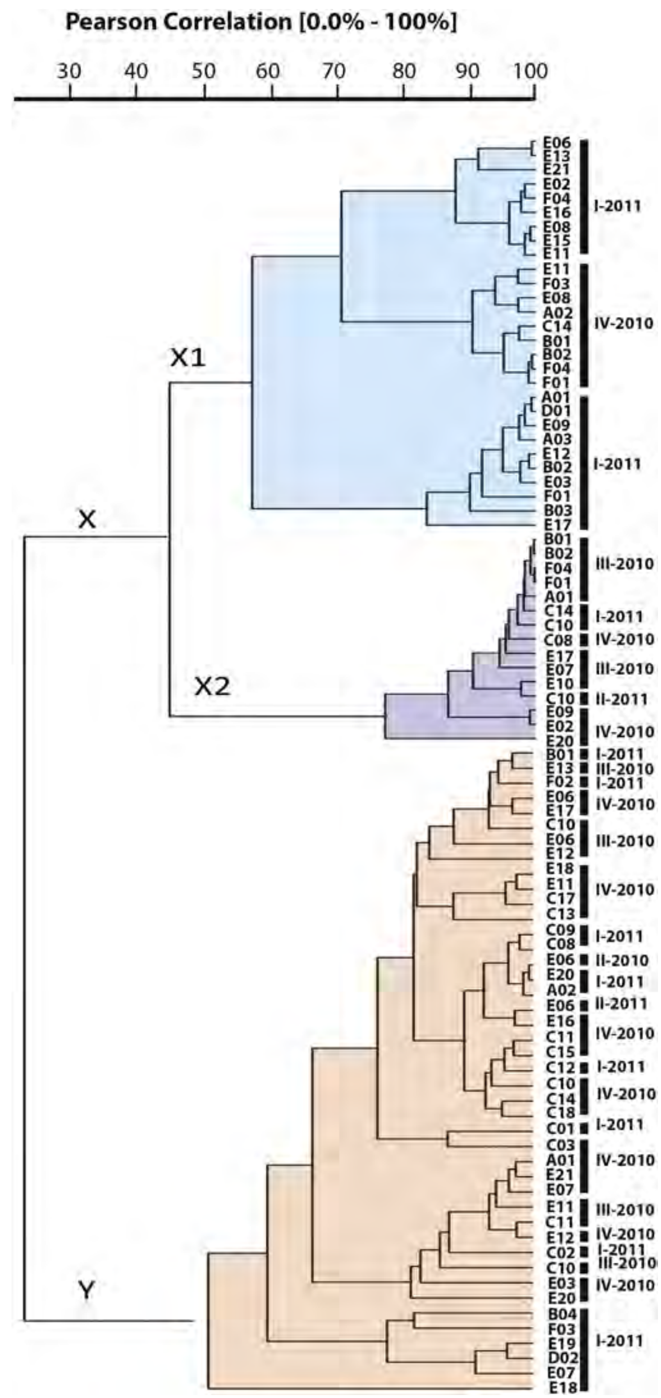

Fig. 12. PCR-DGGE profiles obtained for all samples in the years 2010–2011 (I ⟹ March–April–May; II ⟹ June–July–August; III ⟹ September–October–November; IV ⟹ December–January–February).

associated to agricultural soil (Fazzolari et al., 1990), taxonomically-related to the species *E. cloacae* and also correlated to nitrate presence due to its high capacity of nitrate uptake (Wang et al., 2010).

Nitrate concentration (solid lines) and total coliform distribution (dots) in sinking points (blue colors) and springs (green colors) from two big cave systems (Rio Stella-Basino and Colombaia-Sempal) are shown in Fig. 13. These two systems have been analyzed in detail because of their high nitrate concentrations revealed during the whole period of this LIFE project.

The correlation between nitrates and total coliforms is evident only in a few periods, and in particular when nitrate peaks are clearly visible. High total coliform distributions (>2 log UFC/ml) have been observed

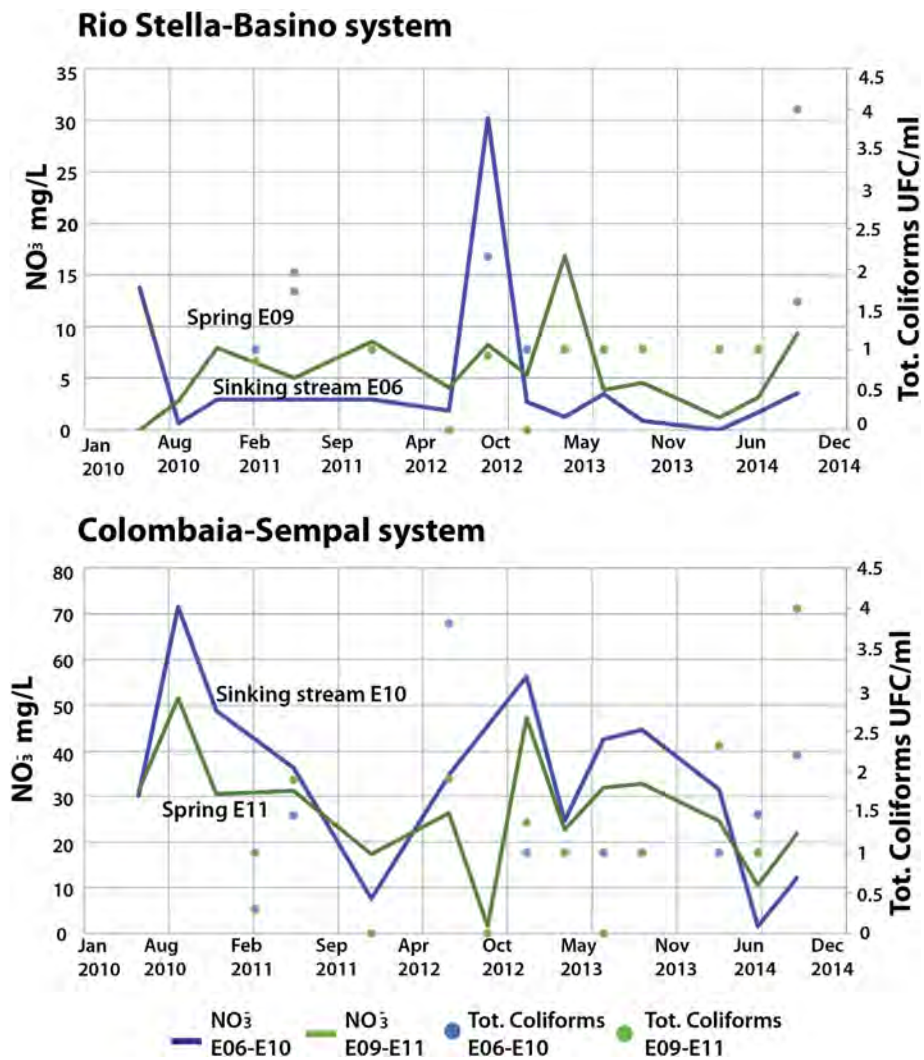

Fig. 13. Nitrate concentration (mg/l) and total coliform distribution from Rio Stella-Basino and Colombaia-Sempal cave systems. In particular in these graphs sinking points (blue colors) and springs (green colors) have been investigated to see if there is a clear correlation between NO_3^- (solid line) and total coliform (dots) concentration. E06 and E10 (blue) represent sinking points, while E09 and E11 (green) represent springs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rarely in Rio Stella-Basino system. The first occurred after a rainy period (autumn 2012) and it is well-visible in E06 (correlated to the high NO_3^- peak) while the second in E09 was registered during autumn 2014.

In Colombaia-Sempal system, sometimes, both sinking stream (E10) and spring (E11) exceed the limit of 2 log UFC/ml of total coliform distributions, especially during rainy periods. E10 reaches total coliform values close to 4 (autumn 2012) and 2.2–2.3 log UFC/ml (autumn 2014), while E11 in autumn 2012 exceeds 4 log UFC/ml.

In an overall view the high total coliform distribution in sinking streams (E06–E10) might be linked to remobilization of by-products related to human activities (cattle raising, agricultural crops, sewage pipes), while the high values in the spring points is probably related to the interaction between water and guano deposits inside the cave along the flow path.

Also in Onferno Cave (F01-04), there are high nitrate concentrations and microbial contamination, mainly due to the presence of bat colonies (Fig. 14). The presence of Proteobacteria, such as *Enterobacter* sp. (Carillo-Araujo et al., 2015; Banskar et al., 2016; Dietrich et al., 2016) are considered the dominant classes in different bat fecal and urine samples.

Moreover, from the microbiological point of view, Gaibola cave (C15–C18) showed the highest total coliform level (2.5–4.1 log UFC/ml) especially in the first three years of monitoring (Fig. 14). Contextually, nitrate concentration presents peaks in the same period or strictly before or after, suggesting a potential relationship between chemical and microbiological contaminations. This could be related to the proximity of this site to anthropic activity from the small built-up area (sewage pipes) and agricultural crops located above or close to the cave system. The presence among the microbial isolates of *Hafnia alvei*, a commensal of the human gastrointestinal tract, able to cause disease in immunocompromised patients and also specifically related to anthropic and agricultural activities, seems to confirm this statement. In fact, the key biochemical reaction of this microorganism is the reduction of nitrate to nitrite due to its nitrate reductase activity (Janda and Abbott, 2006).

Acquafredda-Spipola cave system (C10–C14) presented discontinuous microbial cell load, associated in some cases to nitrate concentration higher than 25 mg/l. The heterogeneity of these data during the project could also be due to the temperature variations: in fact, in some cases it ranged from 0 °C to 20 °C, influenced by seasonal changes. This variation can strongly affect the microbial viability and growth.

The same situation occurred in Rio Stella-Basino system (E06–E09), where the temperature sometimes exceeded 20 °C allowing microbial proliferation and, therefore, nitrate accumulation. In these water samples *Acidovorax facilis*, a Proteobacteria, commonly used as soil inoculant in agriculture and horticulture, has been identified. This microorganism is commonly characterized by nitrate reductase activity (Wu et al., 2007) and therefore could be directly related to nitrate-rich waters affected by anthropic activity.

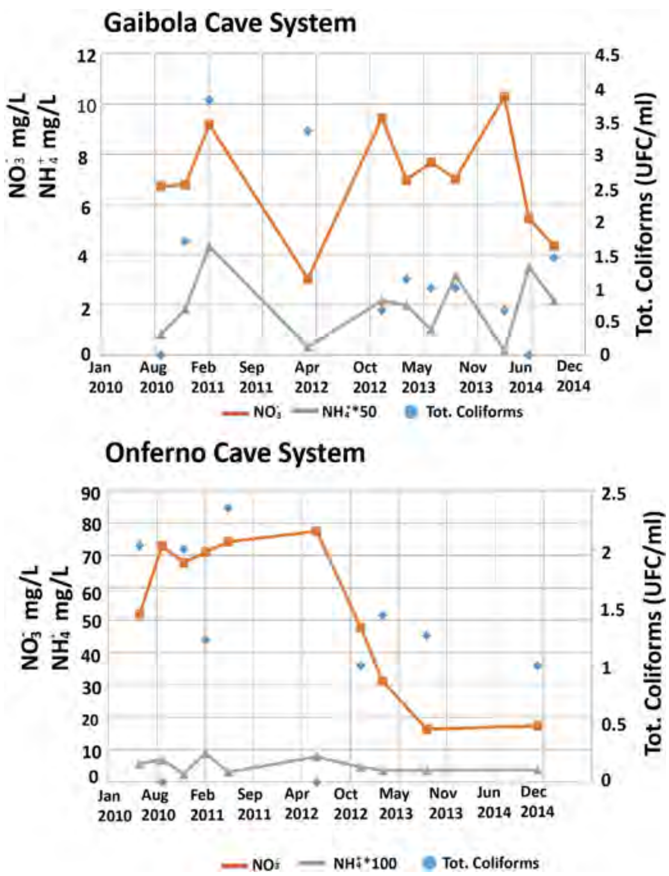

Fig. 14. Nitrate-ammonium concentration (mg/l) and total coliform distribution from Gaibola and Onferno cave systems. In these graphs we decided to use the curves based on mean values of NO_3^- (orange solid lines), NH_4^+ (grey solid lines) and total coliforms (blue dots) because these are small cave systems showing no important changes from sinking points to springs. NH_4^+ concentration is multiplied by 50 or 100 for visibility. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Finally, microbial and chemical data, grouped by sampling season, were elaborated to find out positive correlations between total coliforms and nitrate concentration and total coliforms and ammonium concentration. The results are reported in Table 3, where samples characterized by a significant positive correlation ($p \leq 0.05$) are highlighted in bold. It is possible to observe a positive correlation between nitrates and total coliforms in some sampling seasons such as: II-2011, IV-2011, II-2012, II-2013, I-2014, III-2014 (I = March–April–May; II = June–July–August; III = September–October–November; IV = December–January–February). No positive correlation between microbial counts and ammonium was observed.

None of the karst systems analyzed in the framework of this Project LIFE + can be considered in good status based on the Directive 2000/60/EC (23 October 2000). Poiano springs exhibit the effects of saline intrusion related to diapirism (Chiesi et al., 2010) and almost all the samples exceeded the quality standard limits. All other systems have high sulfate concentrations related to the dissolution of the evaporite bedrock. Notwithstanding, all these karst systems are located in protected areas (Natura 2000 sites), where pollution events seem to be related mainly to natural sources such as dissolution of soluble evaporite rocks and presence of abundant bat guano.

5. Conclusions

The chemical and microbiological analyses of waters entering, flowing inside caves and coming out from the main gypsum aquifers of Emilia Romagna region have been analyzed over a period of five years (2010–2014) with seasonal sampling campaigns. The results have revealed the rapid enrichment of the waters in total dissolved solids (TDS), mainly Ca and SO_4, but also some local episodes of high nitrate and ammonium contents. As suggested by Menció et al. (2016), nitrate pollution may facilitate the incorporation of additional substances and homogenize lithological differences. From an overall standpoint, we didn't observe any variation related to high NO_3 concentration. High concentrations of specific ions can be related not only to anthropic-related pollution (residential areas, septic effluents or agricultural activities and fertilizer lixiviates, livestock waste) but also to biogeochemical processes and natural phenomena (bat guano, degradation of naturally derived organic matter from the soil, wildlife, atmospheric input) (Menció et al., 2016). In these peculiar shallow groundwater gypsum karst areas controlled by multiple inputs, it seems to be fraught to determine a “nitrate background concentration” from only natural sources (Panno et al., 2006; Menció et al., 2016). In fact, these pollutants are variable in concentrations depending mostly on seasonal effects and local precipitation events. PCR-DGGE analyses have shown a wide microbiological variety, both location- and season-dependent. Although high microbial counts were often related to high nitrate contents, these are not always due to pollution events. Microbial counts can vary largely, especially, because of the large temperature changes (related to waters flowing outside of the caves). These changes in microbial abundance and variety occur on a very small (ecological niche) scale.

Table 3
Correlations between total coliforms (log UFC/ml) - nitrate concentration (NO_3^- mg/l) and total coliforms (log UFC/ml) - ammonium concentration (NH_4^+ mg/l) in waters grouped by season. Samples characterized by a significant positive correlation ($p \leq 0.05$) are highlighted in bold.

Sampling season	Correlation tot colif/NO_3^-	Correlation tot colif/NH_4^+
II-10	0.16	−0.01
IV-10	0.25	−0.17
I-11	0.15	0.25
II-11	**0.42**	0.21
IV-11	**0.48**	0.1
I-12	0.26	−0.07
II-12	**0.72**	0.04
I-13	0.12	−0.18
II-13	**0.41**	0.17
III-13	0.32	0.08
I-14	0.41	0.38
III-14	**0.57**	0.14

This study highlights a quite microbially uncontaminated character of almost all the waters analyzed in the gypsum areas, mainly because most of these special habitats are protected by law and, thus, subjected only to minor anthropic activities. However, some local cases have shown a regular high level of both nitrates and microbial cell counts, with clear and continuous episodes of pollution, where remediation measures should be undertaken. In other sites, the natural presence of bat colonies induces a permanent pollution, and remediation measures would not be desirable.

Nevertheless, almost all the waters analyzed for the Project LIFE + 08NAT/IT/000369 “Gypsum” exceed legal limits for natural waters, due to the high solubility of gypsum rock.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2017.03.270.

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