Rainwater pH does not influence retention of *Escherichia coli* cells and *Bacillus subtilis* spores in pyroclastic soil

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Abstract: The main aim of this study was to analyze the influence of rainwater pH on retention of *Escherichia coli* cells and *Bacillus subtilis* spores in a pyroclastic soil. Nine column experiments were performed in intact soil blocks. The experiments were carried out using antibiotic resistant strains, to be distinguished from the natural background of soil. Column tests were performed at three different solution pH (1.5, 5.6, 8.0). The pH range was chosen taking also into consideration pH values measured in rainwater samples collected at the site were soil blocks were carved. Some physical and hydraulic properties of soil were analyzed by means of standard laboratory tests. Despite the various experimental conditions, comparable results were obtained in all tests. It was observed that retention of both *E. coli* cells and *B. subtilis* spores is independent from rainwater pH. This phenomenon is due to a high buffering capacity of the studied soil that inhibits pH-dependent processes that influence microbial transport. The findings presented here have applications within the risk assessment for microbial pollution of groundwater and have the advantage of showing that variations in rainwater pH over time do not cause significant modifications in self-purification processes within the analyzed soil.

Keywords: andosol; *Bacillus subtilis*; *Escherichia coli*; column experiment; rainwater pH.

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Riassunto: *Scopo principale di questa ricerca è stato analizzare l'influenza del pH dell'acqua piovana sulla ritenzione di cellule di* Escherichia coli *e di spore di* Bacillus subtilis *in un suolo di origine piroclastica. Sono stati eseguite prove su Nove colonne in blocchi di suolo intatto raccolto da aree di pascolo all'interno di un'area di studio in Italia Meridionale dove il bestiame al pascolo causa un'inquinamento discontinuo delle acque di sorgente. Gli esperimenti sono stati portati avanti utilizzando ceppi antibiotico resistenti, al fine di essere distinti dal sottofondo microbico naturale, che è stato analizzato prima dell'esecuzione dei test sulle colonne. I test sulle colonne sono stati eseguiti utilizzando soluzioni a tre differenti gradi di pH (1.5, 5.6, 8.0). La gamma di pH è stata scelta prendendo in considerazione valori di pH misurati in campioni d'acqua di ruscellamento raccolti nel sito dal quale sono state estratti i blocchi di suolo. L'intero studio ha preso in considerazione una più ampia gamma di valori di pH al fine di ottenere informazioni utili anche in aree dove le precipitazioni acide interagiscono con le componenti del suolo prima fungere da ricarica per le acque sotterranee. Alcune proprietà fisiche ed idrauliche dei suoli sono state analizzate attraverso prove standard di laboratorio. I risultati sono stati comparabili con quelli ottenuti in studi precedenti, suggerendo inoltre una buona omogeneità dei suoli studiati in un'ampia area all'interno degli Appennini Meridionali. Nonostante le diverse condizioni sperimentali, sono stati ottenuti risultati comparabili in tutte le prove. Si è osservato che la ritenzione di cellule di* E. coli *come anche di spore di* B. subtilis *è indipendente dal pH dell'acqua piovana. Questo fenomeno è dovuto all'elevata capacità di tamponamento dei suoli studiati che inibisce i processi pH-dipendenti i quali influenzano il trasporto microbico. I risultati quì presentati hanno appicazioni nell'ambito della valutazione del rischio dell'acqua di sottosuolo per l'inquinamento microbico e mostrane che cambiamenti di pH dell'acqua piovana nel tempo non causano significanti modificazioni nei processi di autopurificazione all'interno dei suoli analizzati.*

Introduction

In southern Italy, microbial contamination of groundwater is often observed in carbonate aquifers (Celico et al., 2004a; Naclerio et al., 2008). Such a contamination in carbonate systems deteriorates drinking water quality and causes human health to be at risk in Italy as well as in other Countries (e.g., Personné et al., 1998). Since no significant retention of microorganisms is expected in fractured and karstified limestone, due to the wide aperture of discontinuities (e.g., more than 100 µm in a test site; Petrella et al., 2008), an understanding of bacterial transport and attachment in soil media is of utmost importance in the protection of groundwater resources. In porous media, such as soils, the movement of bacteria is mainly controlled by advective–dispersive transport and attachment to solid matrices (Hornberger et al., 1992). The retention of bacteria in soil can be influenced by properties of soil media (e.g., surface charge, grain size), solution chemistry (e.g., ionic strength and pH) and characteristics of bacteria (e.g., cell surface charge and

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hydrophobicity) (e.g., Fontes et al., 1991). With regard to pyroclastic soil in southern Italy, a recent work demonstrated that hydrophobic interaction does not play a significant role in the filtration processes of spores of *Bacillus subtilis* and *Bacillus cereus*, despite the hydrophobic nature of *Bacillus* spores and the very high organic matter content in such a soil (Naclerio et al., 2009a). Retention of *Escherichia coli* cells in the same soil is influenced by adsorption to the clay fraction, while the organic matter content does not play a significant role (Naclerio et al., 2009b). The pore size exclusion phenomenon is another important factor to consider when analyzing retention of both *E. coli* cells and *Bacillus* spores within such soils (Naclerio et al., 2009a, b), with *B. cereus* spores more retained than those of *B. subtilis*.

To date, no information is available concerning the influence of rainwater pH on transport of microorganisms within pyroclastic soil. The important effects of solution pH on bacterial transport was demonstrated in different works in other media. For example, batch experiments showed that the adhesion of *Pseudomonas putida* to goethite decreases gradually in the pH range between 3.0 and 8.0, and sharply between 8.0 and 9.0 (Jiang et al., 2007). Other column experiments demonstrated that even *E. coli* transport is influenced by solution pH in goethite-coated sand, with bacterial mass recovery lower than 7% at pH around 6 and 7, and higher than 75% at pH around 8 and 9 (Kim et al., 2008). The authors suggested that around pH 8 the positively charged goethite-coated sand converted to being negatively charged, causing an electrostatically repulsive interaction between sand and *E. coli* cells. Ams et al. (2004) showed that the presence of Fe-oxyhydroxide-coatings on quartz surfaces significantly enhances the adsorption of *B. subtilis* vegetative cells and *Pseudomonas mendocina* cells and that the extent of adsorption decreases with increasing pH.

The main purpose of this study was to analyze the influence of rainwater pH on retention of *E. coli* cells and *B. subtilis* spores in a pyroclastic soil collected at an experimental field site in southern Italy, taking into consideration the soil chemistry. The pH range for column experiments was chosen taking also into consideration pH values measured in rainwater samples collected close the same site during the research

Methods

Study area

The Acqua dei Faggi experimental site (Longano, Molise region, southern Italy) consists mainly of limestone (Cretaceous-Oligocene). In pasture areas carbonate rocks (R) underlie a pyroclastic soil (Celico et al., 2004a) that can be classified as Vitric Andosol (as defined by the FAO, 1988). The soil is characterized by (Naclerio et al., 2008) high organic-matter content (20% to 35%), which allow it to be classified as organic soils (Pt) (Unified Soil Classification System, ASTM, 2006) according to the fibrous structure. Specific gravity values ranging from 2.06 g cm^{-3} to 2.36 g cm^{-3} are found to be compatible with values of alkali-potassic volcanic minerals typical of such soils of pyroclastic origin derived from eruptive centres of the Campania region (south of the Molise region). The soil is characterized by a profile A/R. Horizon A is usually less than 15 cm thick. Vitric Andosols are widely distributed within the carbonate Apennine chain (di Gennaro, 2002), that is a primary source of groundwater in southern Italy supplying an average volume of 4.1 · 109 m^3 y-1 (Celico, 1983).

The aquifer boundaries are fault zones that act as barriers to groundwater flow and compartmentalize the aquifer system. At basin scale, the groundwater flows westwards towards the perennial spring (Celico et al., 2006). Both infiltration process and groundwater flow are diffuse in a well-connected fracture network, and the fracture spacing is sufficiently dense to apply the continuum approach at the metric scale (Petrella et al., 2007). The fractured limestone is hydraulically similar to granular porous media (Petrella et al., 2008). Thus, during infiltration events, microorganisms are diffusely transported through the soil and then through both unsaturated and saturated limestone (Celico et al., 2004a; Naclerio et al., 2008). Microbial contamination of groundwater at this site is mainly due to cattle grazing (several hundred heads throughout the year), as well as in other sites (Allocca et al., 2008; Celico et al., 2004b). Pasture areas cover 55% of the site, while a beech (*Fagus sylvatica L.*) woodland covers 45%.s

Rainwater sampling and pH measurements

Rainwater samples for pH measurements were collected at a rain sampler located within the Acqua dei Faggi experimental site, where soil samples were collected for column experiments. The sampling was carried out on a daily (during March 2009), weekly (March 2006 to February 2007) or monthly (November 2008 to June 2008) basis. Polyethylene bottles (10 l) containing about 300 ml of vaseline oil to prevent evaporation processes even under very hot summer conditions were used to co trating waters than pure wet-fall compositions (Meijer, 2002). The pH measurements were performed in the laboratory with a pH electrode.

Soil characterization

Properties (organic matter and grain size) of soil samples used for column tests were analyzed by means of standard laboratory tests, according to the ASTM standard procedures (ASTM, 2006). At the end of each column experiment the hydraulic conductivity of soil blocks was calculated through a standard permeameter.

Chemical analyses on saturated paste extracts

Three samples of Vitric Andosol were collected to carry out chemical analyses on saturated paste extracts.

Saturated paste extracts were prepared by adding deionized water to 100-150 g of soil until it reached a condition of complete saturation, according to USDA (2004). Saturated pastes were prepared and left to equilibrate overnight at room temperature. The extracts obtained by centrifugation were filtered through 0.45 µm nylon syringe filters for chemical analyses.

Analyses included electrical conductivity (EC), and major ions. EC was measured through a WTW laboratory conductimeter. Bicarbonate was determined by tritation with HCl. Major ions concentrations were determined by ion chromatography using a DIONEX ICS-100= model, connected to a DIONEX AS40 Automated Sampler. For cation preservation, samples were acidified at pH <3 with a concentrated HNO_3 solution. Soil pH was measured on a soil water suspension (1:2.5, wt:vol).

Column experiments

Nine intact soil blocks (181.36 cm^2 by 11 cm deep) were collected randomly from the Acqua dei Faggi experimental site, according to described method (Celico et al., 2004a; Naclerio et al., 2008). All blocks were covered with plastic and transported to the laboratory where the experimental procedure was commenced immediately at room temperature.

The column experiments were carried out in a standard permeameter. A solution with 0.001M $CaCl_2$ was used as simulated rainwater to prevent dispersion of clays within the soil and the plugging of the column (McMurry et al., 1998). A peristaltic pump was used to constantly force the solution through the soil. A total of 3000 ml was injected at a velocity of 3 mm h^{-1}. The interaction between *E. coli* cells and soil blocks was analyzed through the strain UK19 resistant to ampicillin and kanamicin (Naclerio et al., 2009b). No fecal coliforms resistant to these antibiotics were observed in the natural background population of soil blocks. The interaction between *B. subtilis* spores and the same soil blocks was investigated through the strain ER229, resistant to rifampicin and erythromycin, that was kindly given by E. Ricca (Università degli Studi di Napoli "Federico II"). No microorganisms resistant to these antibiotics were observed in the natural background population of soil blocks. Non-pathogenic *B. subtilis* spores were chosen to carry out these experiments because they are a better indicator than pathogenic *B. cereus* spores for analyzing the interaction between *Bacillus* spores and groundwater at site scale, in the studied aquifer system (Naclerio et al., 2009a). Taxonomic classification of *Bacillus* isolates detected in the studied soil demonstrated that they are the most representative non-pathogenic and pathogenic species, respectively (Naclerio et al., 2009a). *B. subtilis* cells were grown in Difco sporulation medium for 2 days at 37°C; spores were harvested by centrifugation and purified by washing and lysozyme treatment as previously described (Nicholson and Setlow, 1990).

To the top of each of soil blocks, 1.0 · 1010 UK19 cells and 1.0 · 1010 ER229 spores in 0.001 M $CaCl_2$ were applied before simulating infiltration. The number of cells and spores inoculated was in the same order of magnitude of those used in previous studies on the same soil medium, in order to acquire comparable results. Nine experiments were carried out, three for each solution pH (1.5, 5.6, 8.0). The pH values were chosen taking into consideration the pH range in rainwater collected during the observation period, and minimum pH values observed in acid rain throughout the world.

During each experiment, soil block drainage was entirely collected beneath the outflowing holes using sterile plastic tubes. To test retention of *E. coli* cells, 100 μl of each water sample and relative serial dilutions were plated in triplicate on Chromogenic Coliform Agar (CCA, Biolife) supplemented with antibiotic (50 μg ml^{-1} of rifampicin and 5 μg ml^{-1} of erythromycin), after heat treatment (at 80°C for 10 min), and incubated at 37°C for 24 hours. The number of *E. coli* cells and *B. subtilis* spores was estimated as colony-forming units (CFU) using only plates where the number of colonies ranged from 30 to 300.

The *t*-test was applied at a level of significance of 0.05, to determine the effects of solution pH on retention of both microorganisms.

Results

Rainwater pH

Considering 5.6 as the pH of cloud water at equilibrium with atmospheric CO_2 (Charlson and Rodhe, 1982), the pH of rainwater collected at the study area (4.7 to 7.9; volume-weighted mean 6.4; Tab. 1) was in the alkaline range in the 88% of the analyzed samples, and in the moderately acidic range in the 12% of the samples. The acid rain is due to higher concentrations of strong acids that constitute major products of atmospheric pollution of anthropogenic origin (e.g., Herut et al., 2000), even though important contributors may be also natural acid inputs to the atmosphere. For example, acidic rainwater was detected in southern Italy at the Etna active volcano, where the pH value was observed in the 2.4 to 5.6 range, on the summit of the volcano (Aiuppa et al., 2006). In such an area, plume-derived acidic compounds are responsible for the prevailing acidic composition of rainwater samples, with emphasis on those collected on the summit of Mount Etna. A lower pH value (1.5) was measured in rainwater falling over Wheeling in West Virginia in 1979 (Park, 1987).

Differently, the alkaline rainwater at the study area is probably due to carbonate dust particles in the atmosphere that buffers the acidity of rainwater (e.g., Herut et al., 2000). For example, alkaline rainwater was analyzed in northwestern Italy where alkalinity (pH up to 8.0) was related to Saharan dust (Rogora et al., 2004)

Soil blocks properties and saturated paste extracts.

The soil blocks used for column experiments were characterized by high organic matter content (25% to 32%; Tab. 2), that is in the range observed in other blocks collected at the same site (20% to 35% in Naclerio et al., 2008; 20% to 34% in Naclerio et al., 2009a; 21% to 35% in Naclerio et al., 2009b). Grain size analyses show (Tab. 2) a global homogeneity testified by a narrow envelope of grain size curves, with the content in finer-grained pyroclastics (silts and clays) ranging from 16% to 22%. The content of the only clay fraction ranged from 2% to 4%. The hydraulic conductivity of soil blocks ranged between 5.3 · 10^{-6} and 7.9 · 10^{-5} m s-1 (Tab. 2), in good agreement with previous findings at the same site (1.8 · 10^{-5} to 4.5 · 10^{-5} m s-1 in Naclerio et al., 2009a; 6.5 · 10^{-6} to 2.5 · 10^{-5} m s-1 in Naclerio et al., 2009b).

The properties of the analyzed soil are similar to those of other pyroclastic soils in southern Apennine, such as that of the Acqua

Tab. 1 *pH in rainwater samples (dates of collection are given in day/month/year)*

Date	27/04/06	03/05/06	10/05/07	05/06/06	15/06/06	10/07/06	31/07/06	30/08/06	18/09/06
pH	6.71	6.10	6.39	5.84	5.90	6.73	7.12	6.81	6.70
Date	02/10/06	10/10/06	08/11/06	27/11/06	14/12/06	20/12/06	09/01/07	22/01/07	29/01/07
pH	5.90	5.46	6.27	6.29	6.41	5.88	4.70	4.77	5.97
Date	26/02/07	30/11/07	12/12/07	14/01/08	21/01/08	04/02/08	03/03/08	01/04/08	06/05/08
pH	4.90	6.50	6.50	6.31	6.53	6.58	6.51	6.04	7.87
Date	04/06/08	02/03/09	03/03/09	04/03/09	05/03/09	06/03/09	13/03/09		
pH	6.84	7.05	7.05	6.15	6.41	6.80	6.43		

Tab. 2 *Properties of soil blocks used to carry out the column experiments (blocks 1a to 1c were used at a solution pH of 1.5; blocks 2a to 2c were used at a solution pH of 5.6; blocks 3a to 3c were used at a solution pH of 8.0).*

Soil block	Organic matter (%)	Sand (%)	Silt (%)	Clay (%)	Hydraulic conductivity (m s^{-1})
1a	32	76	21	3	$2.0 \cdot 10^{-5}$
1b	25	81	16	3	$5.2 \cdot 10^{-5}$
1c	28	78	18	4	$6.1 \cdot 10^{-6}$
2a	31	78	20	2	$3.1 \cdot 10^{-5}$
2b	25	80	17	3	$1.5 \cdot 10^{-5}$
2c	25	76	22	2	$2.3 \cdot 10^{-5}$
3a	30	76	21	3	$5.3 \cdot 10^{-6}$
3b	28	82	16	2	$7.9 \cdot 10^{-5}$
3c	27	79	17	4	$3.6 \cdot 10^{-5}$

della Madonna experimental site (Naclerio et al., 2009b). In such a site the soil cover is characterized by organic matter content ranging from 12% to 18%, a global homogeneity of grain size (gravel 4% to 5%, sand 83% to 86%, silt 9% to 13%), and hydraulic conductivity ranging from $4.9 \cdot 10^{-5}$ to $6.0 \cdot 10^{-5}$ m s-1.

The results of the analyses on saturated paste extracts are summarised in Table 3. The soil is nearly neutral with pH values ranging from 7.0 to 7.7 (mean 7.4). Regarding the water-soluble ions, the inorganic anion dominating the anion suite was $(HCO_3)^-$, while the dominating cation was Ca^{2+}. A more detailed analysis of soil water chemistry will be described in a dedicated paper, because these details are not useful to discuss about the main scope of the present paper.

Tab. 3 *pH, EC (µS cm^{-1}) and concentrations (µeq l^{-1}) of the ions analysed in saturated paste extracts obtained from soil samples.*

Parameter	Sample 1	Sample 2	Sample 3	Mean
pH	7.0	7.4	7.7	7.4
EC	578	429	585	531
Na^+	287	353	306	315
$(NH_4)^+$	139	12	13	55
K^+	160	50	39	83
Mg^{2+}	140	89	81	103
Ca^{2+}	4178	2972	4449	3866
Cl^-	247	356	370	324
$(NO_2)^-$	58	342	283	228
$(NO_3)^-$	2693	1277	2335	2102
$(SO_4)^{2-}$	269	185	332	262
$(HCO_3)^-$	1160	1160	1391	1237

Column experiments

The proportion of *E. coli* cells recovered at the end of all column tests ranged from 0.02% to 0.30% (Fig. 1), and no significant differences were observed between experiments that were carried out at different solution pH. The mean percent of cells recovered at the end of the experiments was 0.06% (st. dev. 0.04%) at a solution pH of 1.5, 0.14% (st. dev. 0.08%) at a solution pH of 5.6, and 0.13% (st. dev. 0.15%) at a solution pH of 8.0.

Similar findings are observed when analyzing the proportion of *B. subtilis* spores recovered at the end of the same experiments. The percent of spores recovered ranged between 2.00% to 5.80% (Fig. 2) and also in this case no significant differences were observed between experiments that were carried out at different solution pH. The mean percent of spores recovered at the end of the experiments was 3.60% (st. dev. 1.97%) at a solution pH of 1.5, 3.33% (st. dev. 1.10%) at a solution pH of 5.6, and 3.13% (st. dev. 1.35%) at a solution pH of 8.0.

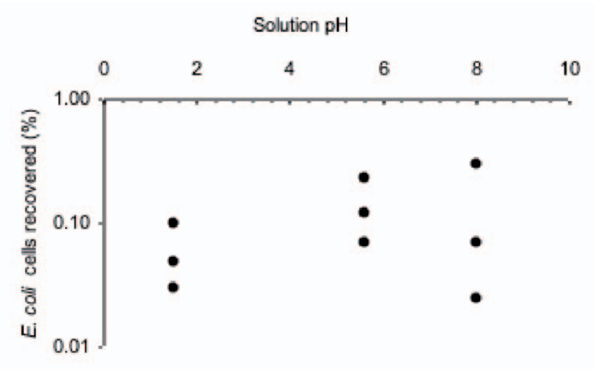

Fig. 1 *Percent of E. coli cells recovered at the end of the column experiments.*

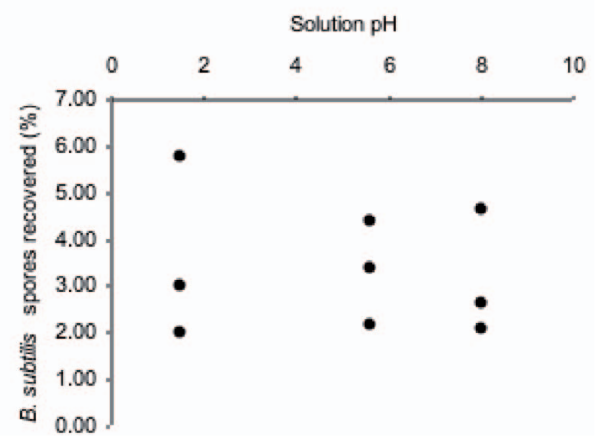

Fig. 2 *Percent of B. subtilis spores recovered at the end of the column experiments.*

Discussion and conclusions

The results of the column experiments suggest that retention of *E. coli* cells and *B. subtilis* spores is independent from pH of the aqueous solution used as rainwater. The said independence is emphasized when comparing these results with those obtained, in the same soil, in previous column experiments that were carried out using the same procedure and a solution pH ~6.0. The overall mean percent of *E. coli* cells recovered at the end of the experiments presented here (0.11%; st. dev. 0.09%; n = 9) is not significantly different from that (0.17%; st. dev. 0.12%; n = 12) obtained in the former experiments (Naclerio et al., 2008; Naclerio et al., 2009b). Concordantly, the overall mean percent of *B. subtilis* spores recovered at the end of the experiments presented here (3.35%; st. dev. 1.33%; n = 9) is not significantly different from that (5.55%; st. dev. 6.30%; n = 6) obtained previously (Naclerio et al., 2009a).

Relationships between solution pH at the top of soil blocks and that measured at the outflowing holes (Tab. 4) clearly suggest that the rainwater-pH-independent transport of both *E. coli* cells and *B. subtilis* spores through such a soil is due to the very high buffering capacity of the medium, which is due to the abundance of carbonate minerals, mainly $CaCO_3$, that buffers the acidity of rainwater. The pH of water samples collected after flowing through the soil columns is always in the pH 6.8 to 7.3 range (Tab. 4), despite the great differences in pH of simulated rainwater at the top of the same columns (pH 1.5 to 8.0). Thus, the independence of microbial transport from rainwater pH can be explained assuming a rapid and significant modification of solution pH early during migration through the medium. This rapid modification inhibits pH-dependent processes that influence microbial transport, such as electrostatic adsorption (depending on soil pH, soil and microbial surfaces can bear net negative, or positive, or no charge) and filtration (with regard to clays, lower pH values lead to the “house-of-cards” structure, that gives the clay aggregates the appearance of “hutches” housing the bacteria; Lünsdorf et al., 2000)

Tab. 4 *pH of simulated rainwater (pH_in) and mean pH of water collected at the outflowing hole (pH_out) during the column experiments (standard deviation given in brackets).*

Soil block	pH_in	pH_out
1a	1.5	6.8 (0.3)
1b	1.5	6.9 (0.3)
1c	1.5	6.8 (0.2)
2a	5.6	7.3 (0.1)
2b	5.6	7.3 (0.1)
2c	5.6	7.3 (0.2)
3a	8.0	7.2 (0.1)
3b	8.0	7.3 (0.2)
3c	8.0	7.2 (0.2)

The findings presented here have applications within the risk assessment for microbial pollution of groundwater, with emphasis on those aquifer systems where topsoils are characterized by high buffering capacity. The results obtained during such a study show that variations in rainwater pH over time, due to natural and anthropogenic inputs to the atmosphere, do not cause significant modifications in self-purification processes within soils similar to the tested one. Taking into consideration the wide distribution of Vitric Andosols within the carbonate Apennine chain in southern Italy, similar findings are expected in a broad range of sites.

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