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Image analysis for quantification of bacterial rock weathering

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Abstract

A fast, quantitative image analysis technique was developed to assess potential rock weathering by bacteria. The technique is based on reduction in the surface area of rock particles and counting the relative increase in the number of small particles in ground rock slurries. This was done by recording changes in ground rock samples with an electronic image analyzing process. The slurries were previously amended with three carbon sources, ground to a uniform particle size and incubated with rock weathering bacteria for 28 days. The technique was developed and tested, using two rock-weathering bacteria *Pseudomonas putida* R-20 and *Azospirillum brasilense* Cd on marble, granite, apatite, quartz, limestone, and volcanic rock as substrates. The image analyzer processed large number of particles (10^7–10^8 per sample), so that the weathering capacity of bacteria can be detected.

Keywords: *Azospirillum*; Image analysis; Rock weathering; *Pseudomonas*

1. Introduction

Weathering of stone is caused by physical (temperature, wedging, crystallization), chemical (air pollution, soil moisture, acid rain), and biological processes (Hirsch et al., 1995a,b; Goudie and Parker, 1999). Microorganisms on rock surfaces, in cracks, and in pore spaces of sand stone and granite sometimes form biofilms (a.k.a. bio-deteriorative patina; rock varnish) (Krumbein and Jens, 1981; De la Torre et al., 1993) that contribute to the disintegration of rocks. Microbial rock weathering is common in all climate zones, usually acts very slowly (Sun and Friedmann, 1999), and has been observed in hot (Adams et al., 1992) and cold deserts (Friedmann and Kibler, 1980), and in every region, such as the Mediterranean Basin (Verges, 1985), Europe (Ascaso et al., 1990), The Americas (Friedmann and Kibler, 1980), Asia (Dahanayake and Subasinghe, 1990), and Antarctica (Friedmann, 1982). Most studies focused on deterioration of

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exposed stones in buildings (Palmer et al., 1991; Flores et al., 1997), churches (Ascaso et al., 1990), monuments (Del Monte et al., 1987; Danin and Caneva, 1990; Arino and Saiz-Jimenez, 1997; Flores et al., 1997), and exposed rocks and cliffs (Danin, 1993; Puente et al., 2004). Most studies described effects (Atlas et al., 1988; Johnston and Vestal, 1993) and the microorganisms involved (Hirsch et al., 1995b; Ferris and Lowson, 1997). Organic weathering mechanisms are poorly understood, except that some microorganisms produce acid in vitro (Hirsch et al., 1995b) or that organic acids were detected in weathered stones (Palmer et al., 1991), making this a likely mechanism. Hirsch et al. (1995a,b) demonstrated that acids produced by microorganisms, as by-products of their metabolism, can dissolve rocks. Microorganisms involved in rock weathering are lichens (Barker and Banfield, 1998), fungi (Hirsch et al., 1995b), cyanobacteria (Ferris and Lowson, 1997), bacteria (Adams et al., 1992; Puente et al., 2004), and microalgae (Hirsch et al., 1995b).

Precise data on weathering rates in most environments are not available (Danin and Caneva, 1990; Danin, 1993), as most methods were descriptive (Hirsch et al., 1995b; Gorbushina et al., 2002), rather than quantitative and analytical.

This study used a quantitative method of electronic image analysis to rapidly measure the weathering potential of soil bacteria of rocks of diverse origins, marble, granite, apatite, quartz, limestone, and basalt.

2. Materials and methods

2.1. Rock sources

As substrate in tests, rocks of diverse character were used. Marble (0.1–500 μm diameter, 3 Mohs); granite (0.1–90 μm, 6–7 Mohs), apatite (0.1–90 μm, 5 Mohs), quartz (0.1–100 μm, 7 Mohs), limestone (0.1–500 μm, 3 Mohs) were purchased from a commercial source (Ward's Natural Science Establishment, USA) and volcanic rock (0.1–120 μm, 5–6 Mohs) was obtained from La Purisima, B.C.S., Mexico. Volcanic rocks were submerged in 1 N HCl solution overnight at 28–33 °C to eliminate organic matter, rinsed several times with de-ionized water, and dried at 160 °C for 2 h. Volcanic rocks were pulverized in a mill (Sprecher and Schun, Industrial Control, Germany). Rock types obtained from the manufacturer were purchased as powdered commercial products. All rock sources were sieved to 90 μm.

2.2. Bacteria

Two plant growth-promoting bacteria (PGPB) previously reported for rock solubilization (Chang and Li, 1998; Puente et al., 2004) were used as model bacteria; specifically *Pseudomonas putida* R-20 (Osburn et al., 1983; Meyer and Lindrman, 1986), and *Azospirillum brasilense* Cd, (ATCC 29710), a well known PGPB (Bashan et al., 2004).

2.3. Bacterial growth conditions

Bacterial strains were initially grown in nutrient broth (Sigma) at 30 ± 1 °C for 18 h, stirred at 120 rpm, and harvested by centrifugation at $1000 \times g$ for 20 min. Inoculum was washed three times in sterile distilled water, and pellets were suspended in saline solution (0.85% NaCl) to a final concentration of 10^9 CFU/ml. Flasks containing (g/l) manitol, 5; glucose, 10; sucrose, 5; and each of the pulverized rock, 1.5, separately, in 135 ml de-ionized water were each inoculated with 15 ml of a bacterial suspension. Flasks were incubated for 28 days at 30 ± 1 °C in a rotary shaker (series 25; New Brunswick, Edison, NJ) at 150 rpm. Samples were taken every week for image analysis.

2.4. Image analysis measurements

Weathering of rock particles, expressed as changes in various parameters, was quantified by measuring the number of powdered particles in a sample, particle diameter, and particle surface areas with an image analyzer (Image ProPlus 4.5, Media Cybernetics, Silver Spring, MD) before and after inoculation with bacteria. The procedure used 0.5 ml aliquots from bacteria–rock suspension diluted 1:10 in de-ionized water. This dilution was based on preliminary empirical determinations of the optimal dilution of suspensions for the image analyzer. To this concentration, 0.5 ml 1% agar, dissolved in 0.06 M phosphate buffer, pH 7.0, was added and mixed at 45 °C. Aliquots of

500 μl agar suspension was placed on a slide containing a shallow well ($23.85 \times 22 \times 1.5$ mm; 787 μl). A cover glass was placed on the rock powder slurry-agar suspension and was slowly pressed down from the sides until the cover glass touched the sides of the slide. This maintained a uniform thickness of the agar film. Direct measurement and counting of particles was performed using the 10× objective lens (UPlan FI) and Olympus Microscope (biological model tri-ocular BX41) with phase-contrast (PH1). The image analyzer used the software Image Pro Plus version 4.5, Camera model Cool Snap Media Cybernetics connected to a standard computer system (Pentium III, Dell, 750 MHZ, hard disk of 80 GB, 128 MB RAM, 32 MB video card, 1.67×10^6 colors of maximum resolution at 1600 pixels). The number of particles and the characteristics in each of five individual fields on each slide was counted. Bacterial cells were excluded by the software of the image analyzer and the maximum numbers of particles per sample was 10^8.

2.5. Calculations of number of particle per sample

The number of particles in a sample was calculated using the following equation:

$$P = \mathrm{PI} \times F \times D$$

where P=particles/ml; PI=number of particles per image; F=number of fields per chamber, and D=the dilution factor of the sample.

PI = the data measured by the image analyzer.

$$F = \frac{\text{volume of counting chamber in mm}^3\text{(area} \times \text{depth)}}{\text{volume of recorded image in mm}^3\text{(area} \times \text{depth)}}$$

$$F = \frac{23.85 \times 22 \times 1.5}{1.2943 \times 0.9616 \times 1.5} = 422(\text{this study})$$

$$D = 20(\text{this study})$$

Therefore, P=PI × 8440 (this study).

2.6. Sample size and statistical analysis

Samples (500 μl) were used and each contained up to 5×10^7 particles/ml of various sizes, all smaller than 90 μm. The initial quantity of particles varied from 10^5 to 10^7/ml among the different rock powders because the sieving process of ground rock controls only particle size, not particle numbers. Therefore, each sample has its own internal "time 0". Triplicate samples were analytically assayed (three samples from each replicate) and experiments were repeated 2 or 3 times. All data were adjusted to normality and then ANOVA or Student's t-test at $P \leq 0.05$ was determined using Statistica™ (StatSoft Co., Tulsa, OK). Numerical data are accompanied by standard error. Data ranged from 0.1 to 90 μm diameter for size and 0.9 to 500 μm^2 for surface area measurements. For simplification of analyses, data are presented only in the range of 0.1–12 μm diameter for size and 1–54 μm^2 surface area.

3. Results and discussion

Capacity of microorganisms for degrading rock is an essential parameter in numerous fields: in agriculture, when rock–phosphate combined with phosphate-solubilizing bacteria is used as fertilizer (Rodríguez and Fraga, 1999); in selecting appropriate stones for construction of monuments and facades of buildings (McDonald and Lewis, 2002; Van Hees et al., 2003); and as a future application of microorganisms in planetary science research and global terra-forming processes (Friedmann and Ocampo-Friedmann, 1995). An accurate, repeatable, and easy-to-use method is fundamental for decision-making. Most evaluations of stone weathering by microorganisms are descriptive or indirect methods, such as techniques that cannot harm monuments or constructions (Gorbushina et al., 2002; Larbi et al., 2003).

We intended to develop an accurate and efficient measurement technique using two PGPBs known to solubilize rocks, insoluble phosphate, and six common rocks used in construction and agriculture. To evaluate the capacity of bacteria to weather rock particles, all rock types were ground and sieved to a similar size, incubated with the bacteria under the same slurry conditions, and analyzed by the same method, employing an image analyzer to enumerate the product. Three parameters were analyzed as indicators of degradation of rock particles: (1) num-

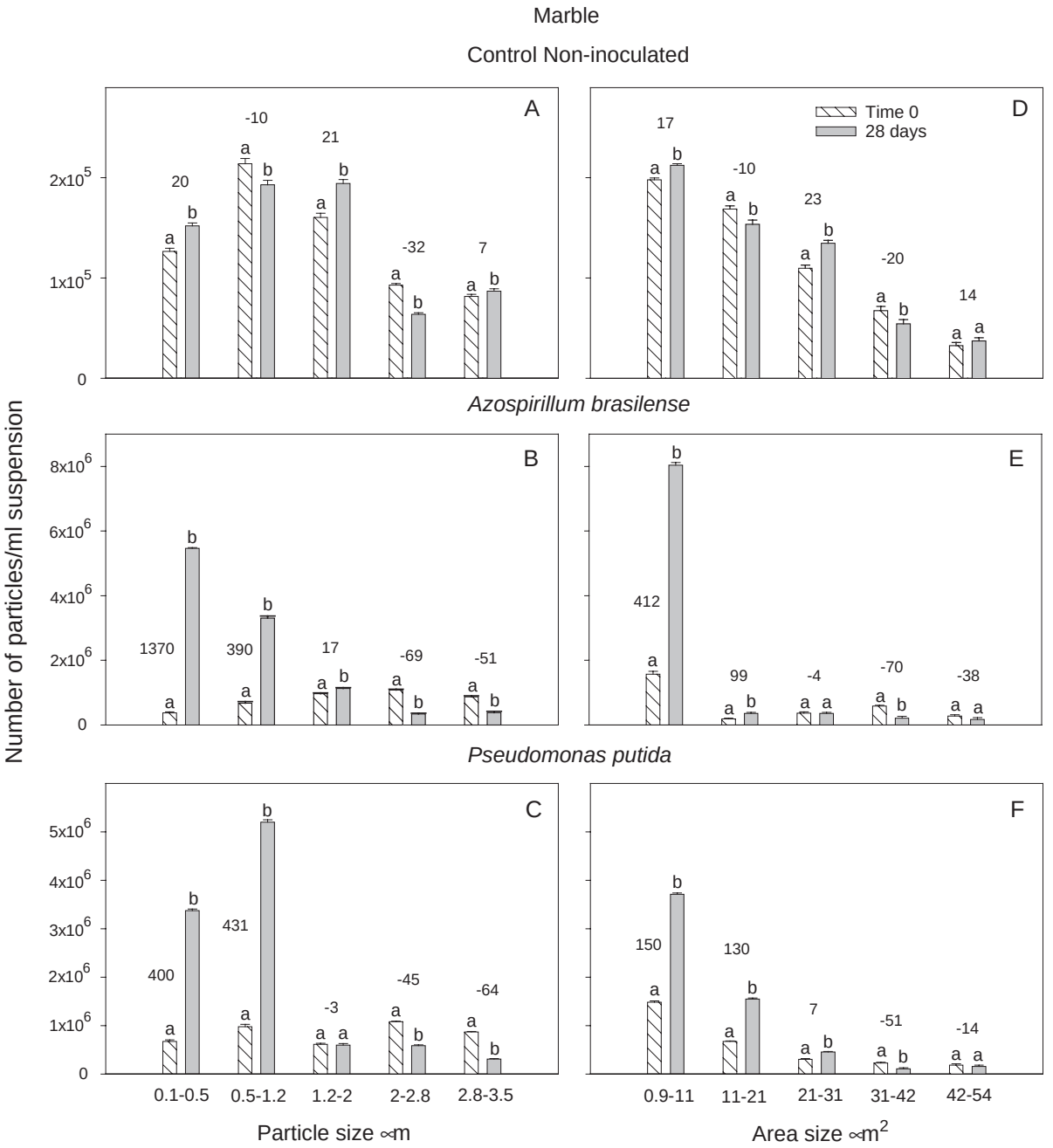

Fig. 1. Reduction in size (A–C) and surface area (D–F) of particles of ground marble by *Azospirillum brasilense* and *Pseudomonas putida.* Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacterial incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

ber of particles before and after bacterial inoculation and incubation, (2) surface area of the particles, and (3) diameter of these particles.

The final total number of particles after 28 days of incubation, although varying among the different rock types, varied greatly and therefore was statistically not

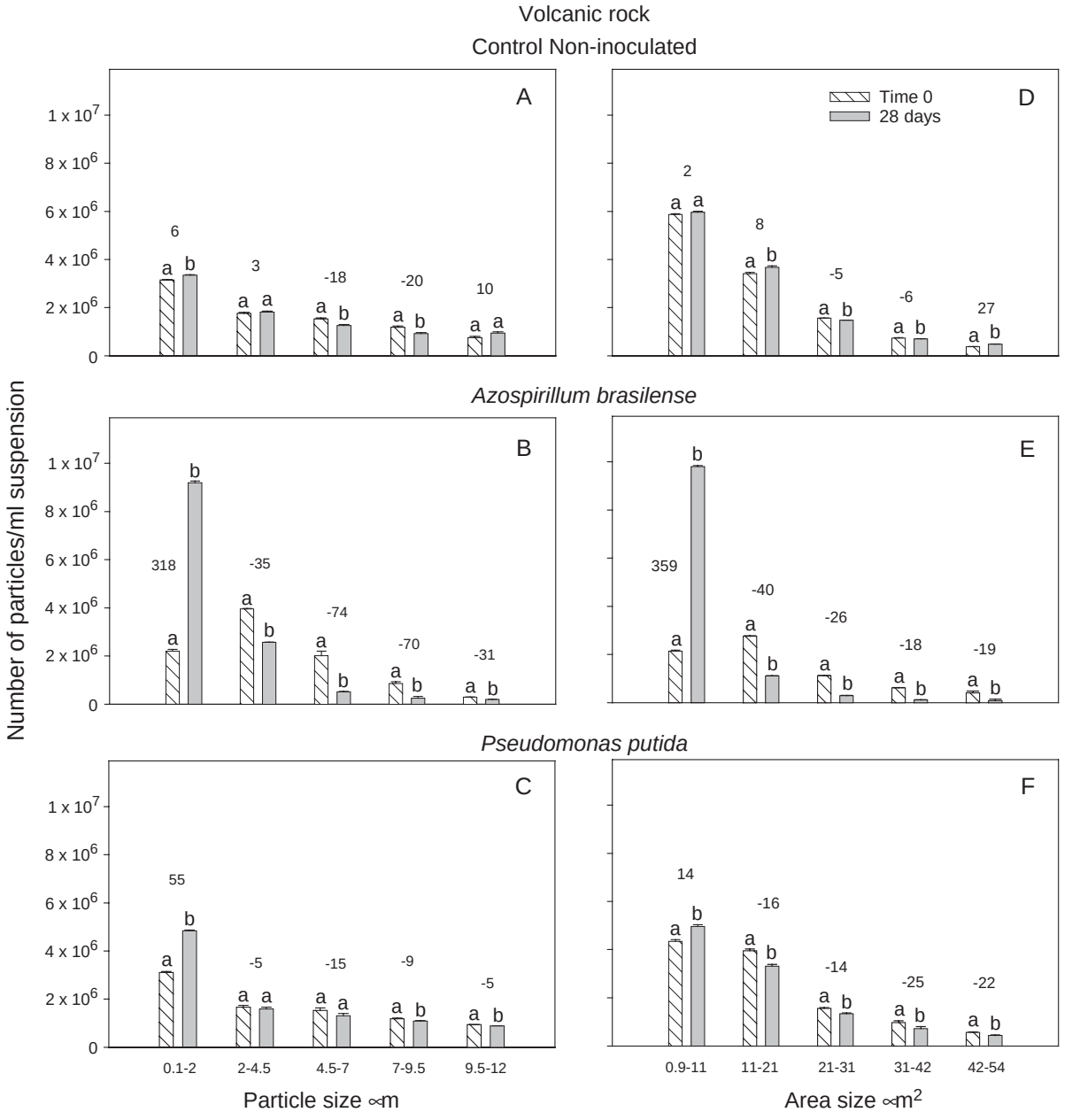

Fig. 2. Reduction of size (A–C) and surface area (D–F) of particles of ground volcanic rock by *Azospirillum brasilense* and *Pseudomonas putida*. Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacteria-incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

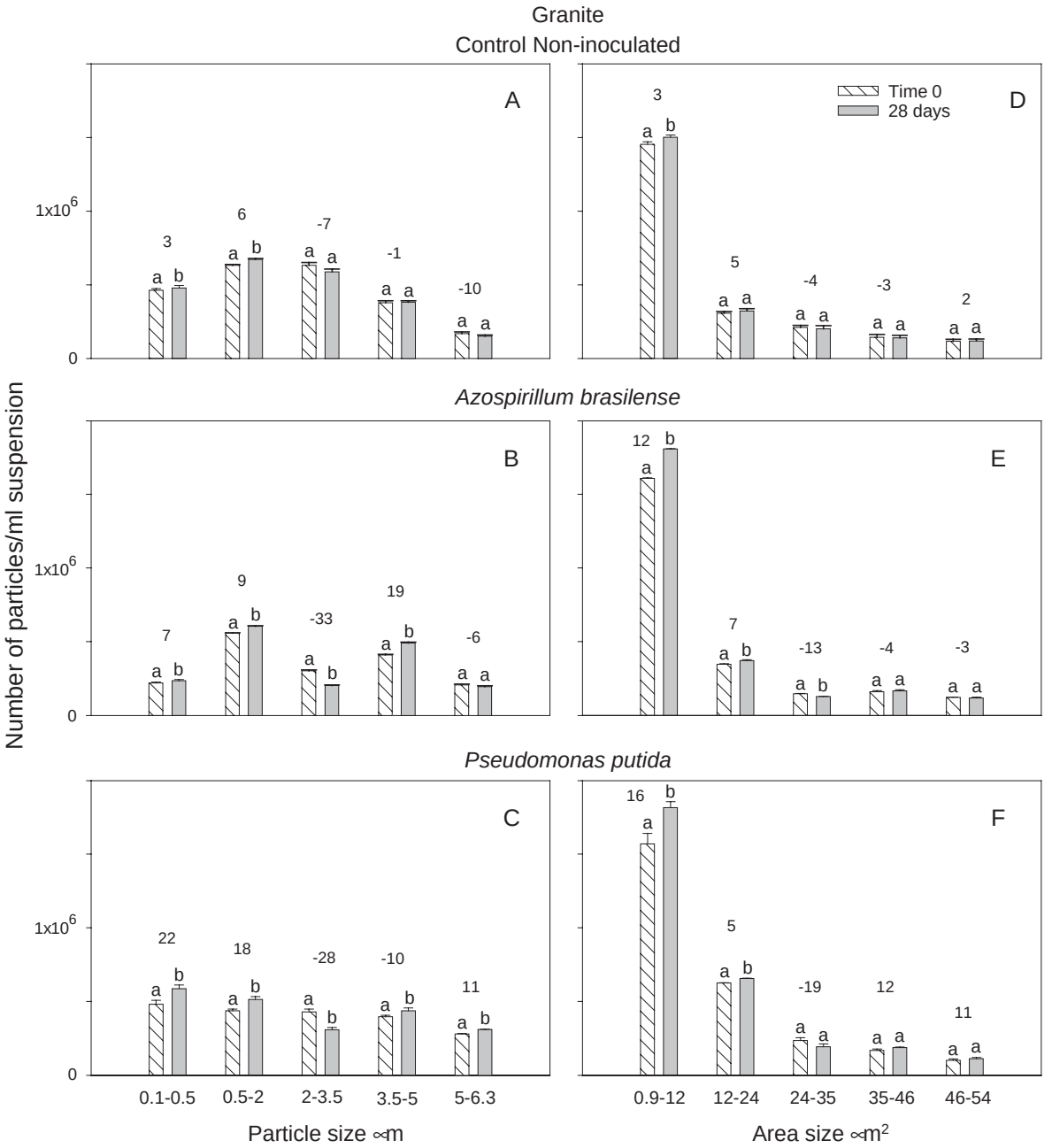

Fig. 3. Reduction of size (A–C) and surface area (D–F) of particles of ground granite by *Azospirillum brasilense* and *Pseudomonas putida*. Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacterial incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

different from non-inoculated particle solution (data not shown). Therefore, this parameter was not evaluated further. Microscopic visual inspection revealed that after inoculation and incubation with both strains of bacteria, the number of small particles dramatically increased in the solution (Fig. 4G, H).

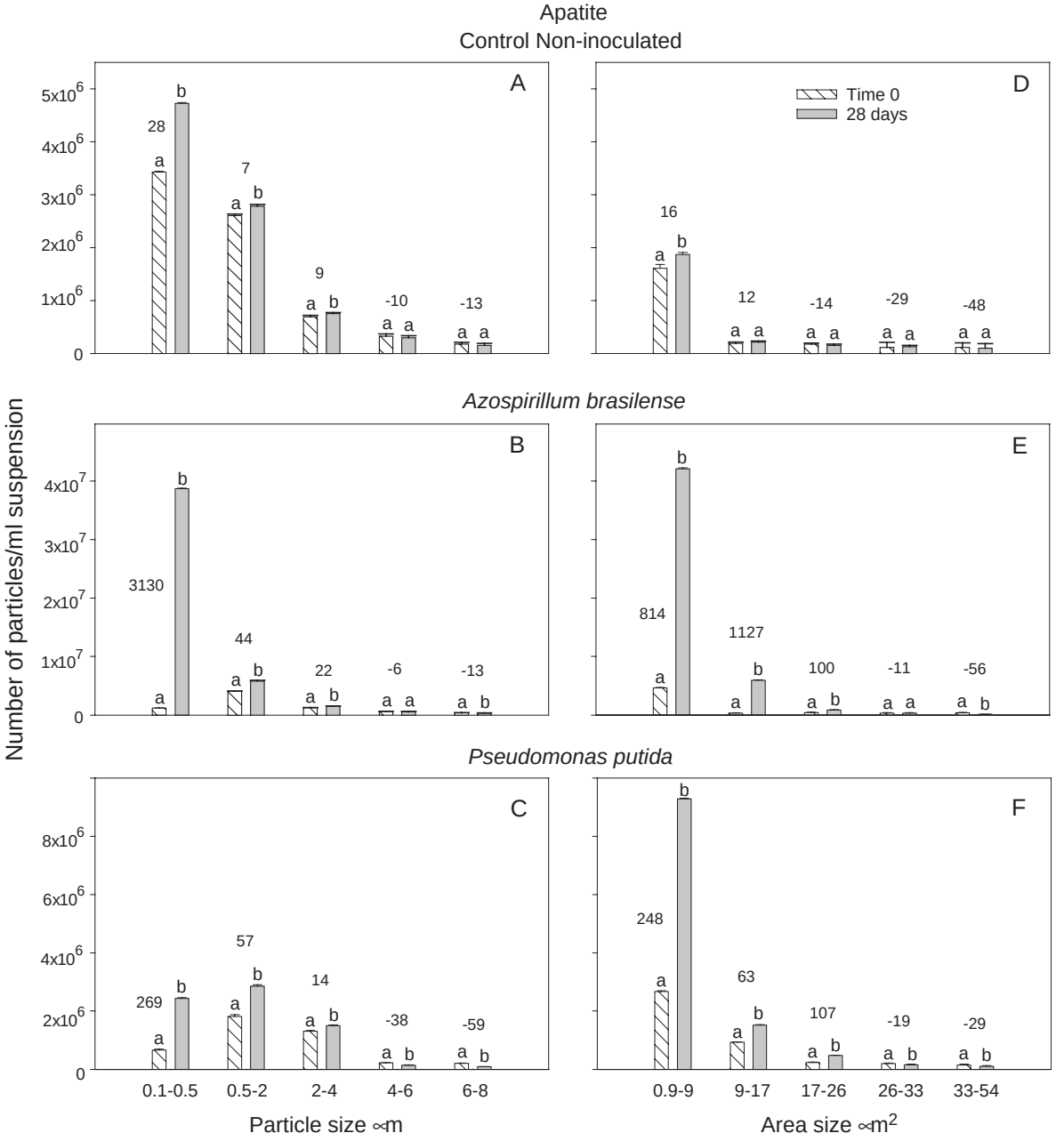

Fig. 4. Reduction of size (A–C) and surface area (D–F) of particles of ground apatite by *Azospirillum brasilense* and *Pseudomonas putida*. Photomicrograph of slurries before (G) and after (H) incubation with *Pseudomonas putida*. Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacterial incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

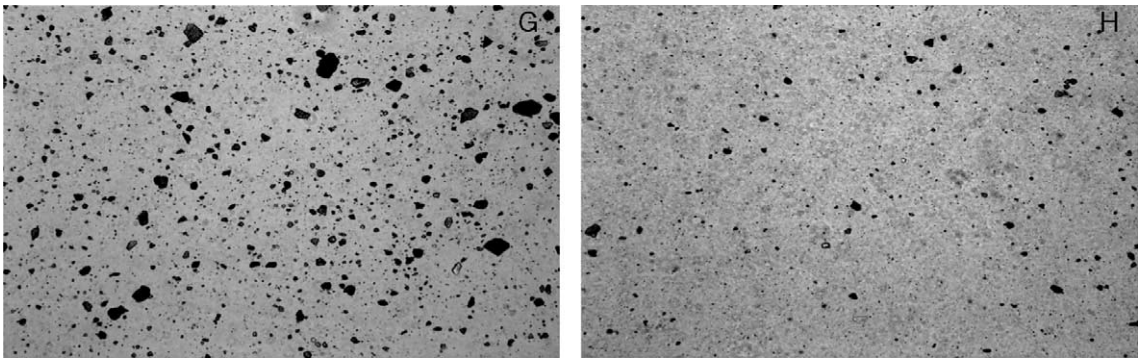

Fig. 4 (*continued*).

For marble powder, image analysis measured a surface area of a particle ranging from 0.1 to 500 μm^2 ($n = 1.03 \times 10^7$ particles measured for *A. brasilense* and 7.13×10^6 for *P. putida*), with the majority in the range of 0.9–54 μm^2 (1.02×10^7 for *A. brasilense* and 6.24×10^6 for *P. putida*). Incubation in the rock media randomly changed the number of particles within this range of surface areas by 10–23% (Fig. 1D). Although all surface area sizes were present after incubation, the fraction between 100 and 200 μm^2 decreased significantly (from 1.85×10^5 to 1.01×10^5 particles, detailed data not shown). This may have occurred from the friction among particles during the long incubation. When the marble slurry was inoculated with *A. brasilense*, the number of small surface area particles (0.9–21 μm^2) significantly increased within a range of 99% (1.8×10^5 to 3. 58×10^5) to 412% (1.57×10^6 to 8.04×10^6), and almost all particles with surface areas greater than 100 μm^2 disappeared (Fig. 1E). Similar trends was observed with *P. putida* ($n_{0.9–21\ \mu m^2,\ time\ 0} = 2.16 \times 10^6$ to $n_{0.9–21\ \mu m^2,\ time\ 28} = 5.26 \times 10^6$) (Fig. 1F), but with an increase in small surface area particles of only 130% to 150%.

Analysis of particle diameter of marble powder revealed particle sizes ranging from 0.1 to 90 μm ($n = 6.9 \times 10^6$ particles measured for *A. brasilense* and 4.34×10^6 for *P. putida*, detailed data not shown), with the majority in the range of 0.1–3.5 μm ($n = 4 \times 10^6$). Incubation in medium randomly change the number of particles within this range by 7–32% (Fig. 1A), although all diameter sizes were present after incubation. When the marble slurry was inoculated with *A. brasilense*, the number of particles with small diameters (0.1–1.2 μm) dramatically increased ($n_{time\ 0} = 9.86 \times 10^5$ to $n_{time\ 28} = 8.77 \times 10^6$ in 28 days). Particles > 2 μm declined significantly and almost all particles with surface area > 10 μm disappeared (Fig. 1B). Similar trends were observed with *P. putida* ($n_{0.1–1.2\ \mu m,\ time\ 0} = 1.65 \times 10^6$ to $n_{0.1–1.2\ \mu m,\ time\ 28} = 8.57 \times 10^6$) (Fig. 1C).

Similar results were obtained when other rock types, volcanic rock (Fig. 2), granite (Fig. 3), apatite (Fig. 4), limestone (Fig. 5), and quartz (Fig. 6) were used. This validated the accuracy of the method we developed and extends its usefulness to construction and agriculture.

It is notable that the degree of weathering by these two model bacteria directly correspond to the hardness of the rock, in increasing order, quartz < granite < igneous < apatite < limestone < marble. The harder the rock, the less weathering occurred within the 28-day incubation time (compare Figs. 1–6, formation of small particles). Although the bacterial populations declined over incubation time, substantial populations (in the range of $5.16 \pm 0.1 \times 10^7$ to $1.6 \pm 0.27 \times 10^8$ CFU/ml for *A. brasilense* and $6.53 \pm 0.67 \times 10^7$ to $5.27 \pm 0.3 \times 10^8$ for *P. putida* for marble; and $3.49 \pm 0.17 \times 10^7$ to $1.73 \pm 0.07 \times 10^8$ CFU/ml for *A. brasilense* and $1.2 \pm 0.12 \times 10^7$ to $1.33 \pm 0.24 \times 10^8$ for *P. putida* for granite, for example) were present at the end of the incubation periods in all rock suspensions (Puente et al., unpublished data). We suggest that the mechanism involved in weathering of the rock particles by these specific PGPBs are caused by the formation of organic acids because both species are known to produce numerous organic acids in cultures containing sugars as the carbon source (Puente et al., 2004; Rodriguez et al., 2004).

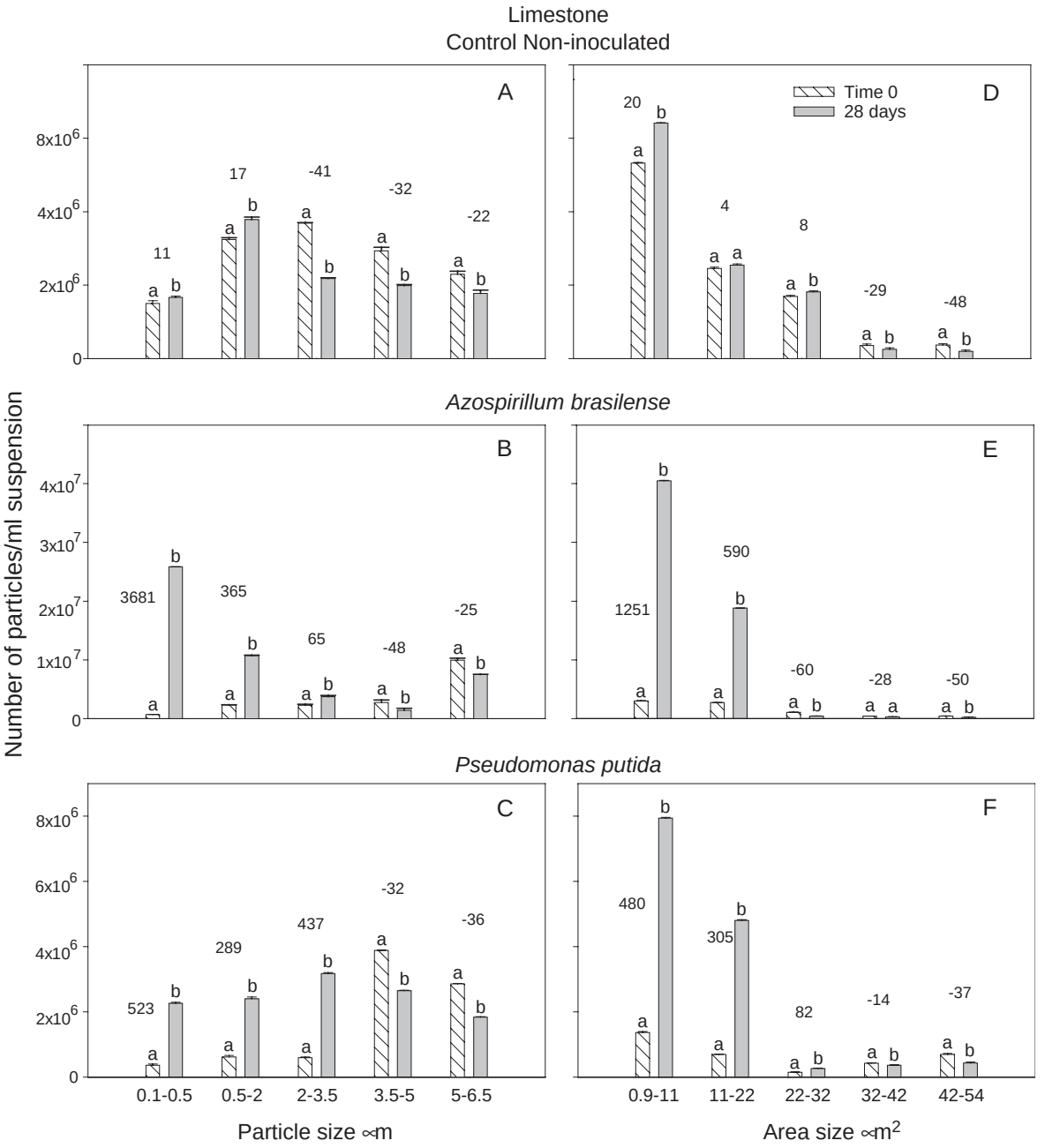

Fig. 5. Reduction of size (A–C) and surface area (D–F) of particles of ground limestone by *Azospirillum brasilense* and *Pseudomonas putida*. Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacterial incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

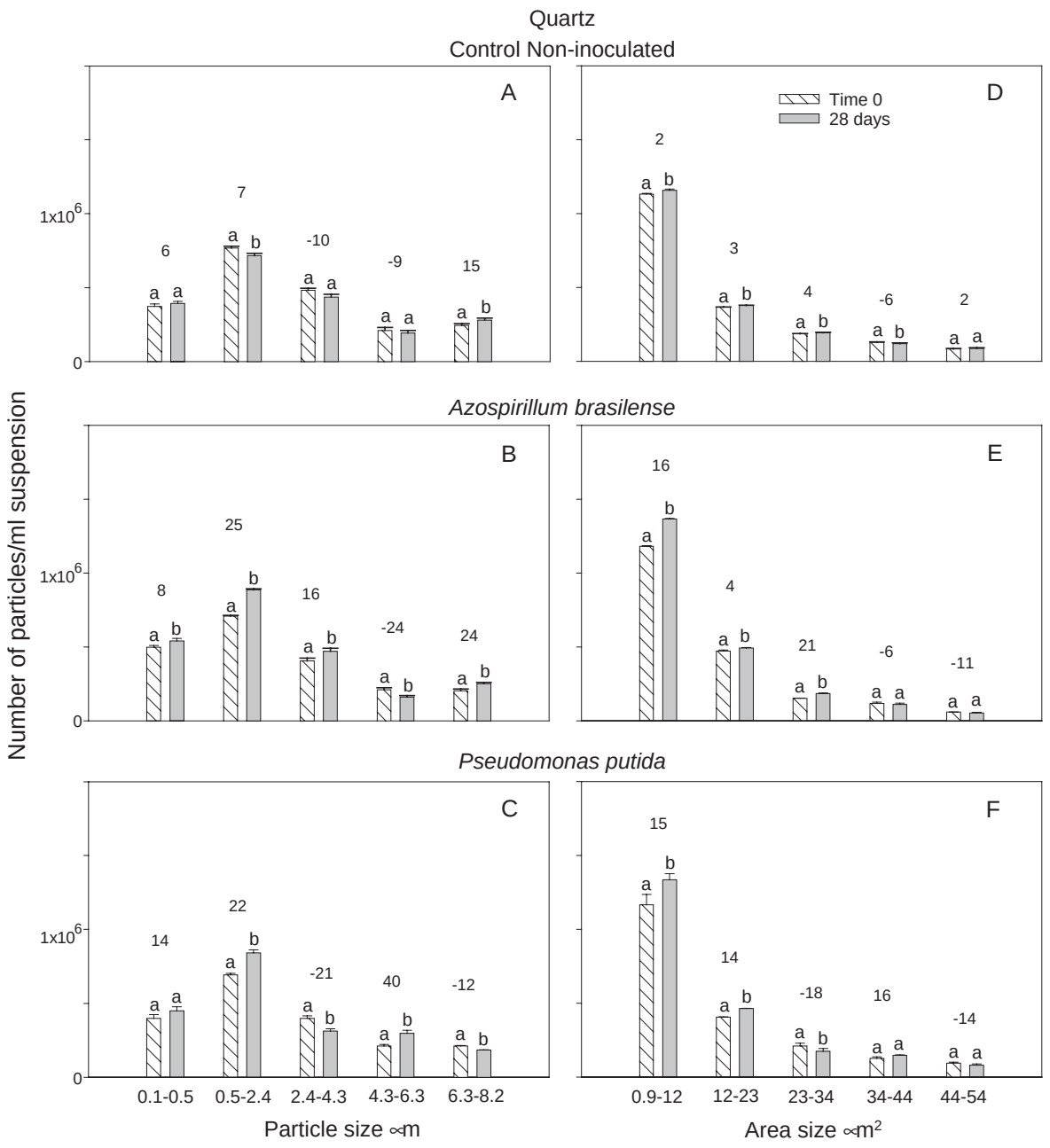

Fig. 6. Reduction of size (A–C) and surface area (D–F) of particles of ground quartz by *Azospirillum brasilense* and *Pseudomonas putida*. Pair of columns denoted by a different letter differs significantly at $P \leq 0.05$ in Student's *t*-test. The number above columns represents the difference, in percentage, between bacterial incubated slurry after 28 days and slurry without incubation. Bars represent standard error (S.E.). Absence of bar represents negligible S.E.

This method can give an accurate evaluation of the *potential damage* of local microorganisms residing on stone can inflict on the stone when proper conditions for bacterial growth occur, such as irrigation of agricultural land or bird droppings and dust particulates combined with rain on monuments and buildings.

However, this method does not evaluate the actual rate of weathering of stone under conditions that are not conducive to microbial growth, a condition that prevails for long periods in dry areas.

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