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Microbially-mediated glass dissolution and sorption of metals by *Pseudomonas aeruginosa* cells and biofilm

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Abstract

A basaltic glass and a vitrified bottom ash were incubated at 25 °C in a growth medium (based on casaminoacids) inoculated with *Pseudomonas aeruginosa*. Bacterial growth and mineral concentrations in different compartments (bacterial cells, growth medium and biofilm) were monitored in short-term (3 days), and long-term experiments involving repeated renewals of the culture medium during 174 days.

In short-term experiments, while the concentration of iron increased in the presence of bacteria, a decrease in Ni and Zn was observed in the growth medium compared to the sterile condition. During long-term experiments, such differences gradually decreased and disappeared after 78 days. On the contrary, iron concentration remained higher in the biotic condition compared to the sterile one. Bacterial growth resulted within a few days in the formation of a biofilm, which lead to the cementation of the altered glass grains. Most of the constituents of the glass (Si, Mg, Fe, Ti, Ba, Co, Zn, Cu, Ni and Cr) were found in the biofilm, while the chemical composition of the bacterial cells was very different.

Keywords: Bioalteration; Basaltic glass; Bottom ash; Biofilm; *Pseudomonas aeruginosa*

1. Introduction

Today it is generally accepted that alteration of rocks as well as anthropogenic products like window glass, cement, slag or waste glass is not only driven by the interaction with water or mineral aqueous solutions. Organic compounds like humic acids, and also the activity of microorganisms are of importance for material degradation and also for the secondary mineralisation of materials [1–6]. However, the exact role of microorganisms in these processes remains unclear. Some authors suggested that they play an important role in the degradation of cathedral window glasses [7], but without precisely describing the alteration mechanisms nor the type of microorganisms involved in the process (bacteria, fungi or lichens). Other authors related the presence of "hair channels" in submarine basaltic glass to bacterial activity, resulting in an acceleration of glass alteration [8–12]. However, it has never been shown that these channels are the result of bacterial action. The role of microorganisms in the degradation of particular material like books degraded by fungi, metallic tubes corroded by bacteria, and cement by *Thiobacillus* has been well documented [13–16]. It remains, however, that there is no convincing experimental validation of such phenomenon for silicate phases. A major difficulty in such complex systems is to precisely measure the alteration rate with help of reliable tracers. Another problem remains to precisely define the microorganism involved and its contribution to the alteration process. However it must be noted that a biologically-mediated K-feldspar dissolution seems to be related to an increased alteration rate of the mineral in the presence of bacteria [17]. Finally, the question rises if the observed degradations are directly related to microbial activities or to the water action or the acidification of the solution. In such cases, the microorganism would take advantage of nutrient supply during ion exchange between proton from solution and alkalis from solids [2,3] without accelerating the alteration rate. Microbes could also play a role in the sequestration of toxic metals via

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biosorption processes [18,19], especially due to the production of exopolysaccharides [20,21].

The aim of the present study was to experimentally evaluate the influence of one type of bacteria on the chemical composition of material leachates with a potentially negative effect on the environment. *Pseudomonas aeruginosa* was chosen because it is widespread in nature (e.g. [22–24]) and it has been often used in bioremediation studies [23,25–28]. It is an aerobic, motile Gram-negative bacterium, heterotroph that produces large amounts of exopolysaccharides, which form the so-called biofilm. These poly-anionic matrix allow the bacteria to adhere at the surface of solid materials [29,30]. Two types of glasses have been studied: one basaltic glass from the Fiji Island in the South Pacific [31,32], and one vitrified bottom ash [33,34], respectively called Basalt and VBash in tables and figures.

2. Materials and methods

In order to preserve the alteration layers and the biofilm at the surface of the glasses, experiments were performed without shaking. For long-term experiments (174 days), an experimental design was developed, allowing successive cycles of bacterial growth, with the formation of a biofilm and a possible alteration layer at the surface of the materials. A Soxhlet-based procedure was developed for that purpose. The glass grains were placed in a perforated polypropylene reactor allowing a free contact of the sample with the medium (Fig. 1a). The reactor was then placed in a larger (150 ml) polypropylene flask (Fig. 1b and c) filled with 50 ml of a commonly used bacterial growth medium named casaminoacid (CAA) medium containing casaminoacids 5 g/l (DIFCO, #223050), K_2HPO_4 1.18 g/l, $MgSO_4{\cdot}7H_2O$ 0.25 g/l (final pH 7.1). This medium has been analysed by ICP/AES using a spectrometer Jobin–Yvon (type JY 124) (Table 1). It must be noticed that it contains appreciable amounts of Si, Mg, Na, K and P. Chemical analysis of the glasses is given in Table 2, the size of the glass grains ranged between 100 and 125 μm. The specific surface area was 600 cm^2/g for the basaltic glass and 595 cm^2/g for the vitrified bottom ash (Coulter SA3100 device, multipoint N2, BET data and Langmuir surface area). The ratio between glass surface and volume of solution was 20 cm^{-1}. Polypropylene flasks and medium were autoclaved at 121 °C during 20 min, while glass grains were heat-sterilized at 180 °C during 2 h in a Pasteur oven. Growth experiments were performed at 25 °C with CAA medium inoculated or not with *Pseudomonas aeruginosa* (2.10^4 colony forming units/ml) in assays supplemented or not with vitrified bottom ash (VBash medium) or basaltic glass (Basalt medium). For long-term experiments, inoculation was done at time 0, a self bacterial inoculation occurring at each renewal.

Table 1
Chemical analysis of CAA medium (ppm)

Si	3.40
Al	0.02
Mg	20.40
Ca	0.33
Fe	0.01
Na	726
K	496
P	258

2.1. Renewal, sampling and analytical methods

Preliminary experiments were performed during 3 days without renewal of the medium. A second set of experiments with periodic medium renewal was conducted during 174 days on vitrified bottom ash. Assays for short- and long-term experiments were done in duplicate. At each 15-day period, the reactor (Fig. 1a) was transferred into a new flask (Fig. 1b) containing 50 ml of sterile medium, thus starting a new cycle. At the end of each cycle, the pH of the growth medium was measured using "WTW pH 340i" pH-meter, then the suspension was centrifuged (20 min, 10,000 rpm). The supernatant was filtered using 0.45 μm filter and then acidified with HNO_3 10N (suprapur) at a ratio of 9:1.

The bacterial pellet was washed by resuspending in ultra-pure water, centrifuged, dried for two days at 90 °C before determining its dry weight. Finally, the residue was mineralized at 500 °C in an oven during 24 h for dissolution in HNO_3 1N. The three 1N HNO_3 solutions, two representing the dissolved material in the remaining growth medium in sterile and biotic conditions, the third one the elements bound through sorption or/and uptake by the cells, were analyzed by ICP-AES.

2.2. Electron microscopy

Grains were examined by scanning electron microscopy (SEM) (JEOL JSM 840 equipped with a spectrometer with dispersion of energy EDS Tracor TN 5500). When needed, samples were treated with osmium oxide according to a slightly

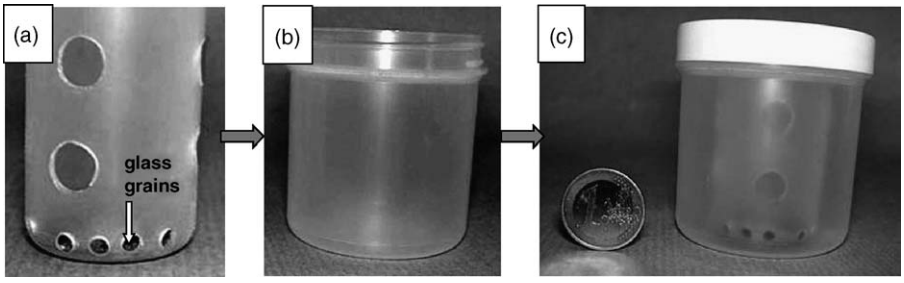

Fig. 1. Experimental design. The glass grains are filled in the reactor (a) and placed in a polypropylene flask (b, c) containing the growth medium.

Table 2
Chemical analysis of the glasses

	SiO_2 (% mass)			Al_2O_3 (% mass)			CaO (% mass)			MgO (% mass)			Na_2O (% mass)			K_2O (% mass)			Fe_2O_3 (% mass)			MnO (% mass)			TiO_2 (% mass)			P_2O_5 (% mass)
Basalt	50.10			14.60			12.00			7.42			2.39			0.11			11.50			0.18			1.31			0.12
VBash	39.90			14.20			14.49			2.87			2.97			0.31			19.30			0.21			1.28			1.67
mg/kg	Rb (mg/kg)	Cs (mg/kg)	Sr (mg/kg)	Ba (mg/kg)	Sc (mg/kg)	V (mg/kg)	Cr (mg/kg)	Co (mg/kg)	Ni (mg/kg)	Cu (mg/kg)	Zn (mg/kg)	Y (mg/kg)	Zr (mg/kg)	Nb (mg/kg)	Mo (mg/kg)	Cd (mg/kg)	Sn (mg/kg)	Sb (mg/kg)	W (mg/kg)	Pb (mg/kg)	La (mg/kg)	Ce (mg/kg)	Nd (mg/kg)	Th (mg/kg)	U (mg/kg)	C (mg/kg)	S (mg/kg)	Sum %
Basalt	0.67	0.07	99	35.00	40.60	294	293	45.49	82	65	81	29.60	75	1.61	0.42	<0.01	0.95	0.14	0.37	1.78	2.63	8.11	8.07	0.28	0.07	0.02	0.14	99.73
VBash	8.97	0.31	845	3749	6.60	297	553	49.00	466	15580	7866	12.60	210	18.10	41.85	1.10	396.79	376.31	14.83	891.53	16.99	81.69	19.3	2.29	1.14	<0.02	<0.02	100.35

Analysis by ICP-AES (major and some minor elements) and ICP-MS (for trace elements). Basalt: basaltic glass. VBash: vitrified bottom ash.

modified Jones et al. [35] method, which requires less osmium oxide. Briefly, samples were fixed in glutaraldehyde to maintain cell structure and to secure attached cells to the glass surface. Samples were immersed overnight in 2% glutaraldehyde in a 0.1 mol/l sodium cacodylate (CAC), rinsed in CAC 0.1 mol/l, and postfixed for 1 h in 50 ml of 0.5% OsO_4 in 0.1 mol/l CAC at 4 °C. The osmium oxide solution was then analysed by ICP-AES before and after sample treatment. Samples were then dehydrated sequentially in 50, 70, 85, 95, and 100% graded ethanol solutions. Finally, the samples were dried for 1 week at 45 °C and stored in a desiccator until examination at the SEM.

3. Results

3.1. Short-term experiments

After 3 days interaction, the pH of the incubation medium was 7.2 for assays without bacteria and 7.5 for assays with bacteria. Concerning solution analysis, an almost undetectable level of iron characterized assays in CAA medium, with or without bacteria (Fig. 2). Addition of VBash or Basalt grains slightly increased the iron concentration in sterile conditions, while a high final iron concentration was reached after 3 days in presence of bacteria. It represented seven times more solubilized iron for VBash and thirteen times more for Basalt compared to the sterile assays.

On the contrary, two metals presenting some potential toxicity, nickel and zinc, behaved differently compared to iron. Their respective concentrations in biotic condition were usually lower than in sterile condition. As shown in Table 2, Basalt contained much less nickel than VBash. Consequently, the nickel concentration remained unchanged in comparison to the CAA medium. Also, chromium concentrations remained unchanged whatever the conditions (Fig. 2).

Table 3 shows that bacteria grown in presence of VBash or Basalt have a much higher content of metals compared to cells grown in CAA medium. This is particularly true for iron and zinc which concentrations increased 35- and four-fold, respectively for VBash, and 13- and 1.4-fold, respectively for Basalt. Similarly, nickel and chromium concentrations increased but remained at a lower level.

3.2. Long-term experiments

Concerning each 2 week-incubation period, the following observations related to bacterial growth, cell yield, siderophore production and pH evolution were raised. A pronounced bacterial growth was observed together with an important production

Table 3
Fe, Ni, Zn and Cr concentrations found in bacteria expressed in μg/g of bacterial dry weight as found in the short-term experiment

Bacteria grown in	Fe	Ni	Zn	Cr
CAA	31.6	<0.02	40.8	1.2
CAA + Basalt	408	1.8	57	3
CAA + VBash	1104	5.4	160.8	5.4

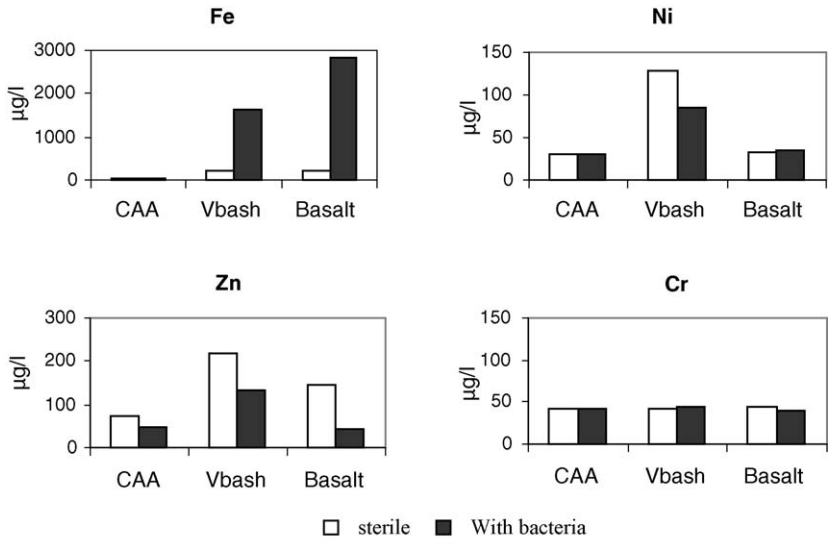

Fig. 2. Fe, Ni, Zn, Cr concentrations as determined by ICP-AES in CAA, VBash and Basalt media inoculated (black symbols) or not (open symbols) with bacteria after 3 days interaction.

of pyoverdine, the yellow green fluorescent pigment and siderophore of *P. aeruginosa* [37]. Cell yield reached during growth was constant within each incubation period representing an average value of $740 \pm 6\,mg\,l^{-1}$ dry weight bacterial cells, while pyoverdine synthesis corresponded to $73 \pm 12\,mg\,l^{-1}$. Such growth and pyoverdine production values are in accordance with commonly obtained growth characteristics in standardized conditions (data not shown). As soon as the first renewal, it could be observed that glass grains became glued suggesting besides a planctonic growth, the development of a biofilm. A pH increase from 7.1 to 8.4 was concomitantly observed with the bacterial growth due to the release of ammonia during the aminoacids catabolism in such medium.

The evolution of the iron, nickel, zinc and chromium concentrations in VBash medium inoculated or not with bacteria during each 2 week-incubation period, is shown in Fig. 3. As with the results of the short-term experiments, the iron concentration in the presence of bacteria was always higher than in sterile conditions. Also the resulting nickel and zinc concentrations confirmed results of the short-term experiments: metal concentrations of the VBash medium with bacteria were always much lower at the beginning of the experiments. At long term, the difference in concentrations between sterile and biotic conditions tend to disappear. The chromium concentration did not change whatever the conditions. As can be deduced from Fig. 3, no evident relationship

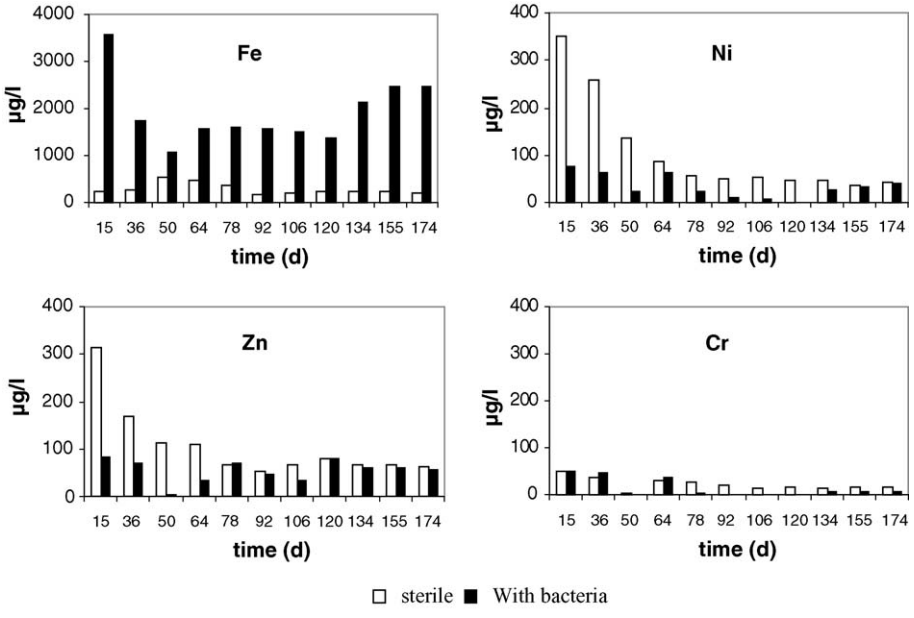

Fig. 3. Fe, Ni, Zn, Cr concentrations during 174 days interaction as determined by ICP-AES in VBash medium inoculated (black symbols) or not (open symbols) with bacteria.

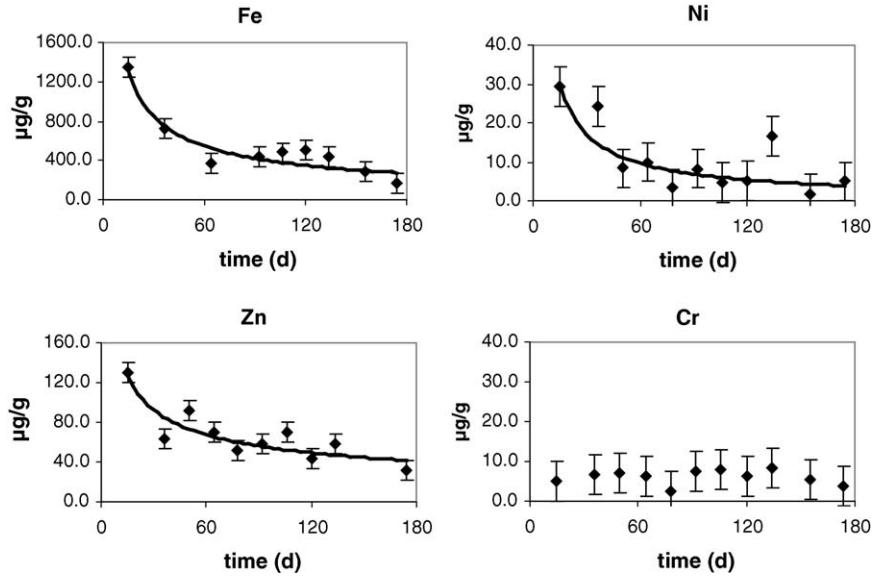

Fig. 4. Fe, Ni, Zn, Cr concentrations as a function of time in bacteria harvested from solution represented in µg/g of bacteria dry weight during 174 days interaction.

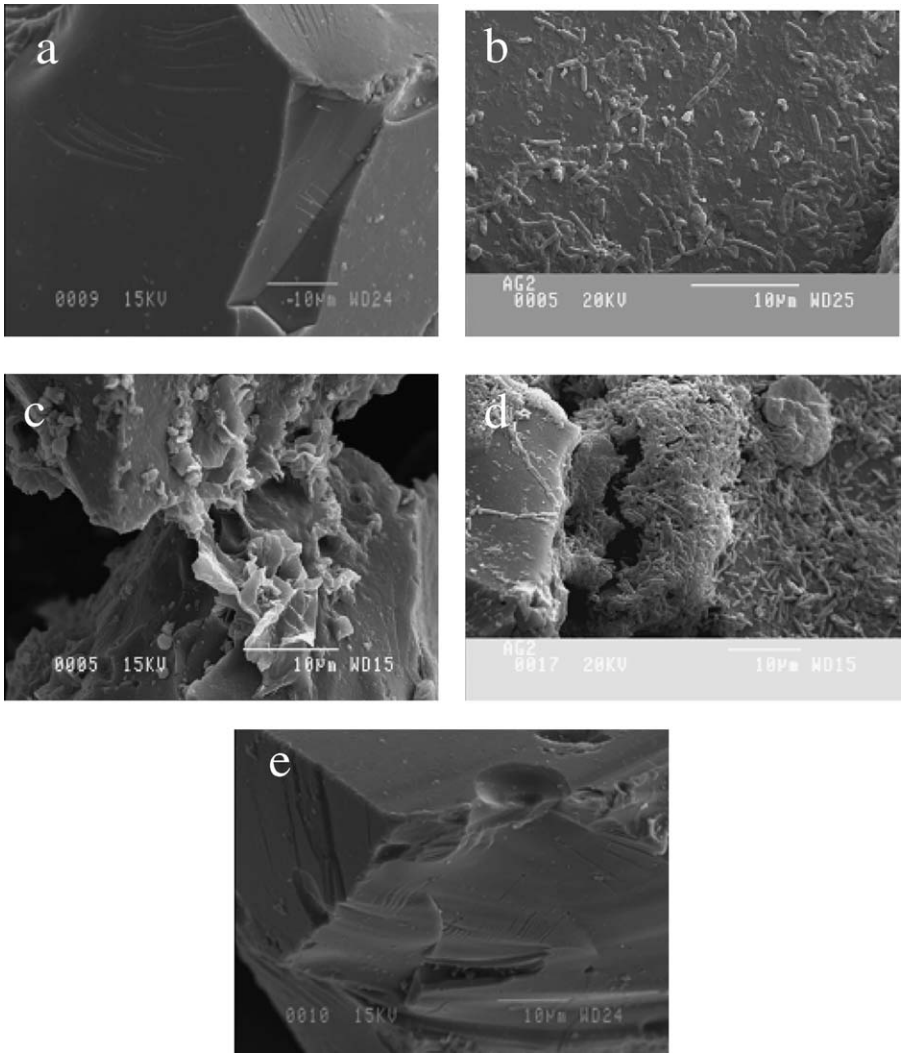

Fig. 5. Scanning electron micrographs showing (a) unaltered VBash grain. (b) Bacteria adhering on the surface of a VBash grain after 3 days. (c, d) Grains cemented by bacterial biofilm after 174 days without (c) and with (d) treatment by osmium oxide. (e) VBash grain altered in CAA medium after 174 days.

Table 4
Amounts of elements (μg/50 ml OsO_4 solution) found in the biofilm after 174 days of incubation

	Si	Mg	Fe	Ti	Sr	Ba	Co	Zn	Cu	Ni	Cr
OsO_4 initial solution	262.8	2.8	4.4	0.2	0.3	0.2	0.8	1.4	3.3	0.7	0.2
OsO_4 after treatment	567.6	141.6	193.4	18.0	1.6	5.8	12.8	15.1	24.6	0.9	1.6

in metal release from Vbash could be defined for the four elements.

The evolution of the four elements in bacteria expressed in μg/g of bacterial dry weight is shown in Fig. 4. Fe, Ni and Zn concentrations decreased rapidly during the first four cycles and then remained at a low level; Cr concentrations however did not change and were constantly at low levels.

Scanning electron microscopy (SEM) showed that bacteria after 3 days incubation adhered to the surface of glass grains (Fig. 5b). After 174 days, the altered glass grains were glued together (Fig. 5c) under the presence of bacteria, whereas grains altered in a sterile environment remained free. From the comparison of Fig. 5c and d, it could be deduced that the osmium oxide treatment dissolved the biofilm matrix allowing a better observation of the bacterial cells (Fig. 5d). Chemical composition of the osmium oxide solution before and after grains treatment is given in Table 4. It was enriched in Si, Mg, Fe, Ti, Ba, Co, Zn, Cu, Ni and Cr compared to the initial solution. It is important to note that Co concentrations were below detection limit (1 ppb) in the growth medium of biotic experiments and bacteria (data not shown). The osmium oxide solution after treatment, however, contained large quantities of Co (Table 4). Thus, the biofilm seems to behave as an important sink for Co.

Finally, the surface of glass grains taken out from the sterile solution (Fig. 5e) looked similar to that of unaltered glass (Fig. 5a), thus, suggesting that no detectable secondary mineral phase occurred during the long-term incubation period.

4. Discussion and conclusion

(1) Bacterial development led to a rapid chemical modification of the solution in contact with glasses. Compared to the sterile conditions, Fe concentrations strongly increase in the presence of bacteria. This phenomenon could be related with the production of pyoverdine since this molecule is able, by its chelation capacities, to increase the level of iron solubilization [36–38].

(2) Ni and Zn concentrations were lower in biotic than in sterile conditions. In the former case, results showed a trapping of these elements in both bacterial cells and biofilm. Total amounts of Ni solved from materials were calculated from Table 4 and from Figs. 3 and 4. In biotic conditions, absolute masses of Ni solved after 174 days of incubation are 18.5 μg in growth medium, 4.2 μg in bacterial cells and 2 μg in the biofilm, corresponding to 24.7 μg of total dissolved Ni. In sterile condition, mass of Ni solved in growth medium was 58.0 μg. This difference of 33.3 μg of Ni between sterile and biotic conditions could represent the amount of element present under the form of a secondary mineral phase at the glass surface. However, as mentioned in the result section, no such precipitates could be visualized by SEM. Thus, a second hypothesis would be that, for such element, a lower dissolution occurred in biotic conditions. To solve the problem, we need to define an ultrapure medium, to select a reliable tracer in order to better evaluate the alteration rates of materials and to better analyse of the biofilm composition.

(3) The development of a biofilm at the surface of the glass grains was evidenced during long-term experiments. It is generally accepted that biofilms constituted of polyanionic polymers have a strong affinity to heavy metals [39–42]. Our results showed effectively that the biofilm was able to retain most of the elements present in the system (Table 4). It is important to note that the bacteria have a short life time, while the biofilm may cover quasi permanently the surface. Thus, for a better understanding of the interactions between complex silicates and bacteria, it appears essential to focus the studies on the properties of the extracellular products.

(4) One of the critical points is to precisely evaluate the mass balance of potentially toxic elements between bacterial cells, solution and biofilm. Another point is to evaluate the role of bacteria on the alteration rate of the materials. To do this and to analyse more precisely the various trace elements a culture medium made of ultrapure reagents has to be elaborated.

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