

# Characterization of attachment and growth of

## *Thiobacillus denitrificans* on pyrite surfaces

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## Abstract

Anaerobic growth and attachment of the autotrophic denitrifying bacterium *Thiobacillus denitrificans* on pyrite surfaces were studied. Polished pyrite slabs were exposed to *T. denitrificans* for 1 to 9 weeks. The reacted pyrite surfaces were imaged with scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Cells were observed as isolated attached cells, cells in division and cells forming microcolonies embedded in organic films. Bacteria began to colonize pyrite surfaces after 1 week, forming microcolonies after 3 weeks. The rate of colonization of the pyrite surface was around 35 cells mm<sup>-2</sup> h<sup>-1</sup> for the 3-week period. After 9 weeks, larger areas of the pyrite surface were covered by organic films. Bacterial enumeration on the pyrite surface and in solution showed that most of the cells were not attached to the mineral surface. Nevertheless, both attached and free-living bacteria probably contributed to pyrite-driven denitrification. The results may be applied to the natural environment to better understand pyrite-driven denitrification in aquifers and to improve the long-term performance of bioremediation processes using pyrite.

**Keywords:** *Thiobacillus denitrificans*, pyrite, bacterial attachment, denitrification, bioremediation, confocal laser scanning microscopy

## 1. INTRODUCTION

Earlier studies on the distribution of microorganisms in the subsurface have shown that most of the bacterial biomass in aquifers is attached to solid material and only a small fraction is free-living in solution (Harvey et al. 1984; Bekins et al. 1999; Lehman et al. 2001; Griebler et al. 2002). It is commonly believed that bacteria attached to surfaces in aquatic environments are more active than free-living bacteria (Paerl 1985). However, the experimental observations are not always consistent (van Loosdrecht et al. 1990). Moreover, physiological studies of single bacterial populations have demonstrated substantial differences between cells in attached and unattached states in terms of cell size, growth rate, enzyme activity and exopolymer production (van Loosdrecht et al. 1990; Marshall and Goodman 1994). Attachment may

promote a number of advantages in groundwater, including a predictable nutrient flux, access to solid phase nutrients and resistance to toxic molecules (Stewart 1994; Dunne 2002; Teitzel and Parsek 2003). Attached bacteria occur on surfaces as dispersed monolayers, microcolonies, or three-dimensional biofilms (Costerton et al. 1995).

Currently, there are few data on the relative contributions of attached and planktonic bacteria to denitrification in aquifers. Iribar et al. (2008) studied the denitrification capability of sediment-attached and free-living bacteria in an alluvial aquifer and found that bacterial densities and denitrifying capability were greater in sediment than in groundwater. Furthermore, the bacterial composition of the sediment-attached consortium differed from that of the groundwater free-living consortium. Teixeira and Oliveira (2002) compared the activity of cells of the heterotrophic denitrifying bacterium *Alcaligenes denitrificans* grown in suspension with that of cells grown on a solid surface. Their results showed that nitrate consumption by cells in a biofilm was significantly higher than by planktonic cells.

Autotrophic denitrification linked to pyrite oxidation has been shown to occur in aquifers (Otero et al. 2009) and in laboratory studies using both the indigenous bacteria of a nitrate-contaminated aquifer (Torrentó et al. 2010b) and pure cultures of an autotrophic denitrifying bacterium, *Thiobacillus denitrificans* (Torrentó et al. 2010a). However, there are few data on the nature of the reaction and on the mechanism by which denitrifying bacteria reduce nitrate using pyrite as the electron donor. Moreover, it remains unclear whether or not denitrifying bacteria need to colonize a pyrite surface for pyrite-driven denitrification. The results of the experiments carried out by Torrentó et al. (2010a) showed that the nitrate reduction rate was dependent on the exposed surface area of the pyrite grains. This finding provides support for two different hypotheses in relation to the relative roles of planktonic and attached cells in pyrite-driven denitrification. On the one hand, a high pyrite surface area could lead to a better mass transfer from solid to liquid phases, releasing larger quantities of Fe- and S-compounds into the aqueous phase and thus resulting in a high pyrite dissolution rate. This would indicate that free-living bacteria play a major role in pyrite-driven denitrification by oxidizing the dissolved Fe<sup>2+</sup> and thus regenerating the oxidant. On the other hand, a large pyrite surface area could also lead to an increase in bacterial growth

and colonization on the pyrite surface, highlighting the role of attached bacteria, which would directly oxidize the mineral. After biostimulation with pyrite in batch experiments performed with sediments and groundwater from a nitrate-contaminated aquifer, Torrentó et al. (2010b) showed an increase in the number of sediment-attached and free-living denitrifying bacteria, but the increase in sediment-attached denitrifiers was greater.

To date, research in microbial attachment to mineral surfaces has dealt mainly with adhesion of acidophilic iron-oxidizing bacteria (e.g. *Acidithiobacillus ferrooxidans*) to pyrite (e.g. Solari et al. 1992; Ohmura et al. 1993; Dziurla et al. 1998; Edwards et al. 1998; Mielke et al. 2003; Harneit et al. 2006; Pisapia et al. 2008) and a few studies have assessed adhesion of iron-reducing bacteria (e.g. *Shewanella oneidensis* and *Shewanella putrefaciens*) to iron (oxy)(hydr)oxides (Neal et al. 2003; Roberts et al. 2006; Zhang et al. 2010). Different pathways have been proposed to accomplish electron transfer to/from solid-phase minerals: (1) the enzymatic pathway in which adhesion of bacteria to the solid is necessary (Silverman and Ehrlich 1964; Leang et al. 2003; Lovley et al. 2004; Gorby et al. 2006); and (2) the indirect pathways in which electron transfer occurs by means of shuttle compounds (Silverman 1967; Sand et al. 2001; Crundwell 2003; Lovley et al. 2004). The indirect mechanisms avoid the need for direct contact between cells and mineral. An indirect contact mechanism has also been proposed as a pathway for pyrite oxidation by acidophilic iron-oxidizing bacteria (Schippers and Sand 1999; Sand et al. 2001; Crundwell 2003; Sand and Gehrke 2006). In this case, attached bacteria oxidize ferrous ions to ferric ions within a biofilm made up of bacteria and exo-polymeric material (EPS), and the ferric ions generated within this layer oxidize the sulfide mineral. Thus, bacteria play an important catalytic role in regenerating the oxidant, and also in concentrating the ferric ions in the exo-polymeric layer where the chemical processes take place. Currently, the most accepted pathway for pyrite oxidation by iron-oxidizing bacteria is a combination of the indirect non-contact and the indirect contact mechanisms (Edwards et al. 1998; Pisapia et al. 2008) with the result that both attached and free-living bacteria contribute to pyrite dissolution. However, direct attachment of iron-reducing bacteria is still accepted as the predominant mechanism for accessing Fe(III) minerals in environmental settings (Lovley et al. 2004).

The present study focused on the ability of *T. denitrificans* to colonize pyrite surfaces. The results could help us to better understand the mechanism of the nitrate-dependent pyrite oxidation reaction, and to determine whether *T. denitrificans* requires attachment to the pyrite surface. Given that quantitative measurements of the adhesion of denitrifying bacteria onto minerals are lacking in the literature, it is necessary to quantify cell attachment density and colonization kinetics of *T. denitrificans* onto pyrite surfaces. Results from such determinations would provide an indication of the contribution of attached and free-phase denitrifying bacteria to pyrite-driven denitrification in aquifers. This could improve insight into the long-term performance of bioremediation processes based on enhancing denitrification by pyrite addition (Torrentó et al. 2010b) and could also lower their maintenance costs. To this end, we performed batch experiments examining surface colonization of pyrite slabs under anaerobic conditions.

## **2. MATERIALS AND METHODS**

### **2.1. Pyrite preparation**

Pyrite was purchased from Wards Scientific. Single, whole pyrite crystals were used to prepare thin polished films of 1 mm of thickness. Polished thin sections were prepared by Spectrum Petrographics, Inc. (Vancouver, WA). Epoxy resin was used to mount the thin sections on glass slides. A slow-speed saw was used to cut 3 mm-square blocks of the thin film. Individual pyrite slabs were removed from the glass slide with acetone (approximately  $3 \times 3 \times 0.1$  mm slabs, 19 mm<sup>2</sup> surface area and 0.2-0.4 mg each). The slabs were then washed individually with ethanol (Edwards et al. 2000) and sterilized by autoclaving at 121°C for 15 min before the start of the experiments.

### **2.2. Culture preparation**

*Thiobacillus denitrificans* (strain 12475 from the German Collection of Microorganisms and Cell Cultures, DSMZ) was cultured in an anaerobic (pH 6.8) nutrient medium specially designed for *T. denitrificans*, following Beller (2005). The liquid medium contained (in mM concentration): Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> · 5H<sub>2</sub>O (20 mM), NH<sub>4</sub>Cl (18.7 mM), KNO<sub>3</sub> (20 mM), KH<sub>2</sub>PO<sub>4</sub> (14.7 mM), NaHCO<sub>3</sub> (30 mM), MgSO<sub>4</sub> · 7H<sub>2</sub>O (3.25

mM),  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  (0.08 mM),  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.05 mM) plus sterile vitamin, trace element and selenate-tungstate solutions (stock solutions 1, 4, 6, 7 and 8 of Widdel and Bak 1992, 1 mL each). The solutions used to prepare the medium were sparged with  $\text{N}_2$  for 15 min before the sterilization. Cultures were maintained under anaerobic conditions at 30°C and unshaken by 5-weekly sub-culturing. The cultures were harvested by centrifugation and washed and resuspended in a sterile saline solution (Ringer 1/4 solution) immediately before the start of the experiments.

### **2.3. Colonization experiments**

Colonization and growth on pyrite surfaces were studied over a 9-week period. Three polished pyrite slabs were placed into 100 mL glass bottles and 48 mL of sterile modified medium were added to the bottles containing minerals. Each bottle was inoculated with 2 mL of the cell suspension ( $2.0 \times 10^8$  cells  $\text{mL}^{-1}$ ). Abiotic control experiments consisted of pyrite slabs in sterile modified medium (50 mL).

The modified medium consisted of the *T. denitrificans* nutrient medium without thiosulfate and iron and the sulfate salts being replaced by chloride salts. Therefore, the solution contained (in mM concentration):  $\text{NH}_4\text{Cl}$  (18.7 mM),  $\text{KNO}_3$  (16.1 mM),  $\text{KH}_2\text{PO}_4$  (14.7 mM),  $\text{NaHCO}_3$  (30 mM),  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$  (3.25 mM) and  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (0.05 mM). This modified medium ensured that pyrite provided the only source of electrons available for the cells.

The colonization experiments were performed under anaerobic conditions, in an anaerobic glove box with a nominal gas composition of 90%  $\text{N}_2$  and 10%  $\text{CO}_2$  and at  $28 \pm 2$  °C. The oxygen partial pressure in the glove box was maintained between 0.1 and 0.3%  $\text{O}_2$  and was continuously monitoring by an oxygen partial pressure detector with an accuracy of  $\pm 0.1\%$   $\text{O}_2$ .

### **2.4. Aqueous samples**

The bottles were manually shaken once a week and 3-mL aqueous samples were taken once a week using 3 mL sterile syringes. From each sample, a portion was used to determine the density and viability of the bacterial cell population in suspension as described below. The remainder of each 3-mL sample was filtered through 0.22  $\mu\text{m}$  syringe filters and a portion of each filtered sample was preserved in nitric acid

solution for cation analyses. Concentrations of cations (total Fe, total S, Mg, Ca, Na, P, and K) were measured by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The uncertainty in the measurement of Mg, Ca, Na, K, P, Fe and S was estimated to be around 5% with detection limits of 2.1, 2.5, 4.4, 2.6, 3.2, 0.4 and 3.1  $\mu\text{mol L}^{-1}$ , respectively. The remaining part of each filtered sample was used for anion and ammonium analyses and pH measurement. Concentrations of nitrate, nitrite, chloride, and sulfate were determined by High Performance Liquid Chromatography (HPLC), using a IC-Pack Anion column and borate/gluconate eluent with 12% of HPLC grade acetonitrile. The error was estimated to be 5% for nitrate, chloride and sulfate and 10% for nitrite. Samples for ammonium analysis were acidified to  $\text{pH} < 2$  with  $\text{H}_2\text{SO}_4$ . Ammonium concentrations were measured using an Orion ammonium ion selective electrode with an analytical uncertainty of 10% and a detection limit of 0.01 mM. pH was measured with a calibrated Crison pH Meter at room temperature ( $22 \pm 2$  °C). The pH error was 0.02 pH units.

Bacterial enumeration was performed by epifluorescent direct counting. Samples were diluted with 0.2- $\mu\text{m}$ -pore-size filtered double distilled water to achieve a final liquid volume of 10 mL, which was added to a black polycarbonate membrane filter (Nuclepore filters, 0.2- $\mu\text{m}$  pore size) placed in the filter tower apparatus support. To each sample, we added 100  $\mu\text{L}$  of a solution containing 10  $\mu\text{g}$  of DAPI (4'-6-diamidino-2-phenylindole dihydrochloride, Sigma-Aldrich) per mL. The mixture of sample and stain was left undisturbed on the filter for 5 min, after which it was subjected to filtration with a vacuum of less than 200 mbar. For a total direct count, the number of DAPI-stained cells deposited on the filter was determined with an epifluorescence microscope using suitable optical filters. The number of cells was counted within squares of an ocular grid at a magnification of  $\times 1000$ . Counts were obtained from randomly located fields covering a wide area of the filter. A minimum number of 200 cells were counted per filter.

The viability of the bacterial cell population in suspension was determined by using the LIVE/DEAD® BacLight™ bacterial viability kits (Invitrogen, Molecular Probes). These kits contain green-fluorescent nucleic acid stain SYTO® 9 and red-fluorescent nucleic acid stain Propidium Iodide (PI). When used alone, the SYTO® 9 stain generally labels all bacteria that have both intact and damaged membranes.

In contrast, PI stain only penetrates those bacteria with damaged membranes, causing a reduction in the SYTO® 9 stain fluorescence when both dyes are present. For this reason, bacteria with intact cell membranes (viable cells) exhibit green fluorescence, whereas bacteria with damaged membranes (dead cells) exhibit red fluorescence.

## **2.5. Solid samples**

Once a week, two experiments were sacrificed and the pyrite slabs were taken out of the solution with sterilized forceps and rinsed with a pH 7.1 sterile phosphate buffer.

The initial pyrite slabs and some slabs retrieved after 1, 2, 3 and 4 weeks were observed by confocal laser scanning microscopy (CLSM) to quantify cell attachment onto pyrite surface. The slabs were fixed in paraformaldehyde (3% in the pH 7.1 phosphate buffer solution) at 4°C overnight. Thereafter, the slabs were stained with DAPI. After staining, the slabs were mounted on glass slides and examined with a Leica TCS SPE inverted stage confocal microscope. The images were captured with LAS AF software and the cells attached onto surfaces were counted using ImageJ software. Averages were obtained from 10 to 20 images (80.09 × 80.09 μm) depending on cell density and colonization heterogeneity. In each image, the apparent statistical surface covered by bacteria was calculated as the ratio of the number of pixels corresponding to bacteria with respect to the total number of pixels. The number of surface-attached cells was estimated assuming that a single cell had a relative surface coverage of 1 μm<sup>2</sup> (each *T. denitrificans* cell can be regarded as a rod that is 0.5 by 1-3 μm; Kelly and Harrison 1989). The standard deviation between counts made for separate images was calculated. Other DAPI-stained slabs were observed on an epifluorescence microscope using suitable optical filters.

The initial pyrite slabs and some of those retrieved after 1, 2, 3, 4, 5, 7 and 9 weeks were examined with scanning electron microscopy (SEM) to study the distribution of cells on the pyrite surface. The slabs were fixed with 2.5% glutaraldehyde in the pH 7.1 phosphate buffer solution (at 4°C, overnight) to maintain the cell structure and to fix the attached cells to the pyrite surface. Subsequently, the slabs were washed with the phosphate buffer and dehydrated sequentially in graded ethanol solutions (25, 50, 75,



and twice in 100% EtOH - 10 min each). After dehydration, the slabs were chemically dried by soaking twice in hexamethyldisilazane (HMDS) for 5 min and air dried in a fume hood for at least 2 h. Thereafter, the slabs were mounted on stubs and gold palladium coated. Samples were examined by scanning electron microscopy (Stereoscan S360 Cambridge Electron Microscopy) and energy-dispersive microanalysis (EDS; INCA Energy 200) using a beam potential of 15 kV.

### **3. RESULTS AND DISCUSSION**

#### **3.1. Chemical results**

No changes in nitrate, sulfate, iron and ammonium concentrations or in pH were detected during the course of the runs (8 to 65 d) of the inoculated and the abiotic control experiments (data not shown). Therefore, nitrate reduction did not occur or was too insignificant to be detected under the experimental conditions.

Torrentó et al. (2010a) found that *T. denitrificans* was able to reduce nitrate using pyrite as the electron donor and that nitrate reduction was markedly influenced by the pyrite surface area. The fact that nitrate reduction was undetected in the present experiments could be attributed to the small surface area of the pyrite slabs. Pyrite slabs were used instead of crushed pyrite that has a higher surface area since the aim was to determine the ability of *T. denitrificans* to colonize pyrite surfaces. The use of polished thin sections facilitated imaging using SEM and CLSM because of their flat surfaces.

#### **3.2. Pyrite surface colonization**

Figures 1 and 2 show SEM and CLSM images of the surfaces of the pyrite slabs before and after the colonization experiments. After 1 week, the SEM (Fig. 1B) and the CLSM images (Fig. 2A) showed single attached rods that started to colonize the pyrite surface. Scratches from polishing on the surface can also be optically distinguished. Single attached cells and cells in division were observed in both 1-week and 2-week old samples (Fig. 1C and 2B), which reveals direct microbial growth on the pyrite surface. The localization of attached cells on pyrite surfaces was random and no preferential orientation related to

crystallographically controlled surface features or surface defects was observed. Nevertheless, in the 7-week old DAPI-stained slabs examined with the light microscope, groups of cells were observed preferentially attached to surface features, such as grooves and microcracks (data not shown). We observed that attached cells and new cells formed by growth and cell division remained attached to their parent cells and gave rise to microcolonies or biofilms. In 3-week old samples microcolonies in addition to actively dividing single cells were detected on the pyrite surface by SEM (Fig. 1D and 1E) and by CLSM (Fig. 2C and 2D). After 4 weeks, small areas of the pyrite surface were covered by the microcolonies embedded in films, presumably of an organic nature (Fig. 1F, 2E and 2F), obstructing the detection of individual cells by SEM. These organic films cover small areas of the pyrite surface and are also observed by SEM in the 5-week old samples (Fig. 1G). After 9 weeks, SEM images demonstrated that the pyrite surface areas covered by organic films were more extended (Fig. 1H and 1I). EDS analysis on the covered and uncovered pyrite surfaces revealed differences in their elemental compositions. The uncovered pyrite remained unchanged (around 39% for S, 21% for Fe, 32% for C and 8% for O), whereas in the covered pyrite the signals of S and Fe were weaker (25-29% and 12-13%, respectively) and the signals of C and O were stronger (42-51% and 8-13%, respectively). In accordance with SEM observations, 9-week old samples examined with the light microscopy showed large areas of the pyrite surface covered by organic films (data not shown). The development of a uniform and continuous biofilm was not observed during the 9-week period. Single cells and cells in division were still observed after 9 weeks (Fig. 1I).

### **3.3. Colonization rate and coverage area**

The rate of colonization of the pyrite surface by *T. denitrificans* was estimated by measuring the increase in number of cells attached to the pyrite surface over time. The density of adhered cells as determined by analysis of the CLSM images of 1, 2 and 3 weeks following the start of the experiments is shown in Table 1. It should be noted that microcolonies are three-dimensional structures. As a result, the way in which the number of attached cells was determined using image analyses of the two-dimensional CLSM pictures could lead to an underestimation of the density of adhered cells. As three-dimensional

microcolonies were observed in the 3-week old samples (Fig. 1D), the number of attached cells calculated for these samples should be considered as an underestimation. Hence, this method was not used to determine the density of attached cells in older samples.

The number of attached cells increased slowly over time (Table 1 and Fig. 3). This increase could be due either to the attachment of bacteria growing in solution or to the growth and division of attached bacteria. SEM images supported both assumptions. On the one hand, the images showed attached cells dividing and multiplying, leading to the formation of microcolonies. On the other hand, individual attached cells were observed after 9 weeks, when microcolonies were mature, indicating new attachments of planktonic cells. Surface colonization rate was found to be  $34.9 \pm 17.6$  cells  $\text{mm}^{-2} \text{h}^{-1}$  for the 3-week period (Fig. 3). Observations by light microscopy of 7-week old samples stained with DAPI suggest that the density of attached cells was of the same order of magnitude as that after 3 weeks (data not shown). This suggests that the attachment sites on the pyrite surface became saturated with cells. Experiments of longer duration are warranted to corroborate this assumption.

After 3 weeks, the surface cell density was  $2.01 \times 10^4$  cells  $\text{mm}^{-2}$ , which is equivalent to approximately one cell per  $50 \mu\text{m}^2$  of pyrite surface. The area covered by attached *T. denitrificans* cells was approximately 2% of the total pyrite surface available (Table 1).

### **3.4. Viability of cells in solution and percentage of attached cells**

Table 2 shows the number of *T. denitrificans* cells in solution, including viable and dead cells, during the 8-week experimental period. As is shown in Figure 3, the number of cells in solution remained almost constant over time. High viability was observed over this experimental period. Therefore, planktonic cells survived over time but did not multiply. The percentage of viable cells with respect to total cells in solution tended to decrease slightly over time, suggesting attachments to the pyrite surface or cell death. After 9 weeks, attached single cells and cells in division in addition to mature colonies were observed on the pyrite surface by SEM, indicating that new attachments of free-living cells occurred over time.

Using the calculated densities of both attached and planktonic cells, about 0.2% of total cells were estimated to have attached to the pyrite surface in 3 weeks. After 9 weeks, this percentage was expected to have been of the same order of magnitude as after 3 weeks because light microscope observations suggested that the density of attached cells did not increase further after 3 weeks, and because the density of planktonic cells remained almost constant over time

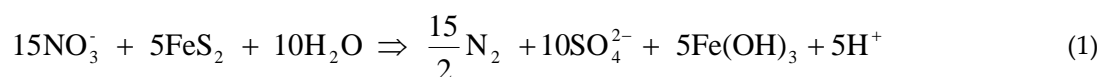
### **3.5. Role of attached and free-living cells in denitrification**

It may be postulated that both planktonic and attached cells were metabolically active for the following reasons: (1) most of the cells remained planktonic, (2) the number of attached cells increased throughout the experiment, and (3) the number of viable cells in solution remained almost constant over time. Cell-mineral contact, for at least a small number of cells, was necessary for *T. denitrificans* to oxidize pyrite. The planktonic cells stayed alive and active over time, probably at the expense of the products released from pyrite dissolution by attached cells, such as dissolved Fe<sup>2+</sup>.

Despite the need for further research into the mechanism by which *T. denitrificans* cells use pyrite as the electron donor, it may be assumed that ferric ion is the pyrite-attacking agent. This has been suggested for pyrite oxidation by acidophilic bacteria and for chemical pyrite oxidation by oxygen or ferric ion even at neutral pH (Moses et al. 1987). This assumption is supported by the fact that *T. denitrificans* are also Fe<sup>2+</sup>-oxidizing bacteria (Straub et al. 1996). Thus, bacteria would oxidize the ferrous ions dissolved from the pyrite and using nitrate as the oxidant.

Although nitrate reduction was not corroborated by chemical analyses in the present study, bacteria probably were metabolically active and thus contributed to the pyrite-driven denitrification process for two reasons: (1) colonization and direct growth of bacteria on the surface was observed, and (2) the number of planktonic cells remained almost constant. Nitrate reduction was not detected because of the small surface area of the pyrite slabs (approximately 30 mm<sup>2</sup> and 0.2-0.4 mg, which correspond to approx. 0.075 to 0.150 m<sup>2</sup> g<sup>-1</sup>). Additional experiments were performed adding crushed pyrite in order to provide sufficient surface area and roughness for reactions to be detected. In these experiments, in addition to the

three pyrite slabs, 1 g of 50-100  $\mu\text{m}$  sterilized powdered pyrite ( $0.4259 \pm 0.0156 \text{ m}^2 \text{ g}^{-1}$ ) was added to each bottle. The results showed that after 45 d, approximately 12% of the initial nitrate was reduced concurrently with the release of sulfate (Fig. 4), corroborating the link between nitrate reduction and pyrite oxidation. Iron concentration in solution was below the detection limit. This suggests that ferrous iron was also involved in nitrate reduction, being oxidized to ferric iron and precipitated (eq. 1).



Therefore, we assumed that in the present experiments planktonic bacteria oxidize the ferrous ions dissolved from the pyrite, and using nitrate as the oxidant. *T. denitrificans* attached cells reduce nitrate and oxidize pyrite directly by using appropriate enzymes or indirectly by oxidizing ferrous ions. A direct pathway for pyrite oxidation by attached cells has been demonstrated for *Acidithiobacillus ferrooxidans*, which has a complex and highly developed respiratory chain that covers the outer and the inner membranes and transfers electrons from pyrite to dissolved oxygen (Appia-Ayme et al. 1999; Yarzabal et al. 2002; 2004; Castelle et al. 2008). In the case of *T. denitrificans*, attached bacteria would remove electrons directly from the pyrite surface or indirectly by  $\text{Fe}^{2+}$  oxidation and transfer them to nitrate. Although the specific mechanism of the process remains unclear, it is assumed that in the present study both attached and planktonic cells of *T. denitrificans* contributed to denitrification linked to pyrite oxidation.

### 3.6. Implications

The results of the present study, with a pure culture of a denitrifying bacterium and using polished and small-area pyrite samples, could be qualitatively applied to the natural environment to better understand pyrite-driven denitrification in aquifers and to optimize bioremediation. However, it is uncertain whether the required attachment to pyrite surfaces of at least some cells is specific to this strain or whether it is a common behavior of denitrifying bacteria. Furthermore, the relative roles of attached and planktonic cells probably depend on the composition of the bacterial community, on the geochemical conditions (e.g. pH, redox potential, ionic strength, nutrient availability, dissolved solutes), and on the mineralogy, surface area, heterogeneity and availability of the solid phase to be colonized. In the natural

environment, pyrite surfaces with a higher surface area and greater roughness than the ones used in the present experiments will probably promote bacterial attachment. Further *in situ* colonization experiments could be performed to apply these results to the natural environment of nitrate-contaminated aquifers. For example, microcosms containing pyrite could be placed into the native contaminated groundwater and maintained undisturbed to study the interaction between the mineral, groundwater and the indigenous microorganisms. In groundwater, *in situ* colonization experiments have been used to study bacterial attachment onto natural sediments (Hirsch and Rades-Rohkohl 1990; Griebler et al. 2002; Herrmann et al. 2008). Other *in situ* microcosms have been employed to evaluate microbial colonization, biofilm development and the diversity of bacterial communities colonizing artificial substrates such as glass beads or polymers (Claret 1998; Dang and Lovell 2000; Peacock et al. 2004; Iribar 2007). The utilization of *in situ* microcosms seems to be the most suitable method for avoiding the uncertainty of the results obtained exclusively from laboratory studies.

Given that attached cells have a number of advantages over free cells, the use of immobilization biosystems for bioremediation purposes has been evaluated in order to optimize biotechnological processes (Cassidy et al. 1996; Singh et al. 2006). Two approaches have been adopted (Cohen 2001): (1) the addition of a bedding material to provide surfaces for attachment of indigenous bacteria; and (2) the addition of desired microbial species entrapped in a bedding material. Immobilized cells could improve bioremediation processes by preventing bacteria from leaving the system and by protecting them against unfavorable environments. However, most of the research has been performed on a laboratory scale, and applications of immobilized cells for *in situ* bioremediation are still in the early developmental stage (Singh et al. 2006). As regards nitrate contamination, few laboratory studies have assessed nitrate removal by immobilized denitrifying bacteria. Tal et al. (1997, 1999, 2003) and Liu et al. (2003) evaluated the use of heterotrophic denitrifying bacteria entrapped within alginate beads with starch and tartrate, respectively. Zhang et al. (2009) assessed *T. denitrificans* immobilized on polyvinyl alcohol (PVA) beads to remove nitrate using thiosulfate as the electron donor. Gómez et al. (2000) evaluated the use of submerged filters

inoculated with an activated sludge and amended with different carbon sources for the removal of nitrate from contaminated groundwater.

The results of the present study using *T. denitrificans* suggest that a small number of cells must adhere to the pyrite surface, and that both attached and free-living cells seem to contribute to pyrite-driven denitrification. Therefore, bioremediation strategies based on denitrification with pyrite can be improved by creating optimal growth conditions for the autotrophic denitrifying bacteria in bioreactors, e.g. promoting attachment. For example, immobilized cells could be of considerable use under low hydraulic retention times (i.e. high flow rate and/or small bioreactor size) and could reduce the start-up period. Further research with denitrifying bacteria is needed to optimize the bioremediation strategies in order to improve the nitrate removal efficiency and bacterial attachment to the mineral surface and to avoid cell leakage or nitrite accumulation.

#### 4. CONCLUSIONS

Growth and attachment of the autotrophic denitrifying bacterium *Thiobacillus denitrificans* onto pyrite surfaces was studied by means of 9-week colonization experiments using polished pyrite slabs.

A small number of cells attached to the pyrite surface, grew and divided. Single cells attached to the pyrite surface were detected first, and after 3 weeks, microcolonies were observed. The microcolonies were observed as islands. Less than 2% of the available pyrite surface was estimated to be covered by cells. The rate of colonization of the pyrite surface was around 35 cells mm<sup>-2</sup> h<sup>-1</sup> during the 3-week period. In 4-week old samples, the cells were found to be surrounded by an organic film. After 9 weeks, organic films covered larger areas of the pyrite surface. About 0.2% of total cells were estimated to be attached to the pyrite surface. Planktonic cells survived during the course of the runs and new attachments of free-living cells were observed over time.

The results suggest that under the experimental conditions, a small number of *T. denitrificans* cells had to attach to pyrite surface in order to reduce nitrate coupled to pyrite oxidation. However, both

attached and free-living cells probably contributed to denitrification. At present, the relative contribution of the free and attached cells and the nature of their contribution remain unresolved.

These results may be applied to the natural environment to predict the spatial distribution of denitrifying bacteria in subsurface environments in an attempt to better understand pyrite-driven denitrification in aquifers. However, further *in situ* colonization experiments are warranted to assess interaction between indigenous bacteria and pyrite in nitrate-contaminated aquifers where mixed natural microbial populations and pyrite surfaces with a larger reactive area, greater roughness and more microtopographic features exist. Furthermore, the results could prove useful in optimizing bioremediation strategies based on the stimulation of pyrite-driven denitrification, e.g. promoting the attachment of denitrifying cells to pyrite in bioreactors.

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## Figure captions

**Figure 1.** SEM images of the initial pyrite surface (A) and after 1 week (B), 2 weeks (C), 3 weeks (D and E), 4 weeks (F), 5 weeks (G) and 9 weeks (H and I) of the start of the colonization experiments. After 1 and 2 weeks, single attached cells are clearly discernable. After 3 and 4 weeks, microcolonies developed. The coherent action of an organic film is visible in the contact areas between the cells. After 9 weeks, organic films covering larger areas of the pyrite surface are observable. Scale bars are shown.

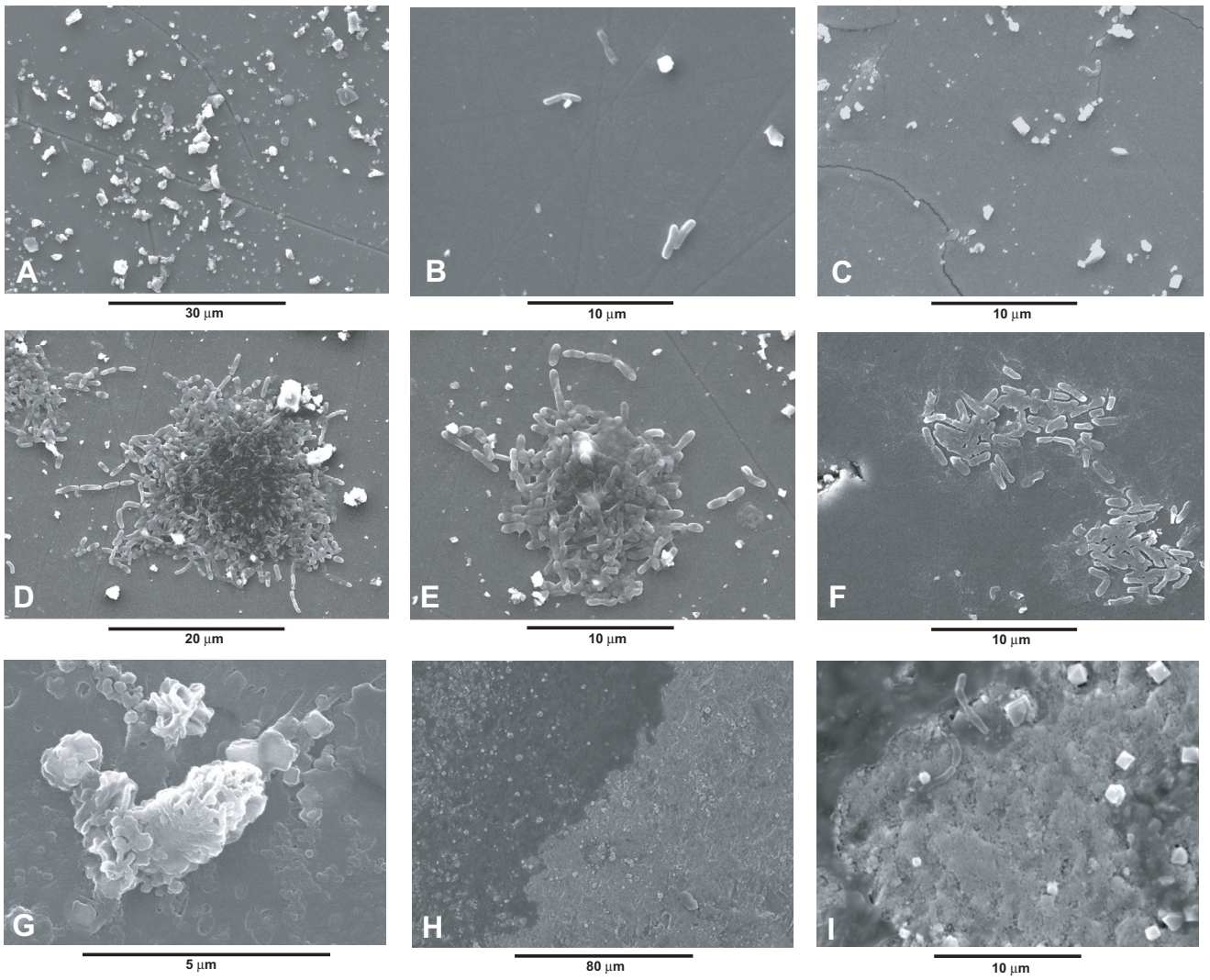
**Figure 2.** Organisms adhering to the surface of pyrite slabs after 1 week (A), 2 weeks (B), 3 weeks (C and D) and 4 weeks (E and F) of the start of the colonization experiments. Cells shown on the left are stained with DAPI and imaged in a CLSM. Images to the right of each fluorescent image correspond to the same site in transmitted light. Scale bars are shown.

**Figure 3.** Cell number in solution and adhering to the pyrite surface over time. The number of viable and total cells in solution was obtained from LIVE/DEAD and DAPI counts of aqueous samples obtained after 1, 2, 3, 4, 5, 6, 7, 8 and 9 weeks and based on 50 mL volume. Attached cells were estimated by using CLSM images of the surface of the pyrite slabs retrieved from the experiments after 1, 2 and 3 weeks and based on 19 mm<sup>2</sup> available surface. The number of attached cells after 3 weeks was probably underestimated, since three-dimensional microcolonies were detected by SEM. Surface colonization rate was estimated from the regression of the data.

**Figure 4.** Variation of nitrate concentration (A) and sulfate concentration (B) over time in the additional experiments performed with powdered pyrite. In these experiments, three

sterilized pyrite slabs and 1 g of 50-100  $\mu\text{m}$  sterilized pyrite were added to 48 mL of the sterile modified medium solution and 2 mL of the *T. denitrificans* culture ( $2 \times 10^8$  cells  $\text{mL}^{-1}$ ).

**Figure 1**



**Figure 2**

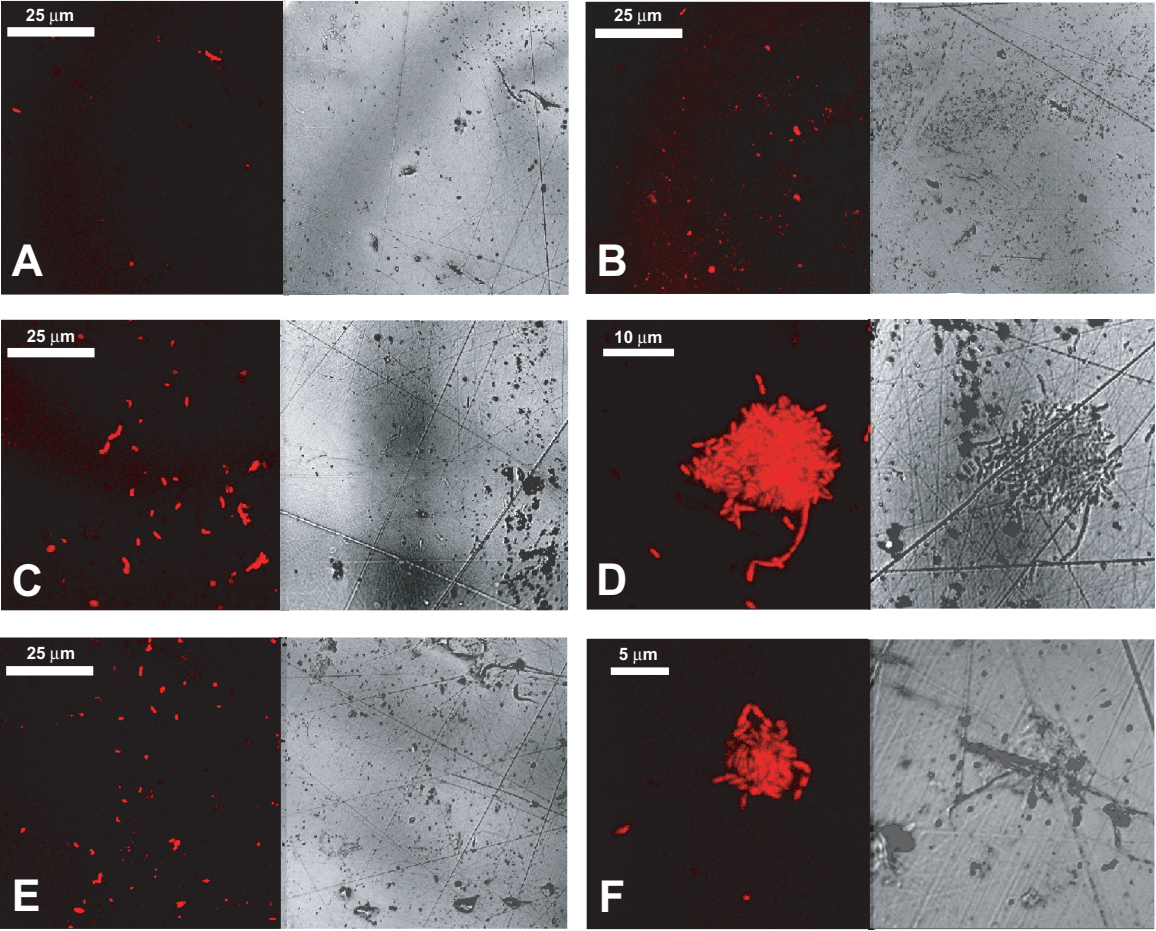
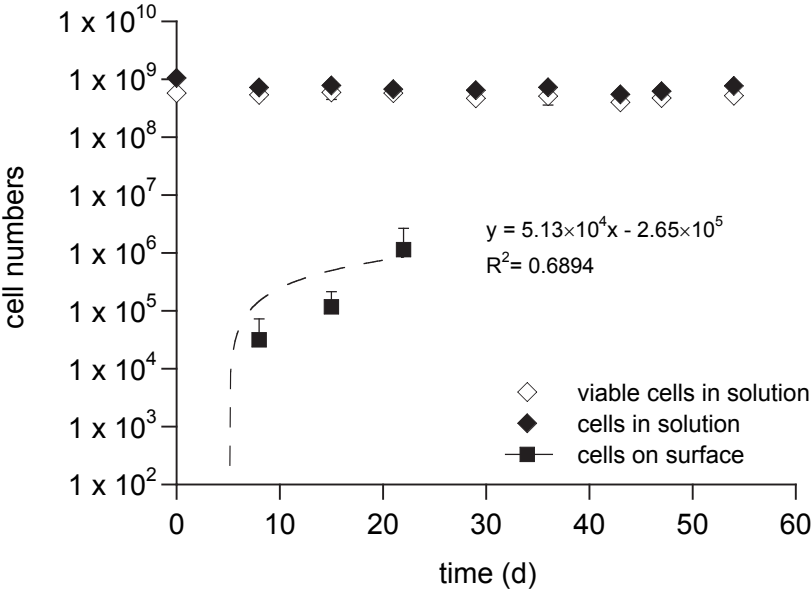
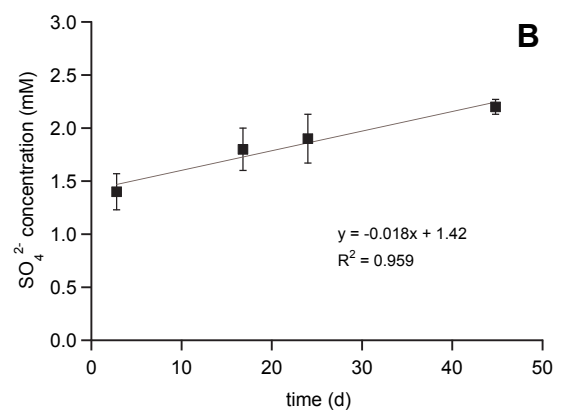
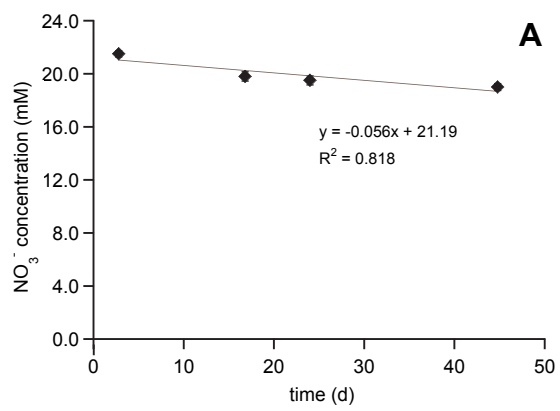




Figure 3



# Figure 4



**Table 1.** Average cell coverage on pyrite surface after 1, 2, 3 and 4 weeks of the beginning of the experiments. Attached cells were estimated by counting from CLSM images.

	time (d)	% apparent statistical coverage area	cells mm <sup>2</sup> on surface <sup>(1)</sup>
1 week	8	0.06	5.55×10 <sup>2</sup>
2 weeks	15	0.21	2.07×10 <sup>3</sup>
3 weeks	22	2.01	2.01×10 <sup>4</sup> <sup>(2)</sup>
4 weeks	27	0.87	n.d.

(1) Based on that the relative surface coverage of one bacterium is 1 μm<sup>2</sup>

(2) The calculated value probably is underestimated (see text)

n.d. = not determined (see text)

**Table 2.** Number of cells in liquid suspension and their viability during the 9-week experiments.

sample	time	cells in solution	viable cells	dead cells	% of viability
	d	$\times 10^7$ cells mL <sup>-1</sup>			
initial	0	2.12	1.16	0.96	54.7
1 week	8	1.45±0.24	1.08±0.17	0.37±0.10	74.3
2 weeks	15	1.57±0.25	1.19±0.29	0.37±0.08	76.1
3 weeks	21	1.35±0.23	1.16±0.15	0.19±0.08	85.8
4 weeks	29	1.30±0.15	0.94±0.06	0.36±0.01	72.6
5 weeks	36	1.45±0.22	1.03±0.31	0.43±0.01	70.6
6 weeks	43	1.10±0.06	0.80±0.07	0.30±0.01	72.7
7 weeks	47	1.25±0.13	0.95±0.13	0.29±0.00	76.5
8 weeks	54	1.55±0.12	1.05±0.07	0.50±0.00	68.0