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Isolation of bacteria capable of reducing ferric iron coupled with oxidizing elemental sulfur

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Abstract

Bacteria capable of reducing ferric iron and oxidizing elemental sulfur were isolated from the inoculums of sediments of Sippewissett salt marsh with marine basal medium, synthetic iron hydroxides (FeOOH) and elemental sulfur. AQDS was also added to investigate if the redox reaction between two insoluble substrates can be stimulated. The initial results showed that the concentrations of ferrous iron and sulfate continuously increased during the incubation for the samples with inoculation of sediments. The addition of anthraquinone-2,6-disulfonate (AQDS) enhanced the production of ferrous iron but not obviously increased the sulfate concentration. The metabolic pathway responsible for this microcosm can't be determined yet based solely on the available data. The ratio of Fe(II)/S higher than 30 coupled with increasing pH value might indicate the total conversion of sulfur to sulfate. On the contrary, the total sulfur concentration is not balanced before and after incubation. More chemical measurements are required to provide the additional constraints. DGGE analyses showed different band patterns for different incubation durations. This might imply a community change as a function of time. No different band pattern was observed for different enrichments with the same incubation time.

1. Introduction

Iron reduction is considered an essential metabolism in many aquatic anaerobic environments because of abundant iron-bearing substrates as well as its impact on the other cycling of elements (e.g. cycling of sulfur or nitrogen). The mobilization of iron or other metals (U, Cr, Tc) by iron reducer also provides a remediation strategy for the metal contamination in the aquifer (Fredrickson et al., 2000; Nevin and Lovely, 2000). The major metabolic pathways for iron reduction are coupled with the oxidation of organic matter or hydrogen to generate a relatively higher yield of energy for bacterial utilization. The extent and rate of iron reduction are controlled by several factors, including the reactivity of iron–bearing substrates, the electron donors and the presence of humic acids (Lovely and Blunt-Harris, 1993).

Many previous studies have shown that the participation of humic acids or hydroquinone can stimulate the iron reduction several factors higher than the controls (e.g. Lovely, 1993). Iron reducing bacteria transfer the electron from the electron donor to the soluble humic acid or hydroquinone group. The subsequent oxidation of reduced humic acid or quinone groups transfers the electrons to the insoluble electron acceptors. These intermediates, on the other hand, can be cycled numerous times and act as an electron shuttle between electron donor and acceptor. In addition to the species mentioned above, U^{6+}, cystein, and S^{o} have also been reported for effective stimulation of iron reduction in the laboratory batch experiment (Nevin and Lovely, 2000). The existence of electron shuttle would especially facilitate the microbial-mediated redox reactions in the sediments since the porosity is relatively low compared to that in the aqueous environments. Instead of direct contact between two substrates for bacteria, the soluble electron shuttle can, therefore, transport electron from the electron donor to the electron acceptor.

The microbial transformation of sulfur species in marine environments is known extensively as the sulfur cycle. Sulfate is anaerobically reduced to sulfide by sulfate reducer. In the oxic environments, either sulfide or elemental sulfur is oxidized to sulfate with the participation of oxygen. In the absence of oxygen, the nitrate can be used as a terminal electron acceptor. An important intermediate, thiosulfate, is found during the anaerobic oxidation of sulfide. Another pathway for transformation of thiosulfate can happen through the disproportionation to sulfate as well as sulfide.

Previous studies have demonstrated several metabolic pathways between sulfur and iron cycling in the aquatic sediments (Aller and Rude, 1988; King, 1990). However, only one study was found to link the iron reduction with sulfur oxidation to explain the relatively low concentration of sulfate when the marine sediment was added with ferric hydroxides (Thamdrup et al., 1993). A model was proposed that the hydrogen sulfide reduced from sulfate was re-oxidized to elemental sulfur and formed iron sulfide with iron hydroxide as an electron acceptor (Thamdrup et al., 1993). Subsequent disproportionation of elemental sulfur resulted in the formation of hydrogen sulfide as well as sulfate. Totally, two-third of sulfur was converted into sulfide where only one-third was oxidized to sulfate. Since the disproportionation of elemental sulfur is

energetically unfavorable (41 KJ/mol) (Thauer et al., 1977), the scavenger of hydrogen sulfide by reduced iron can turn the energetic budget into a favorable condition.

A question, therefore, arises that if there is any metabolic pathway more favorable for bacterial oxidation of elemental sulfur coupled with metal reduction? An alternative pathway for sulfur oxidation other than that proposed by Thamdrup et al. (1993) might be the conversion of all the sulfur into sulfate with reduction of ferric iron. The oxidized iron has to donate a higher reducing power for total oxidation of sulfur compared with that for the sulfur disproportionation. In addition, hydroquinone is frequently used to stimulate the iron reduction coupled with oxidation of soluble organic matter or hydrogen. Is it possible that any catalyst is able to stimulate the metabolism between these two insoluble substrates (S^0 and Fe(III))? Several sulfate reducers, like *Desulfocapsa sulfoexigens sp.*, were reported responsible for the disproportionation of elemental sulfur and thiosulfate (Bak and Cypionka, 1987; Finster et al., 1998). No study has demonstrated the community structure and its changes associated with the oxidation of elemental sulfur and reduction of ferric iron.

In order to answer these questions, an experiment was designed to isolate the bacteria capable of reducing ferric iron and oxidizing elemental sulfur with inoculation of Sippewissett salt marsh sediments. The anthraquinone-2,6-disulfonate (AQDS) with different concentrations was added to investigate the stimulated effects on iron reduction and sulfur oxidation. Denaturing gradient gel electrophoresis (DGGE) was performed for the enrichment sampling at different incubation time to investigate the community changes as a function of time or concentration of AQDS.

2. Experimental design

Media and substrates. The basal medium was consisted of 20 g of NaCl, 3 g of $MgCl_2 \cdot 6H_2O$, 0.15 g of $CaCl_2 \cdot 26H_2O$, 0.2 g of KH_2PO_4, 0.5 g of KCl, 0.25 g of NH_4Cl, 10 ml of bicarbonate solution (10 mM finally), 1 ml of trace element solution, 1 ml of vitamin mixture, and 1 ml of sodium sulfide solution (1 mM finally, as a reducing agent) per liter of deionized water. Trace element solution contained 5.2 g of EDTA, 2.1 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of H_3BO_3, 0.1 g of $MnCl_2 \cdot 4H_2O$, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.024 g of

$NiCl_2 \cdot 6H_2O$, 0.002 g of $CuCl_2 \cdot 2H_2O$, 0.144 g of $ZnSO_4 \cdot 7H_2O$, 0.036 g of $Na_2MoO_4 \cdot 2H_2O$, 0.025 g of sodium vandate, 0.006 g of $Na_2SeO_3 \cdot 2H_2O$, and 0.008 g of $Na_2WO_4 \cdot 2H_2O$ per liter of deionized water. Vitamin mixture solution contained 100 ml of 10 mM phosphate buffer at pH=7.2 and 0.1 g of riboflavin, thiamine-HCl, L-ascorbic acid, D-Ca-pantothenate, folic acid, niacinamide, nicotinic acid, 4-aminobenzoic acid, pyrioxine-HCl, lipoic acid, NAD, and thiamine pyrophosphate, respectively. The medium was either autoclaved and purged with nitrogen and carbon dioxide mixture or sterilized by filtering through 0.2 μm filter.

Artificial ferric iron was obtained from a stock solution of FeOOH with a concentration of 400 mM. Elemental sulfur was prepared from polysulfide with a concentration of 2.52 M by bubbling with carbon dioxide to convert sulfide into sulfur. The final concentration could be lower than 2.52 M due to loss of hydrogen sulfide into the air. AQDS stock solution with a concentration of 1 mM was prepared by dissolved powders of AQDS into deionized water and sterilized through 0.2 μm filter.

Enrichment and cultivation. Two sets of enrichments were designed using different S/Fe(III) ratio. The first set contained of S^0 of 25 mM and Fe(III) of 20 mM while the second set had S^0 of 5 mM and Fe(III) of 40 mM. Each set consisted of 4 samples where a control sample was prepared only with basal medium and electron donor and acceptor, and three additional samples contained (i) control medium and sediments, (ii) control medium, sediments and AQDS of 10μM, and (iii) control medium, sediments and AQDS of 50μM. The inoculated sediments were taken from Sippewissett salt marsh, which included 2 g of black fine-grained sediment and 2 g of brown coarse sediment (oxidized iron). The headspace was continuously purged with nitrogen and carbon dioxide mixture (80:20) during inoculation. The sole carbon source is the carbon dioxide in the headspace. Incubation was performed in dark at 30 °C.

Analytical methods. Ferrous iron was analyzed by measuring the absorbance of the extract at a wavelength of 562 nm by the spectrophotometer and calibrated to the standards. 0.1 ml of the sample was taken out by the syringe and injected into 4 ml of 0.5 M HCl. After 15 to 30 min, 0.1 ml of the acid extracts was transferred to 4 ml of ferrozine reagent (in 50 mM HEPES buffer). After anotehr 15 to 30 min., the ferrozine reactant was filtered through 0.2 μm filter to the vial for the absorbance measurement by

the spectrophotomer. Sulfate concentration was measured by HPLC using lithium borate gluconate eluent. pH was measured by full scale pH paper.

DGGE analysis. DNA was extracted by using MoBio Soil Kit and stored at –20 °C beofre PCR amplification. The primers used for PCR were DGGE341F (GM5F) (5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCC CCC TAC GGG AGG CAG CAG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3'). Each 25 μL reaction product contained 10X reaction buffer, 25 mM MgCl2, dNTP (10 mM), primers (30-40 pmol), taq (5U/ml), and 0.5 μL of DNA extraction. The cycling of the program consisted of an initial denaturation step of 94 °C for 5 min, followed by 10 cycles of denaturation at 95 °C for 1 min, annealing at 66 °C (decreasing in each cycle by 1 °C) for 1 min, and an elongation step for 72 °C for 3 min. Another 15 cycles of 95 °C for 1 min, 56 °C for 1 min, and elongation at 72 °C for 3 min were performed as suggested by Moeseneder et al. (1999). Each run ended with a final elongation step of 72 °C for 5 min.

DGGE was performed using a Dcode Universal Mutation Detection System (Bio-Rad). Two polyacrylamide gel media with different proportions of urea and formamide were mixed together to achieve the 30% denaturation at the top and 70 % denaturation at the bottom of the gel. A well cast was placed into the gel before solidification. TAE buffer was added to immerse the whole gel. In order to prevent the diffusion of urea and formamide into the injection well, all wells were washed by the TAE buffer for at least two times before the injection of PCR products. DGGE gel was run at a constant voltage of 200 V and 60 °C for five hours. The gels were poststained and read in the UV lamp filtered at a wavelength of 520 nm.

3. Results

Incubation and cultivation. Different samples exhibited different colors after 10 days incubation (Fig. 1). The color of the control sample remained in orange to brown no matter the ratio of sulfur to ferric hydroxide is. The samples with the inoculation of sediments turned into black by the end of the incubation.

Observation of the wet mounts showed various types of morphology for the microorganisms, including S-shape, rod, and rounded shape. No significant difference in the morphology of bacteria was found among different samples. An interesting and prevalent phenomenon was observed where a rod-shaped organism attached to the ferric hydroxide particles at one end with several high reflectance spots at another end (Fig. 2). These high-reflectance spots are potentially the elemental sulfur. This observation indirectly attested that the metabolism involving two insoluble substrates favors a direct contact between bacteria and the substrates.

Ferrous iron, sulfate and pH analyses. The concentration of ferrous iron of control samples (with high S/Fe(III) ratio or low S/Fe(III) ratio) increased slowly from 0 to 20 mM during the whole incubation (240 hrs) (Fig. 3 and 4). On the contrary, the samples with an inoculation of sediments increased abruptly at around 80 hrs of incubation. The concentration of ferrous iron increased to ~40 mM at 80 hrs and to ~90 mM at 240 hrs. The addition of AQDS of 10 μM further enhanced the iron reduction as revealed from that the concentration of ferrous iron increased to ~60 mM at 80 hrs and to larger than 120 mM at 240 hrs. The addition of AQDS of 50 μM, however, didn't proportionally stimulate the iron reduction. The concentration of ferrous iron was similar to that of the samples with the inoculation of sediments only.

Sulfate analyses were not successful at the first two samplings because the higher concentration of chloride from seawater medium swarmed the signals. Only one set of results was obtained at the end of incubation. The concentrations of sulfate for high S/Fe(III) samples were around 3 to 5 mM while those for low S/Fe(III) samples were below 2 mM (Fig. 5).

pH analyses were performed at the end of the incubation. The pH values for the samples inoculated with sediments were all between pH=8 and 9.

DGGE analyses. Five DNA extractions were obtained at the fifth days of incubation for DGGE analyses. Only two lanes (for the sample (i) with control medium and sediments (high S/Fe(III) ratio) and (ii) with control medium, sediments and 10 μM of AQDS (high S/Fe(III) ratio)) showed distinctive bands (Fig. 6). Two to three bands were identified at the top of the gel while another band was identified near the middle of the gel. The difference of the band pattern between two samples was not feasible to

determine. Another five DNA extraction were obtained at the ninth days of incubation. Three lanes showed a consistently distinctive band (for the sample (i) with control medium, sediments, and 50 μM of AQDS (high S/Fe(III) ratio), (ii) with control medium and sediments (low S/Fe(III) ratio), and (iii) with control medium, sediments and 10 μM of AQDS (low S/Fe(III) ratio)). The bands, on the contrary, located near the bottom of the gel.

4. Discussion

Possible metabolic pathways. Our analytical results showed that bacteria can grow with reduction of iron and oxidation of elemental sulfur. There might be two potential metabolic pathways utilized as shown below:

$$3S^0 + 2\,FeOOH \rightarrow 2FeS + SO_4^{2-} + 2H^+ \qquad (1)$$

$$S^0 + 6FeOOH + 10H^+ \rightarrow 6Fe^{2+} + SO_4^{2-} + 8H_2O \qquad (2)$$

The first reaction involves the disproportionation of elemental sulfur into sulfide and sulfate. The reduction of ferric iron is coupled with the oxidation of sulfide to elemental sulfur (Thamdrup et al., 1993). However, the second reaction directly converts the elemental sulfur into sulfate.

The Gibbs free energy at pH=7 for reaction (1) and (2) are –49.8 KJ/mol of S and –85.7 KJ/mol of S^0, respectively. Energetically, the second reaction is more favorable than the first one. Another potential indicator for discriminating the possible reaction pathway is the stoicmetric ratio of Fe(II) to sulfate. The ratio is 2:1 in reaction (1) while 6:1 in the reaction (2). The analyses of ferrous iron and sulfate indicated that the ratios of Fe(II)/S for samples with inoculation of sediments were all larger than 30. pH analyses also showed an increasing even with phosphate buffer. These two evidences seemed point to the possibility that the total conversion of elemental sulfur to sulfate coupled with ferric iron reduction might be the predominantly metabolic pathway in this case. However, the contradiction could be found where a gap between the initial sulfur concentration and the final sulfate concentration occurred. If the reaction (2) is the predominately metabolic pathway, then the total sulfur concentration should be conserved. There are two possible explanations: (1) the concentration of elemental sulfur

at time zero is overestimated, and (2) the reaction (1) is still the controlling pathway. Iron sulfide is precipitated from the reaction between reduced iron and sulfide disproportionated from the elemental sulfur. Additional analyses of sulfide and reducible iron could provide a better constraint.

AQDS as an electron shuttle between two insoluble substrates? The analyses of ferrous iron indicated that AQDS can stimulate the oxidation of sulfur and reduction of iron. This provides an implication that AQDS might act as an electron shuttle between two insoluble substrates. A question therefore can be raised whether bacteria need a direct contact with either or both of these substrates? Our observation showed that bacteria attached to the ferric hydroxides at one end and elemental sulfur at the other end (Fig. 2). It looks like that the bacteria bring the particles of elemental sulfur with themselves and search for the iron source. However, since the medium is in aqueous phase, there should be no strict requirement that bacteria have to contact with two substrates at the same time to acquire the metabolic energy. AQDS is able to transfer electrons between two substrates. However, bacteria might need to have a contact with sulfur to acquire the reducing power. Further experiment using dialysis bag as a confined barrier for separation between two substrates could justify this speculation.

The concentrations of ferrous iron for the enrichments with the addition of AQDS were all larger than 120 mM. The initial concentration of artificial FeOOH added was 20 and 40 mM respectively for different enrichment sets. Obviously, AQDS can not only stimulate the reduction of Fe(III) from artificial iron but also from the natural sediments. This observation is consistent with the previous studies.

An unexpected result was that the higher AQDS concentration (50 μM) didn't stimulate iron reduction. The amount of ferrous iron generated was just about the same as the medium without the addition of AQDS. This concentration was much lower than that (250 μM) reported by Nevin and Lovley (2000). The toxicity of AQDS to the microorganisms might be dependent on the organism species or environments. This factor should be taken into consideration for the artificial stimulation of metal reduction in the contaminated aquifer.

Community changes. The DGGE analyses showed a community change at different incubation duration as revealed from different band patterns. However, the band

patterns were not so clear. It might have some bands invisible. An improvement of PCR procedure is necessary to clarify this implication. In addition, no difference of band pattern was observed among inoculums with or without AQDS. Although AQDS was effective in stimulating iron reduction, the incubation duration is probably too short to develop a community dominated by a specific organism.

Bacteria capable of oxidizing elemental sulfur and reducing ferric iron were only reported from marine enrichments. Whether bacteria from fresh water environments can utilize the same metabolic strategy is still in questions. This could be part of the future work to isolate some potential new species.

5. Conclusion

This study demonstrated that bacteria can reduce iron and oxidize elemental sulfur. The addition of AQDS stimulated the redox reaction as revealed from enhanced production of ferrous iron. However, the exact metabolic pathway responsible is still not clear. More experiments and measurements are necessary to provide additional constraints. The changes of community during the incubation could be revealed from the DGGE analyses with uncertainty. But no significant community structure existed among the enrichments with or without AQDS.

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Figure captions

Figure 1. Pictures showing the color differences between controls and enrichments. (a) High S/Fe(III) enrichments. (b) low S/Fe(III) enrichments.

Figure 2. Picture showing a direct contact between elemental sulfur and ferric hydroxide.

Figure 3. Concentrations of ferrous iron as a function of incubation duration for samples with high S/Fe(III).

Figure 4. Concentrations of ferrous iron as a function of the incubation duration for samples with low S/Fe(III).

Figure 5. Concentrations of sulfate measured at the end of the incubation.

Figure 6. Band patterns from the DGGE analyses.

(a)

(b)

Fig 1

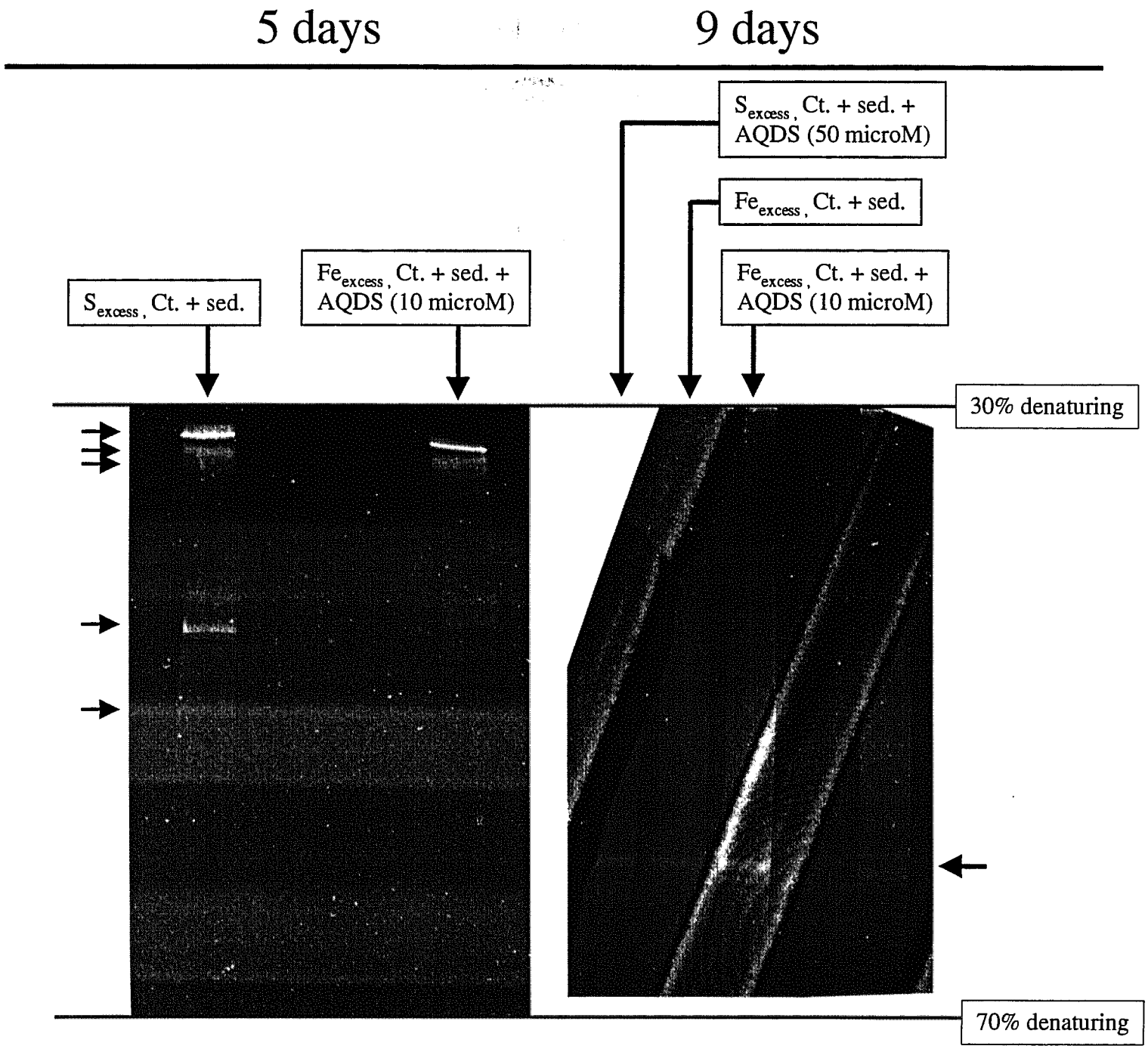
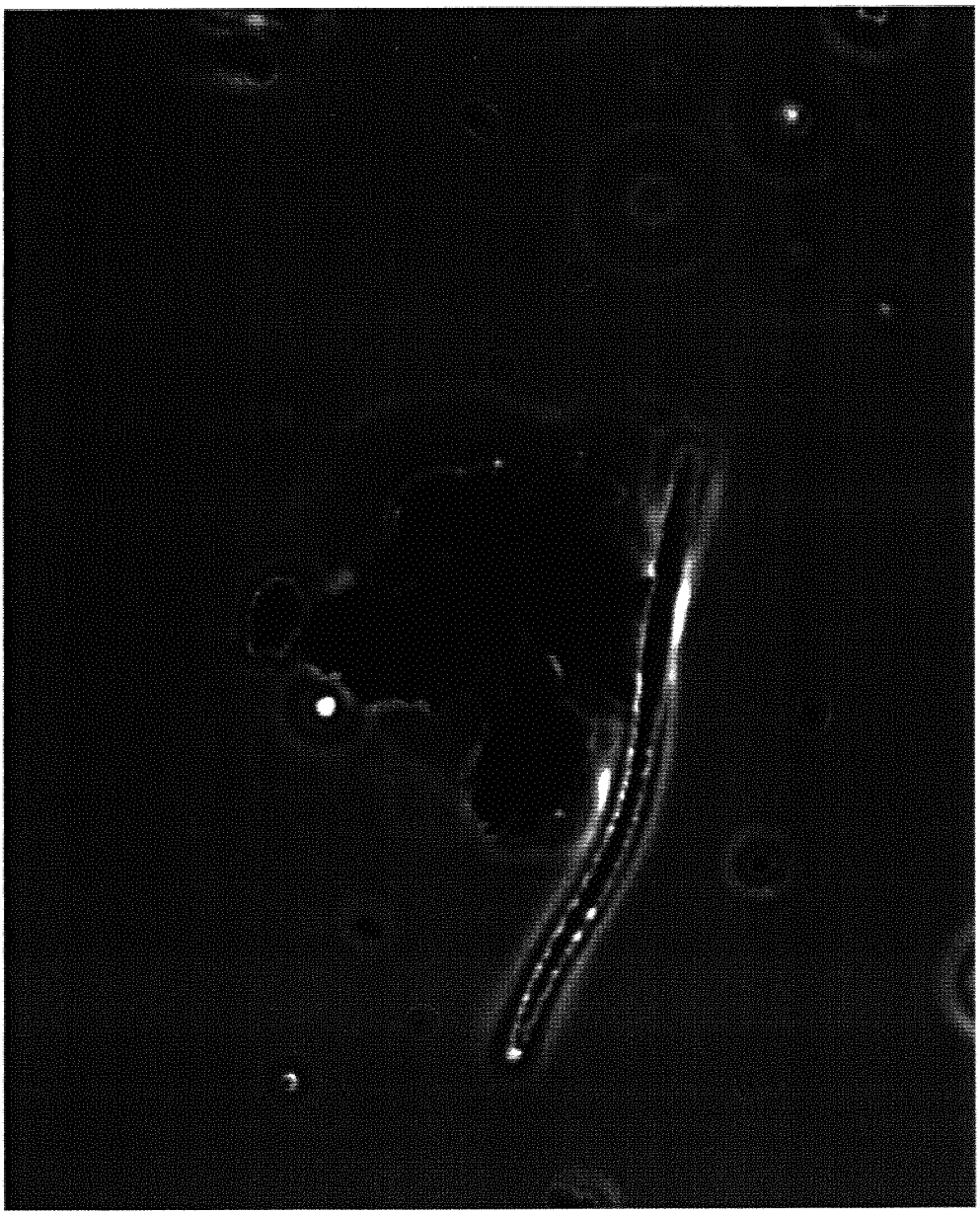

5 days
9 days
S_{excess}, Ct. + sed. + AQDS (50 microM)
Fe_{excess}, Ct. + sed.
Fe_{excess}, Ct. + sed. + AQDS (10 microM)
S_{excess}, Ct. + sed.
Fe_{excess}, Ct. + sed. + AQDS (10 microM)
30% denaturing
70% denaturing

Fig. 6

Fig. 2



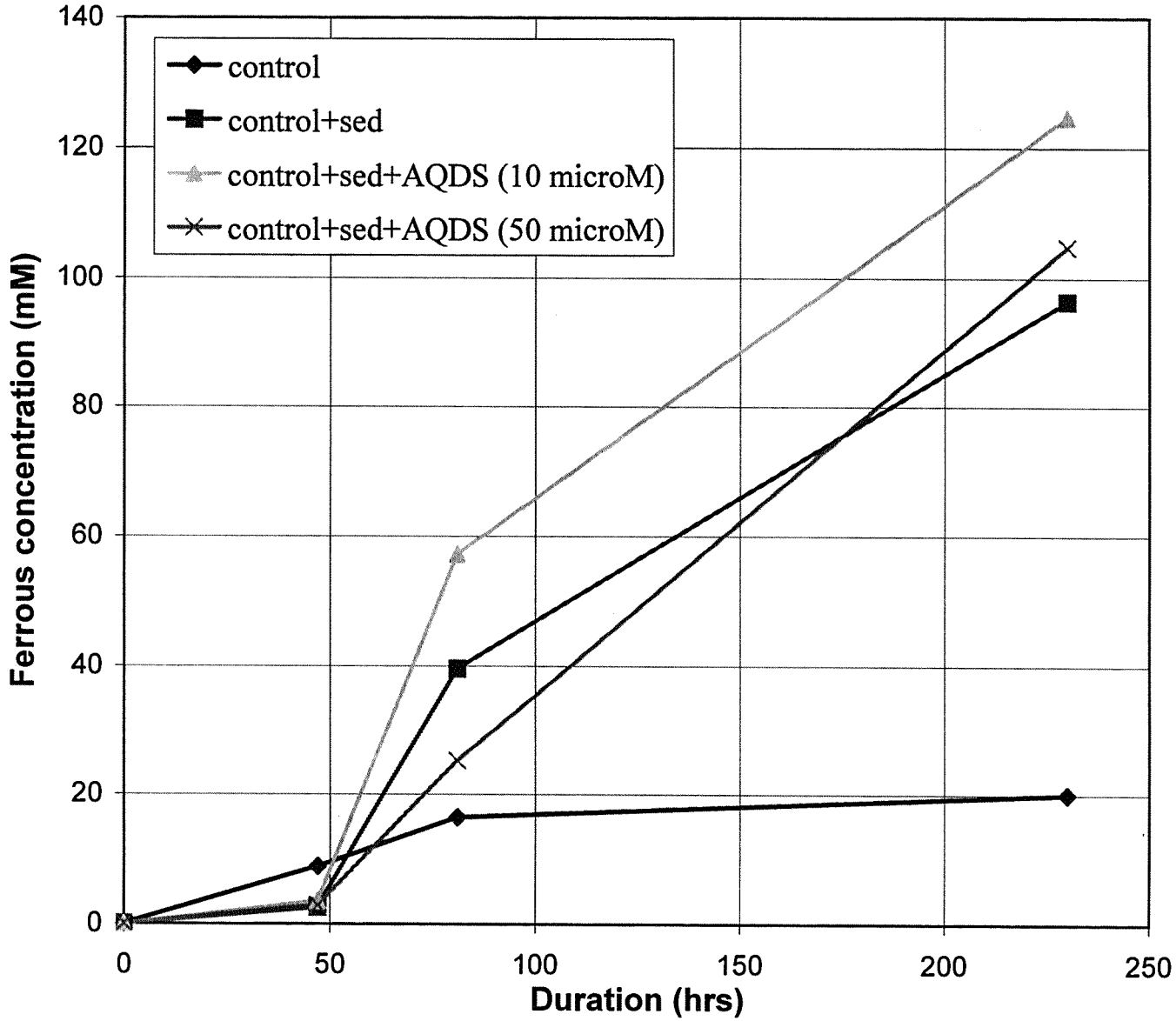

control
control+sed
control+sed+AQDS (10 microM)
control+sed+AQDS (50 microM)
Ferrous concentration (mM)
Duration (hrs)
0
20
40
60
80
100
120
140
0
50
100
150
200
250

Fig 3

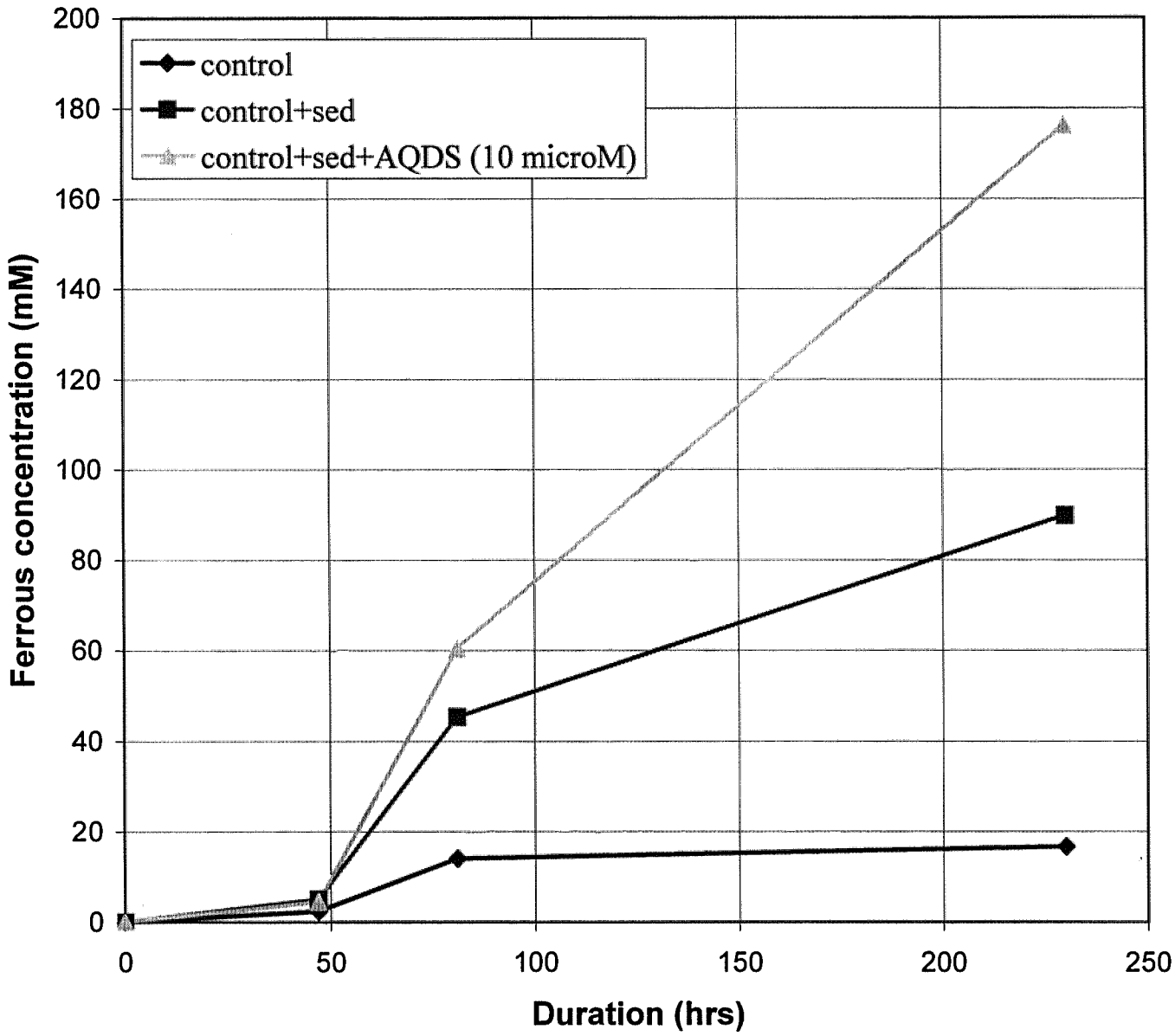

control
control+sed
control+sed+AQDS (10 microM)
Ferrous concentration (mM)
Duration (hrs)
0
20
40
60
80
100
120
140
160
180
200
0
50
100
150
200
250

Fig 4

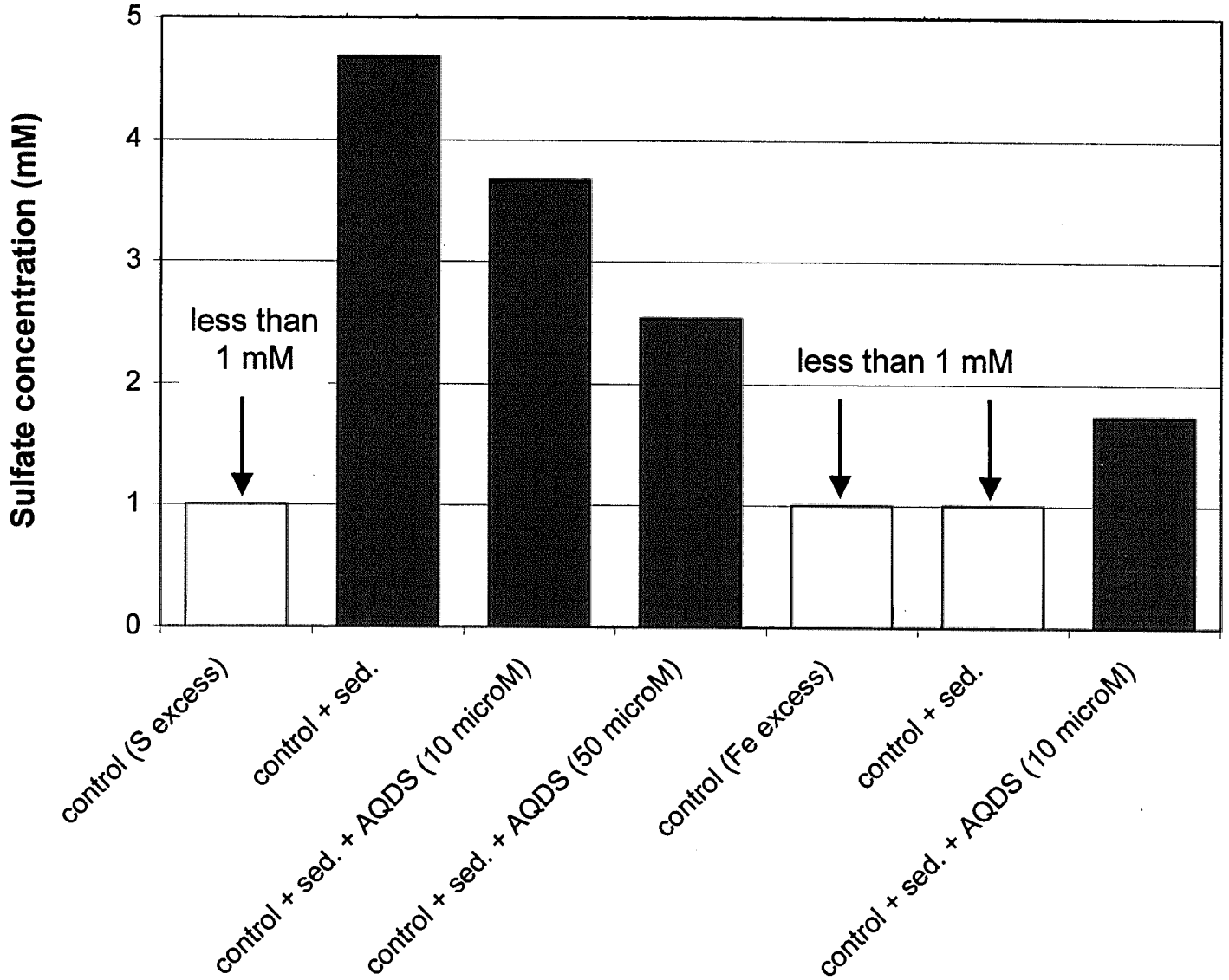

Sulfate concentration (mM)
5
4
3
2
1
0
less than 1 mM
less than 1 mM
control (S excess)
control + sed.
control + sed. + AQDS (10 microM)
control + sed. + AQDS (50 microM)
control (Fe excess)
control + sed.
control + sed. + AQDS (10 microM)

Fig 5