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Deflocculation of Activated Sludge by the Dissimilatory Fe(III)-Reducing Bacterium *Shewanella alga* BrY

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The influence of microbial Fe(III) reduction on the deflocculation of autoclaved activated sludge was investigated. Fe(III) flocculated activated sludge better than Fe(II). Decreasing concentrations of Fe(III) caused an increase in sludge bulk water turbidity, while bulk water turbidity remained relatively constant over a range of Fe(II) concentrations. Cells of the dissimilatory metal-reducing bacterium *Shewanella alga* BrY coupled the oxidation of H_2 to the reduction of Fe(III) bound in sludge flocs. Cell adhesion to the Fe(III)-sludge flocs was a prerequisite for Fe(III) reduction. The reduction of Fe(III) in sludge flocs by strain BrY caused an increase in bulk water turbidity, suggesting that the sludge was deflocculated. The results of this study support previous research suggesting that microbial Fe(III) respiration may have an impact on the floc structure and colloidal chemistry of activated sludge.

Activated sludge treatment of both domestic and industrial wastewater produces sludge which consists of 1 to 5% (wt/wt) total solids and 95 to 99% (wt/wt) bulk water. The disposal of surplus sludge is an expensive process which is ameliorated by reducing the sludge volume through dewatering. Prior to dewatering the sludge is often stored for several days, during which time it rapidly becomes anaerobic. Anaerobic storage has been reported to cause sludge deflocculation (measured as a release of fines), turbid effluent, and reduced dewaterability (17–19). The concentration of fines, i.e., particles less than 10 μm in diameter, has been found to correlate with sludge dewaterability (3, 19, 23). This implies that fines may be an important parameter for determining the dewaterability of activated sludge.

High concentrations of iron, 70 to 90% of which was in the form of Fe(III), have been found in activated sludge (20). This iron accumulation can come from influent wastewater or through the addition of $FeSO_4$, which is used as an agent for phosphorus removal. Almost all treatment plants in Denmark add iron for phosphorus precipitation (9). More than 100 of these contain from 0.2 to 2 mmol of Fe per g of volatile solids (VS) (16a). The number of treatment plants with high Fe contents throughout Europe is expected to increase as greater emphasis is placed on phosphorus removal. In many waste water treatment plants a preclarifying process removes particles through lime-Fe salt precipitation. In these plants, high Fe levels can also be expected in process tanks.

The reduction of Fe(III) in activated sludge is initiated as soon as anaerobic conditions prevail (16a, 20) and may be associated with heterotrophic bacterial activity (18a). A variety of bacteria are capable of coupling organic matter oxidation to dissimilatory Fe(III) reduction (13). It has been suggested that microbial Fe(III) reduction may be responsible for a release of fines from the sludge (20), since organic components, which are common in sludge flocs (6, 21, 25), are flocculated better by Fe(III) than by Fe(II) (11, 24). Fe(III) reduction in activated sludge may also cause a release of phosphorus into the bulk water. In fact, phosphorus accumulation rates observed in the bulk water of the Aalborg East treatment plant (Denmark) during the first 4 h of anaerobic treatment corresponded well to observed Fe(III) reduction rates (20). Fe(III)-reducing bacteria may therefore not only influence the ecology of activated sludge but also have a significant impact on solid/liquid separation, including phosphorus removal, during the treatment of anaerobic sludge.

The purpose of this study was to test the hypothesis that anaerobic reduction of activated sludge-bound Fe(III) by a model Fe(III)-reducing bacterium can result in sludge deflocculation.

Culture conditions and cell preparation. *Shewanella alga* BrY (4, 22) was grown aerobically in tryptic soy broth (30 g/liter) at 25°C on a rotary shaker at 150 rpm for 15 h. Cells were harvested by centrifugation (6,000 × *g*, 4°C, 30 min) during the late exponential/early stationary growth phase. Optimal Fe(III) reductase activity is expressed at this stage of growth (7). Cells were resuspended in 30 ml of 40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer (pH 7.2) that had been made anaerobic by boiling and cooling under a stream of O_2-free N_2.

Sludge preparation. Activated sludge was obtained from the wastewater treatment plant in Bozeman, Mont. This is a traditional nitrification treatment plant serving 23,000 person equivalents. Sludge was collected from the effluent of the aeration tanks, where it has a mean residence time of 9 days. The sludge was immediately transported to the laboratory, autoclaved, concentrated by centrifugation (10,000 × *g*, 4°C, 10 min), and resuspended in 1.5 liters of anaerobic PIPES buffer (40 mM; pH 7.2). Autoclaving the sludge resulted in a release of small particles to the sludge bulk water. Aliquots of the sludge slurry (100 ml) were dispensed into 250-ml Erlenmeyer flasks under a constant stream of O_2-free N_2.

Effects of Fe(III) and Fe(II) on deflocculation. Bulk water turbidity was chosen as a measure of deflocculation in these studies. The dewaterability of sludge, as measured by the specific resistance to filtration, is affected by the particle size distribution. An increase in specific resistance to filtration as-

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TABLE 1. Experimental setup for Fe(III) reduction-deflocculation assay

Treatment	Presence or absence of[a]:				
	Active cells	Killed cells	H_2	Fe(III)	Sludge
a	+	−	+	+	+
b	+	−	−	+	+
c	−	+	+	+	+
d	−	−	+	+	+

[a] +, present; −, absent.

TABLE 2. Characteristics of Bozeman, Mont., sludge[a]

Component	Concn (mean ± SD)[b]
Total solids	2.9 ± 0.03
VS	1.9 ± 0.10
Protein	458 ± 16
Humics	324 ± 9.0
Carbohydrates	339 ± 2.0
Uronate	11.7 ± 1.2
Fe(III)	9.3 ± 16.17
Fe(II)	2.46 ± 0.53

[a] The pH of the sludge was 7.3.
[b] All concentrations except those for total solids and VS (which are in grams per liter) are in milligrams per liter.

sociated with deflocculation, seen as a release of fines, has been observed in anaerobically stored sludge (19). A linear relationship exists between specific resistance to filtration and bulk water turbidity (3, 19). The autoclaved, concentrated sludge slurry was used to determine the effects of various concentrations of Fe(III) and Fe(II) on bulk water turbidity. Samples of the slurry were placed in 1.5-ml microcentrifuge tubes, and various amounts of $FeCl_2$ or $FeCl_3$ were added to give final concentrations of between 0 and 2.5 mM Fe. The samples were vortexed for 10 s and centrifuged in a Fisher Marathon H microcentrifuge at the lowest setting for 15 s. The turbidity of the supernatant was measured at 650 nm with a Hach model DR/2000 spectrophotometer. Duplicate determinations were performed for each iron concentration, and duplicates did not differ from the mean by more than 10%.

Fe(III) reduction-deflocculation assay. Anaerobic $FeCl_3$ was added to each flask of the autoclaved, concentrated sludge slurry to give a final Fe(III) concentration of ca. 2 mM. The sludge-iron mixture was incubated in the dark at 25°C overnight to allow flocculation, i.e., removal of small particles from sludge bulk water. Strain BrY cells were then added to the flocculated sludge to provide a final concentration of ca. 7.2×10^8 cells per ml. Flasks were incubated at 25°C and 150 rpm under a headspace of N_2 and analyzed for turbidity every 15 min. After 1 h, the N_2 headspace was replaced with a headspace of 100% H_2, and flasks were sampled for turbidity, Fe(III), and Fe(II) every 3 h. All treatments, including positive and negative controls, are outlined in Table 1. Each treatment was performed in triplicate.

Analytical techniques. Bulk water turbidity during the deflocculation assay was determined as described above. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) and the loss of Fe(III) over time. The amount of Fe(II) solubilized after 15 min in 0.5 N HCl was determined with ferrozine as previously described (15). The amount of hydroxylamine-reducible Fe(III) was determined as described previously (14). Cell numbers were determined by a modification of the epifluorescence microscopy technique (10) as previously described (15). Cells for microscopy were fixed in 2% glutaraldehyde, stained with 4′-6-diamidino-2-phenylindole dihydrochloride (DAPI), filtered onto a 0.2-μm-pore-size membrane, and enumerated under an Olympus Beta-Max epifluorescent microscope. Neutral hexose levels were determined by the phenol-sulfuric acid method (5) with glucose as the standard. Uronic acids were quantitated by the *m*-hydroxydiphenyl method (2) as modified by Kinter and Van Buren (12) with glucuronic acid as the standard. Levels of protein and humic compounds were estimated by a modification of the Lowry procedure (6, 16) with bovine serum albumin and humic acid from Jansen Chemica as the standards. Levels of sludge total solids and VS were estimated as previously described (1).

Sludge characterization. Protein was the largest fraction of the organic matter in the activated sludge obtained from the wastewater treatment facility in Bozeman, Mont. Significant amounts of carbohydrate and humic compounds were also present (Table 2). This is in accordance with the composition of activated sludges from other treatment plants (6, 25). The Fe(III)-Fe(II) content of this sludge was quite low (5 mg/g of VS) compared with previously reported values in the range of 50 to 70 mg/g of VS for Aalborg treatment plants (20). This is most likely due to the fact that $FeSO_4$ is not used to remove phosphorus in the Bozeman treatment plant. The low concentrations of iron in the sludge required the addition of Fe(III) for flocculation of the fines. The amount of Fe(III) added was similar to that found in water treatment plants which use $FeSO_4$ for phosphorous removal (20).

Effects of Fe(III) and Fe(II) on sludge. Increasing concentrations of Fe(III) caused a decrease in sludge bulk water turbidity (i.e., removal of fines by flocculation), while bulk water turbidity remained almost constant over a range of Fe(II) concentrations (Fig. 1). At Fe(III) concentrations greater than 2 mM, there was an almost complete removal of fines from the bulk water. However, a drastic increase in turbidity was observed at Fe(III) concentrations below 1 mM. These results demonstrated that the addition of Fe(III) caused flocculation of the autoclaved activated sludge and that the impact on flocculation was greatest at some critical Fe(III) concentration between 1 and 2.5 mM. The data suggested that the reduction of Fe(III) to Fe(II) at an initial Fe(III) concentration of approximately 2 mM could lead to a substantial deflocculation of the sludge. Interestingly, 2 mM $FeCl_3$ corresponded to 59 mg/g of VS, which is very close to the intrinsic

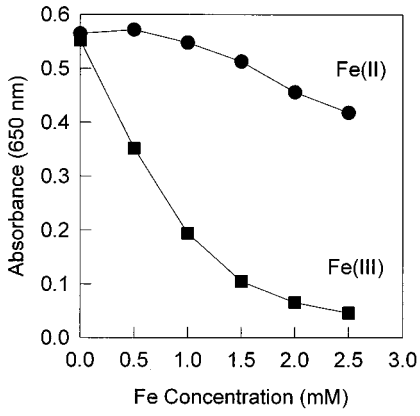

FIG. 1. Effects of different concentrations of Fe(III) and Fe(II) on sludge bulk water turbidity.

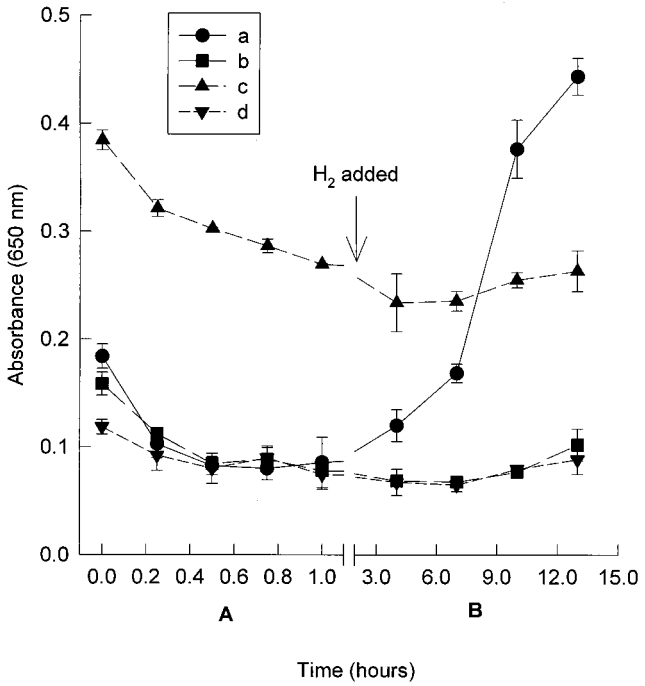

FIG. 2. (A) Cell binding to Fe(III)-sludge flocs prior to electron donor addition. (B) Release of fines into the sludge bulk water as a result of bacterial Fe(III) reduction after electron donor addition. Refer to Table 1 for an explanation of the symbols.

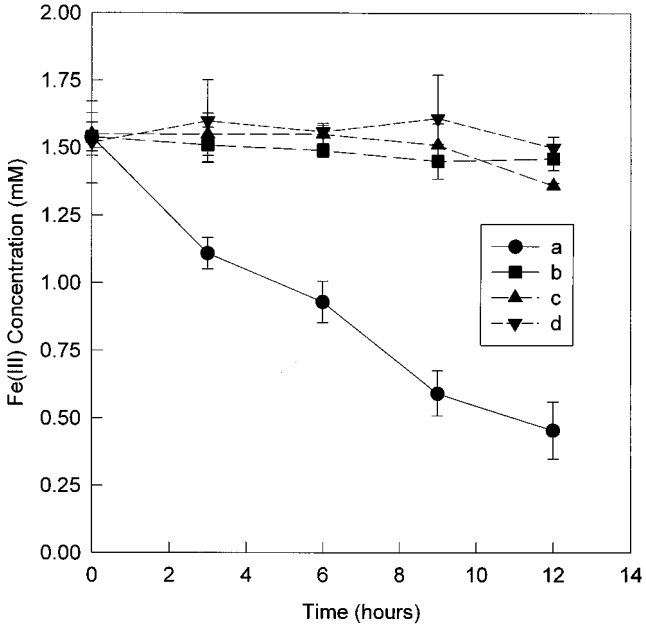

FIG. 3. Fe(III) concentration over time after the addition of H_2. Refer to Table 1 for an explanation of the symbols.

Fe(III) concentrations observed in a treatment plant performing chemical removal of phosphorus (20). The data do not offer an explanation for the apparent differences in flocculation effectiveness between Fe(II) and Fe(III). It has, however, been demonstrated that Fe(III) binds humic substances better than Fe(II) does, and humic substances seem to be a major part of the sludge floc matrix (Table 2) (6, 21). It is also known from the traditional flocculation theory that the valence of the ion causing flocculation is of great importance. This has been quantified by the Schultze-Hardy rule (8), which states that the critical ion concentration necessary for flocculation is proportional with $1/z^6$, where z is the valence of the cation. A change in valence from 3 to 2 thus decreases the flocculation efficiency of the cation (Fe) by a factor of approximately 10.

Fe(III) reduction and deflocculation of sludge. A period of 1 h was required for the binding of the BrY cells to the Fe(III)-sludge flocs (Fig. 2). During this time the flasks were shaken under a headspace of N_2 to facilitate the attachment process. The attachment of the cells to the Fe(III)-sludge flocs resulted in a decrease in bulk water turbidity from 0.180 to 0.085 absorbance units. This decrease in turbidity was considered to be due to cell binding to the flocs because the turbidity of the control without cells did not decrease significantly over this time. Also, a lag of approximately 6 h in turbidity decrease and Fe(III) reduction was observed in parallel experiments in which the flasks were not shaken (data not shown).

After the 1-h period of cell adhesion, the Fe(III) reduction-deflocculation experiment was initiated by replacing the N_2 headspace of treatments a, c, and d with a headspace of 100% H_2. The turbidity of treatment a, with active cells and H_2, increased over time (Fig. 2). A decrease in Fe(III) concentration (Fig. 3) and an increase in Fe(II) concentration (Fig. 4) over time were observed concomitant with the turbidity increase in this treatment. Approximately 86% of the total amount of Fe(III) was reduced. There was no increase in turbidity (Fig. 2) or Fe(III) reduction (Fig. 3 and 4) in controls which lacked an electron donor, contained heat-killed cells, or contained no cells. These results demonstrate that *S. alga* BrY was capable of reducing Fe(III) associated with activated sludge. The reduction of Fe(III) in sludge flocs by strain BrY caused deflocculation, as shown by the release of fines and an increase in bulk water turbidity. The relationship between Fe(III) concentration, as measured by the hydroxylamine

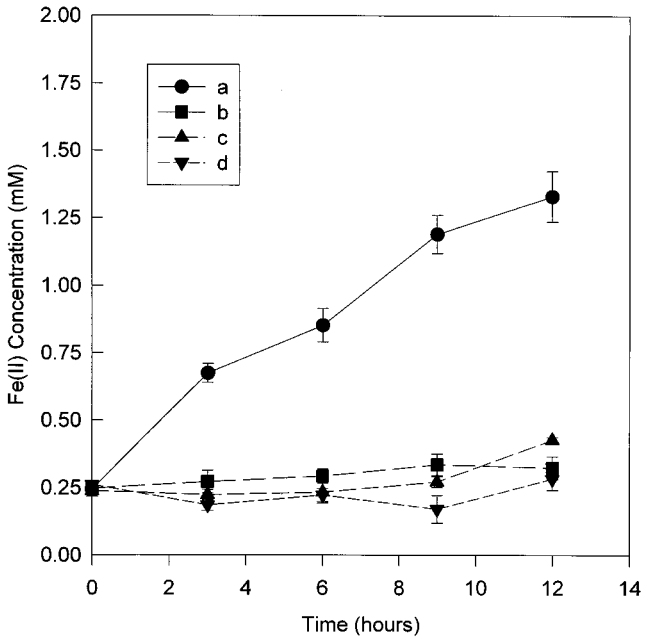

FIG. 4. Fe(II) concentration over time after the addition of H_2. Refer to Table 1 for an explanation of the symbols.

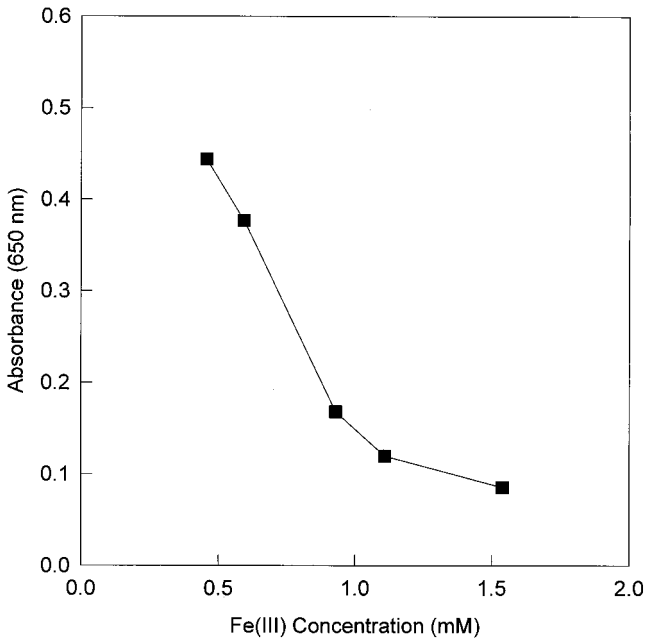

FIG. 5. Relationship between Fe(III) concentration and turbidity in treatment a.

method, and bulk water turbidity in treatment a (Fig. 5) was similar to that observed previously (Fig. 1). The decrease in Fe(III) concentration, resulting from bacterial reduction, led to an increase in turbidity which was most drastic at Fe(III) concentrations below 1 mM.

The increase in turbidity in treatment a was not due to desorption of cells from sludge flocs. The final turbidity in this treatment was much greater than the initial turbidity (Fig. 2). Microscopic analysis also showed that the initial concentration of free cells in the bulk fluid was quite high. As Fe(III) was reduced over time, the concentration of free cells decreased. By the end of the experiment, very few free cells were observed and most of the bulk water turbidity was due to other small particles, e.g., colloids.

Previous studies have implicated microbial Fe(III) respiration as a mechanism for the Fe(III) reduction observed in anaerobically stored sludge (16a, 19, 20). It has also been suggested that anaerobic degradation processes, such as Fe(III) and SO_4^{2-} reduction, may be involved in the disintegration of sludge flocs during anaerobic storage, leading to a decrease in dewaterability (19). The results of this study demonstrate that microbial Fe(III) reduction can cause deflocculation of activated sludge. While this study used an artificial system, consisting of autoclaved sludge and a high cell density of a pure bacterial culture, the maximal Fe(III) reduction rates observed here (30.5 μmol g of organic matter^{-1} h^{-1}) correspond with Fe(III) reduction rates observed in anaerobically stored "natural" sludge (15.9 to 23.9 μmol g of organic matter^{-1} h^{-1}) (20). Dissimilatory Fe(III)-reducing bacteria have recently been isolated from sludge in which Fe(III) reduction is a significant process (3a). The results presented here suggest that Fe(III)-reducing bacteria can have an effect on sludge floc structure during short- or long-term anaerobic storage periods.

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