Evidence for Microbial Iron Reduction in a Landfill Leachate-Polluted Aquifer (Vejen, Denmark)

HANS-JÖRGEN ALBRECHTSEN* AND THOMAS H. CHRISTENSEN
Institute of Environmental Science and Engineering, Groundwater Research Centre, Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 29 April 1994/Accepted 16 August 1994

Aquifer sediment samples obtained from the anaerobic part of a landfill leachate plume in Vejen, Denmark, were suspended in groundwater or in an artificial medium and incubated. The strictly anaerobic suspensions were tested for reduction of ferric iron [Fe(III)] oxides, which was measured as an increase in the concentration of dissolved Fe(II). Iron reduction did not occur when the medium was inoculated with inactive sediment and when the organisms in the inoculated medium were killed by formaldehyde, by chloroform, or by pasteurization, whereas the level of iron reduction was significant when living bacteria were present. Mixed cultures were obtained from the sediment samples, and differences in apparent iron reduction rates among the different cultures were maintained during several transfers. In addition, iron reduction was observed in unamended incubation mixtures containing whole sediment and groundwater. Synthetic amorphous Fe(III) oxides, as well as naturally occurring sediment-bound Fe(III) oxides, could be reduced by the cultures. Together, our results provide evidence that iron-reducing bacteria are present and microbial iron reduction occurs in the polluted aquifer sediments which we studied.

Until recently, microbial reduction of iron [Fe(III)] oxides, in which ferric oxide is used as a terminal electron acceptor, was an overlooked process that is important in the anaerobic turn-over of organic matter (9, 13, 17). Fe(III) oxides are extremely insoluble and in this way differ from most other common electron acceptors used by microbes (e.g., oxygen, nitrate, and sulfate). This characteristic is especially important in aquifers, where the water flows through a porous medium; the soluble electron acceptors are transported with the flowing water, whereas the Fe(III) is fixed on the porous medium.

In aerobic aquifers, sediment-associated Fe(III) oxides may be the predominant compounds responsible for the oxidation capacity of the aquifer (7). Despite the abundance of Fe(III), heterotrophic bacteria probably first use the more oxidized electron acceptors (oxygen and nitrate) in the water phase, since the predicted thermodynamic energy yields obtained with these electron acceptors are the highest yields. In terms of oxidation capacity per unit volume of aquifer, the soluble electron acceptors are often insignificant compared with Fe(III) oxides (7), although of course the soluble electron acceptors can be replenished over time by infiltration of water rich in the electron acceptors or by diffusion (e.g., diffusion from the unsaturated zone).

Landfill leachate contains high concentrations of organic matter and is usually strongly reduced (4). When leachate enters a shallow, often naturally aerobic aquifer, a sequence of redox zones may develop, with a methanogenic/sulfate-reducing zone close to the landfill and a denitrifying zone and an aerobic zone in the outskirt of the plume, if nitrate and oxygen are present in the unpolluted aquifer. If the sediment is rich in Fe(III) oxides, a zone characterized by increased concentrations of dissolved Fe(II) (15) and depletion of Fe(III) in the sediment (6) may develop between the methanogenic/sulfate reducing zone and the denitrifying/aerobic zone. Indications of iron reduction downstream of landfills have been observed in some cases, as summarized by Christensen et al. (4). Furthermore, dissolved organic matter and xenobiotic organic compounds seem to be degraded in the ferrogenic zone (16).

Indications of microbial iron reduction have been observed in deep unpolluted aquifers (3), and iron-reducing bacteria have been isolated from deep unpolluted aquifers (11). Microbial iron reduction also has been observed in a shallow aquifer contaminated by crude oil (2, 10). The relative importance of enzymatic and nonenzymatic iron reduction in sediments has also been studied (14). However, no attempt has been made to distinguish between microbial and abiotic processes as the cause of the increased concentrations of dissolved, reduced iron species that characterize the iron-reducing zones in landfill leachate plumes.

The aim of this study was to estimate the significance of microbial processes in the reduction of sediment-bound Fe(III) in a leachate plume. In this study we focused on the Vejen Landfill leachate plume in Denmark, which has been described previously with respect to water chemistry (15, 16) and sediment chemistry (6).

MATERIALS AND METHODS

Sampling. The sediment samples were obtained 2.5 to 5.8 m below the surface in the anaerobic part of the leachate plume downstream of the Vejen Landfill in Denmark (Fig. 1). Municipal waste was disposed of at the landfill from 1962 to 1981. The shallow aquifer is unconfined and consists mainly of glacial meltwater sand with clay and silt inhomogeneities. The sediments were deposited in the Weichselian glacial period (10,000 to 70,000 years ago) in the late Quaternary.

The sediment samples were collected with a piston sampler (19). Aluminum coring tubes (diameter, 5 cm) were driven into the aquifer with a Cobra jackhammer. After the filled coring tubes were drawn to the surface, they were cut into pieces and sealed at the ends with aluminum foil and plastic caps. The
which was prepared previously described methods (7, 8). Processed sediment samples indicate the nitrogen-flushed bottle (15). The wells consisted of iron pipes (diameter, 2.0 cm), each of which had a Teflon check valve mounted above a 10-cm screen. The iron pipes were driven into the ground with a pneumatic hammer. Groundwater samples were obtained from each well by applying nitrogen pressure through a Teflon tube lowered into the well as described in detail by Lyngkilde and Christensen (15). The other end of the Teflon tube was lowered to the bottom of a 1-liter autoclaved nitrogen-flushed bottle which was filled with no headspace and was sealed with a butyl rubber stopper. The groundwater was characterized (the amount of dissolved iron was determined, etc.) as described in detail by Lyngkilde and Christensen (15).

Experimental conditions. All manipulations were carried out by using aseptic and strictly anaerobic techniques. All of the enrichment culture experiments were performed in 117-ml serum bottles with butyl rubber stoppers; the headspace gas in the bottles was N$_2$-CO$_2$ (80:20). The bottles were incubated at the actual groundwater temperature (10°C). Subsamples were collected with a syringe and a needle.

The medium used was an artificial oligotrophic anaerobic medium (OAM) that was modified after Worakit et al. (21); this medium contained (per liter) 0.25 g of yeast extract, 0.25 g of Bacto Tryptone (Difco), 1.0 g of NH$_4$Cl, 0.04 g of K$_2$HPO$_4$, 3H$_2$O, 0.1 g of MgCl$_2$, 6H$_2$O, 0.05 g of CaCl$_2$, 2H$_2$O, trace metals, and 3.8 g of NaHCO$_3$, and its pH was around 7.2.

Amended sediment incubation experiments. Serum bottles were prepared with 80 ml of OAM. Before the preparations were autoclaved for 1 h at 121°C, 5 ml of sediment that was rich in ferric oxides was added as an electron acceptor.

Sediment samples (5 ml) that were obtained from seven different sample points (samples C1 to C7), which represented different dissolved Fe(II) concentrations in the anaerobic part of the plume (Fig. 1), were used as inocula.

After 33 days of incubation, 0.1 ml from each sediment suspension was transferred to autoclaved OAM supplemented with 5 ml of sediment rich in ferric oxides. After incubation of this first preparation for 206 days, 0.1 ml was transferred to fresh medium.

Enrichment experiments. Several sediment samples (samples E1 to E5) (Fig. 1) were collected from the anaerobic part of the leachate plume, and these samples were enriched with synthetic amorphous Fe(III) oxides that were prepared as described by Lovley and Phillips (12). Seven enrichment cultures were established from these preparations. Inoculum (0.1 ml) was transferred to OAM supplemented with synthetic amorphous iron oxides [final concentration, approximately 400 mg of Fe(III) per liter]. A control was prepared by adding formaldehyde to a final concentration of 2% (wt/wt).

After three to five transfers (the last three transfers on OAM), the cultures were incubated for 128 days and then pasteurized at 80°C for 10 min. After pasteurization the concentration of dissolved Fe(II) was monitored for another 160 days.

Unamended sediment incubation. Adjacent groundwater and sediment samples (samples U1 to U3) were collected from the plume (Fig. 1). The sediment cores were opened in an anaerobic box (Coy Laboratory Products, Inc., Grass Lake, Mich.), and samples (20 g, wet weight) were transferred to autoclaved 58-ml serum bottles, mixed with 30 ml of sterile filtered groundwater, and incubated. Inactivated controls were prepared by adding 150 μl of chloroform to sediment samples prepared as described above.

The concentrations of dissolved Fe(II) were determined by the ferrozine method (20).

Determination of dissolved Fe(II) concentration. Subsamples (approximately 1 ml) were immediately filtered (Minisart SRP 15; pore size, 0.45 μm; Sartorius) and diluted in an HNO$_3$ solution (25 ml of concentrated HNO$_3$ dissolved in 2.5 liters of water). This filtration procedure removed colloidal oxidized iron species, and the Fe(II) that passed through the filter was quantified with a Perkin-Elmer model 370 atomic absorption spectrophotometer at 247.9 nm. The Fe(II) values obtained were verified by comparison with the results of the ferrozine method (20).

Microbial analysis. Subsamples of each suspension were preserved by adding phosphate-buffered formaldehyde to a final concentration of 2% (wt/wt) for counting of bacteria or were frozen at −80°C for ATP analysis.

The bacteria were counted by the acridine orange direct count method as described by Albrechtsen and Winding (1). Dilutions of the samples were mixed thoroughly, filtered onto a black Nucleopore filter (pore size, 0.2 μm), and stained with acridine orange (final concentration, 10 μg/ml). Fading of the fluorescence was reduced by washing the filter with 0.3 M 1,4-diazabicyclo[2.2.2]octane (DABCO) for 0.3 min. All liquids were filtered (pore size, 0.2 μm) before they were used. The bacteria were counted with an epifluorescence microscope.

ATP contents were determined by the luciferin-luciferase method, using purified enzyme reagent obtained from Lumac bv or Boehringer Mannheim GmbH. Subsamples (100 μl) of each suspension were treated with 100 μl of nucleotide releasing reagent NRB (Lumac bv) to extract the ATP. A parallel water sample was treated with 20 μl of an ATP solution (5 ng/ml) as an internal standard. The light output was measured with a BioCounter MZ2010 instrument (Lumac bv), and the values obtained were converted to ATP values on the basis of the internal standard data and a standard curve prepared for...
TABLE 1. Concentrations of bacteria and ATP contents in the sediment incubation mixtures, determined at the beginning of the experiment and during the experiment.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cells (10^6) ml^-1 at zero time</th>
<th>ATP content (ng ml^-1) at:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Zero time</td>
<td>46 Days</td>
</tr>
<tr>
<td>C1</td>
<td>1.7 ± 0.5</td>
<td>0.149</td>
</tr>
<tr>
<td>C2</td>
<td>1.2 ± 0.4</td>
<td>0.046</td>
</tr>
<tr>
<td>C3</td>
<td>3.4 ± 0.9</td>
<td>0.213</td>
</tr>
<tr>
<td>C4</td>
<td>3.0 ± 0.6</td>
<td>0.170</td>
</tr>
<tr>
<td>C5</td>
<td>2.1 ± 0.6</td>
<td>0.050</td>
</tr>
<tr>
<td>C5b</td>
<td>2.3 ± 0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>C6</td>
<td>1.7 ± 0.5</td>
<td>0.085</td>
</tr>
<tr>
<td>C6b</td>
<td>1.5 ± 0.5</td>
<td>0.034</td>
</tr>
<tr>
<td>C7</td>
<td>2.8 ± 0.7</td>
<td>0.034</td>
</tr>
</tbody>
</table>

* Mean ± standard deviation, as determined by the acridine orange direct count method.

* Control treated with formaldehyde.

* - not measured because of interference of the formaldehyde added.

RESULTS

Dissolved iron in the plume. In the leachate-polluted aquifer close to the landfill (Fig. 1) the concentration of dissolved Fe(II) was less than 2.5 mg liter^-1, but 25 to 50 m from the landfill the concentration was more than 30 mg liter^-1; the concentration remained at this level until 100 m downstream and reached values as high as 68 mg liter^-1 (15). Further downstream the concentration decreased to the background level of 0.1 to 0.4 mg liter^-1. The concentrations given above were not corrected for dilution.

Amended sediment incubation experiments. The sediment rich in Fe(III) oxides that was used as the electron acceptor in our experiments was yellowish brown (7.5 YR 4/6; determined by Munsell color charts). The oxidation capacity of this sediment was 90 μeq g^-1 (dry weight)^-1, and Fe(III) was responsible for approximately 90% of this oxidation capacity. The total amount of the iron species (extracted with 5 M HCl at 90°C for 8 h) was 20.8 mg g^-1 (dry weight)^-1. The total amount of manganese was only 0.09 mg g^-1 (dry weight)^-1. Mild extraction with 0.1 M HCl at 70°C for 24 h produced 2.16 mg of Fe(III) per g (dry weight) and only 0.03 mg of Fe(II) per g (dry weight), and long-term extraction (21 days) with 5.0 M HCl at 20°C produced 10.7 mg of Fe(III) per g (dry weight) and only 0.9 mg of Fe(II) per g (dry weight), showing that the sediment was well oxidized.

At the beginning of the sediment incubation experiment, the total numbers of bacteria in the suspensions ranged from 1.2 × 10^6 ± 0.4 × 10^6 cells ml^-1 in sample C2 to 3.4 × 10^6 ± 0.9 × 10^6 cells ml^-1 in sample C3 (Table 1). The ATP contents ranged from 0.034 ng of ATP ml^-1 in sample C7 to 0.21 ng of ATP ml^-1 in sample C3.

The concentration of Fe(II) increased during incubation of all seven sediment samples (Fig. 2). The Fe(II) concentration increased the least in the controls treated with formaldehyde (to 0.02 mg g^-1 (dry weight)^-1 after 215 days). The increase in Fe(II) concentration varied among the sediment samples incubated; the highest Fe(II) concentration (0.66 mg g^-1 (dry weight)^-1) was observed in sample C2 after 215 days of incubation, and this value remained constant for the next 100 days. In the other experiments, the Fe(II) concentration increased continuously. The slowest increases were observed in samples C5 and C6.

After transfer to fresh medium the dissolved iron concentration increased in all of the suspensions incubated (Fig. 3), except the sample C6 suspension. The different iron reduction patterns observed with the different suspensions were to a large extent also recognizable in the following incubations. Generally, the few differences observed were between the first incubation mixture, which was inoculated with original aquifer sediment, and the second and third transfers, which were very similar.

Enrichment experiments. All seven cultures grown in OAM supplemented with synthetic amorphous Fe(III) oxides as electron acceptors exhibited significant iron reduction compared with the formaldehyde-killed control (Fig. 4).

Pasteurization after 128 days of incubation resulted in substantial (5 to 56%) decreases in the concentrations of dissolved iron, but the concentrations remained constant or (in a few bottles) slightly decreased during the 160 days after pasteurization.

Unamended sediment incubation experiments. The unamended sediment incubation samples were collected from the landfill leachate plume in an environment where there were significant markers of the leachate, but where the concentrations had decreased because of dilution and degradation of some of the organic matter but were still elevated compared with the background levels. The samples were characterized by relatively low organic matter contents (6 mg of dissolved carbon per liter and 0.3 mg of sediment-bound carbon per g (dry weight)) and contained no significant organic contaminants (16). Fe(III) oxides (0.1 to 1 mg of Fe(III) per g (dry weight)) when extracted with 0.5 M HCl at 20°C for 24 h) were present in the sediment.

Iron reduction occurred in all three incubation mixtures (Fig. 5). During incubation the concentrations of dissolved Fe(II) in the active incubation mixtures increased to 0.0018 to 0.0076 mg of Fe(II) per g (dry weight), which were distinctly higher than the concentrations in the chloroform-inhibited controls.
DISCUSSION

The observed increases in the concentrations of dissolved Fe(II) in the water phase in the leachate plume (Fig. 1) indicate that Fe(II) was produced, and therefore this zone was considered ferrogenic (15). They hypothesized that this redox zone was formed by reduction and solubilization of iron oxides in the sediment, which were caused by the reduction of dissolved organic matter in the leachate by either chemical or biologically mediated processes. However, the increase in dissolved iron content also may have been due to other processes. In the early stages of the landfill the leachate supposedly contained large amounts of Fe(II) (4), which may have been bound to the sediment in the aquifer either by ion exchange or by precipitation of sulfides or carbonates. When the landfill is older, this Fe(II) might be released from the sediment by dissolution or ion exchange as a result of the changed composition of the leachate. Further downstream (100 to 200 m) from the landfill the concentration of dissolved Fe(II) decreased, probably as a result of precipitation (e.g., as carbonates), ion exchange onto the sediment, oxidation, and dilution.

Other investigators have shown that iron oxides can be reduced abiotically by natural organic matter as fulvic acids (5), but microbial dissimilative iron reduction also may occur, since all seven sediment samples obtained from the polluted aquifer exhibited a potential for microbial iron reduction when they were incubated anaerobically in a synthetic medium containing readily degradable organic matter and naturally occurring Fe(III) oxides (Fig. 2). The significant difference between the active cultures and the formaldehyde-killed controls strongly indicates that some of the bacteria present (Table 1) were involved in the increase in the dissolved iron concentration. The absence of a close relationship between the rate of iron reduction and the number (as determined by the acridine orange direct count method) and growth (as determined by the ATP method) of bacteria is probably due to the occurrence of

FIG. 3. Dissolved Fe(II) concentrations over time in experiments performed with different transfers of cultures grown in OAM and offered naturally occurring Fe(III) from aquifer sediment as an electron acceptor. Symbols: ◯, first transfer; ●, second transfer; ■, original sediment inoculum. All preparations were incubated anaerobically at 10°C. The controls (indicated by asterisks) were inhibited by formaldehyde.

FIG. 4. Effect of pasteurization on the development of dissolved Fe(II) in enriched cultures grown in OAM containing synthetic amorphous Fe(III) oxides as electron acceptors. The cultures were incubated anaerobically at 10°C. The designations of the seven different cultures examined are indicated. The control (indicated by an asterisk) was inhibited by formaldehyde.
a complex population, only a minor part of which is iron reducing.

A chemical bacteriostatic agent (formaldehyde) was able to inhibit active iron-reducing cultures and to almost eliminate iron reduction in the presence of both naturally occurring Fe(III) oxides (Fig. 2 and 3) and synthetic amorphous ferric oxides (Fig. 4 and 5). In addition, thermal inhibition (pasteurization) (Fig. 4) terminated active iron reduction. This is consistent with observations made in marine environments, where pasteurization has been shown to stop the development of dissolved Fe(II) (18). Although it was obvious that the iron species were affected by the heat treatment (Fig. 4), this finding could not explain the effect on iron reduction as reduced availability since the Fe(III) oxides were autoclaved before the experiment was started.

In summary, the absence of significant abiotic iron reduction due to the medium and the inhibition of iron reduction by the addition of chemical bacteriostatic agents or pasteurization strongly indicate that microorganisms were involved in the iron reduction observed.

Another indication that microorganisms are involved in iron reduction is the fact that iron-reducing capability can be transferred. In the sediment incubation experiments as little as 0.1 ml of an active culture transferred to 100 ml of medium was sufficient to initiate iron reduction (Fig. 3), and the apparent iron reduction rates were different. Similar results were obtained in the enrichment experiment, in which many different apparent rates were maintained after at least three transfers (Fig. 4). The stability of iron reduction for several transfers and after substantial dilution indicates that the iron-reducing bacteria grow and that the iron reduction is not just due to transfers of an enzyme, since the enzymatic activity probably would have been reduced by dilution.

The differences in the iron reduction rates in the different sediment incubation experiments (Fig. 2) might be explained by differences in the amount of Fe(III) in the different preparations, but the addition of sediment rich in Fe(III) would probably have eliminated the possible differences in the sediment samples used as inocula. It should also be noted that measurements of dissolved Fe(II) could have underestimated total iron reduction (as discussed in reference 9) as up to approximately 50% of the Fe(II) was bound to the sediment, iron exchanged, or precipitated (e.g., as carbonates) in the actual samples (data not shown). Differences in these parameters among the different sediment samples used as inocula could also have influenced the apparent levels of iron reduction, but it should be noted that in all of the vessels one-half of the sediment present was the same sediment (the sediment rich in Fe(III)), moderating the differences in sediment characteristics among the vessels. Therefore, a possible explanation is that the differences were due to different populations of iron-reducing bacteria.

The observed potential for iron reduction in cultures obtained from the polluted aquifer indicated that iron-reducing microorganisms occur in the aquifer. Since not only synthetic amorphous Fe(III), which is considered to be readily available for microbial iron reduction, but also naturally occurring Fe(III) oxides, which are also present in the actual aquifer, could be reduced, conditions for microbial iron reduction seem to be present in the aquifer.

Nevertheless, because we provided yeast extract and tryptone as artificial organic carbon sources in the enrichment cultures, the balance between microbiologically mediated and nonmicrobiologically mediated iron reduction processes could have been dramatically altered from the in situ processes. The unamended sediment incubation experiment in which only naturally occurring organic matter present showed that iron reduction can occur (Fig. 5), although the rates were much lower than the rates in the amended incubation experiments. Only insignificant, if any, abiotic iron reduction was observed during incubation. This strongly indicates that a substantial part of the iron reduction observed in the landfill leachate-polluted aquifer may be microbiologically mediated.

Although neither oxygen, nitrate, nor sulfate was present in the medium, our results do not indicate that iron was the energy-yielding terminal electron acceptor, as such a conclusion demands further justification (e.g., pure-culture studies with experimental conditions deviating from the field situation). Our results were obtained from experiments carried out under conditions comparable to the conditions in the field, including incubation at the in situ temperature (10°C) with a natural Fe(III)-rich sediment and with a dissolved organic matter content (approximately 250 mg of C per liter) that was within the range observed in the leachate plume, although higher than the content in the proposed iron-reducing zone (1.2 to 72 mg of C per liter) (15) and probably more readily degradable. This clearly suggests that microbial iron reduction is important in aquifers, not only in deep aquifers (11) and shallow crude oil-polluted aquifers (2, 10), but also in shallow landfill leachate-polluted aquifers.

ACKNOWLEDGMENTS

The technical assistance of Gitte Brandt, Mona Reifstrup, and Karin Hansen is gratefully acknowledged. We thank Gorm Heron for providing iron speciation for the characterization of some of the sediments.

This study was part of a major research program focusing on the effects of waste disposal on groundwater. This program is funded by the Danish Technical Research Council, the Technical University of Denmark, and the Commission of the European Communities.

REFERENCES


