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Electron shuttling via humic acids in microbial iron(III) reduction in a freshwater sediment

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Abstract

The biological and chemical potential for electron shuttling via humic acids was evaluated by analyzing the depth distribution of humic-acid-reducing and iron-reducing bacteria in a freshwater sediment, and correlating it to the redox characteristics of humic acids and iron. Physicochemical analysis of profundal sediments of Lake Constance revealed a distinct stratification, with oxygen respiration, microbial iron and sulfate reduction, and methanogenesis allocatable to defined layers. Among the acid-extractable iron in the surface layer, ferric iron (Fe(III)) was dominant, whereas ferrous iron (Fe(II)) prevailed below 2 cm depth. Humic acids showed a higher electron-accepting (oxidizing) capacity in the surface layer and a higher reducing capacity in deeper layers. The more reduced redox state of humic acids in deeper layers was probably due to reduction by humic-acid-reducing microorganisms. Most-probable-number analysis revealed that the sediments contained populations of humic-acid-reducing bacteria that (i) were substantially larger than those of the iron-reducing bacteria in the respective sediment layers and (ii) were in the same range as those of the fermenting bacteria. Our results suggest that microbial reduction of humic acids and subsequent chemical reduction of poorly soluble iron(III) minerals by the reduced humic acids represents an important path of electron flow in anoxic natural environments such as freshwater sediments.

Keywords: Humic acid reduction; Microbial iron reduction; Electron shuttling; Sediment

1. Introduction

Humic substances are a chemically heterogeneous class of polymeric organic compounds that are widespread in aquatic and terrestrial environments. They contain redox-active functional groups, such as quinones, and have the ability to form humic–metal complexes [1].

Iron-reducing bacteria can use humic acids, a major subfraction of humic substances [1], as electron acceptor in the biological oxidation of acetate, lactate, or H_2 [2,3]. The reduction of quinoid constituents is probably responsible for the electron uptake by humic acids [4,5], but also participation of complexed metal ions (e.g., Fe(III) [6]) and conjugated aromatic systems [7] in electron uptake has been suggested. Initially, the ability to reduce extracellular quinones in mesophilic microorganisms was thought to correlate directly with the ability to reduce Fe(III) [8]. A study from our laboratory, however, revealed that also pure cultures of fermenting bacteria are able to use humic acids as alternative electron acceptors, thus shifting their fermentation product pattern towards more oxidized products [9]. Subsequent studies by other authors have shown that humic acids can also be reduced by sulfate-reducing, halorespiring, methanogenic, and hyperthermophilic microorganisms [10,11].

Reduced humic acids can transfer electrons to ferric iron and oxidized manganese species in a purely chemical reaction [2,7,8,12,13]. Accordingly, the transfer of electrons from microbial oxidation processes to ferric iron would not be restricted to iron-reducing microorganisms, but could be an activity caused also by fermenting bacteria as well as by sulfate-reducing, halorespiring, and methanogenic microorganisms via humic acids as electron shuttle.

Very little is known about the ecological significance of humic acid reduction in natural environments. To date, it is not possible to determine rates of humic acid reduction in soils or sediments directly. It has been shown recently

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that humic substances and other extracellular quinones can serve as electron shuttles to Fe(III) oxides in aquifer sediments [14]. Another study evaluated the potential for humic acid reduction in several environments, focusing on the phylogenetic relationships between the iron-reducing microorganisms and the humic-acid-reducing microorganisms recovered [3]. All humic acid reducers isolated with anthraquinone-2,6-disulfonate (AQDS), a model compound for quinone moieties in humic acids, are members of the family *Geobacteraceae* and are able to reduce both humic acids and Fe(III) oxides.

In the present study, we determined the biological potential for humic acid reduction in a freshwater sediment, comparing the population sizes of acetate-oxidizing and lactate-oxidizing humic-acid-reducing microorganisms with those of iron-reducing and fermenting bacteria. By assessing the vertical distribution of humic acids and iron oxides and their respective redox capacities, we were able to correlate the biological potential for humic acid reduction at different depths with the chemical capacities of humic acids and ferric iron to serve as electron acceptors in this profundal sediment.

2. Materials and methods

2.1. Sampling site, field procedures, and core processing

Samples were taken from undisturbed profundal sediments (Lake Constance, Germany; coordinates: 47°37.675′N and 9°18.352′E) outside the influence of the River Rhine from a water depth of ~100 m. Sediment cores (8 cm diameter, 20–30 cm long) were taken with a four-tube multicorer from the research vessel 'Robert Lauterborn', yielding four parallel cores per sampling site. The cores were cooled immediately after sampling and processed within a few hours after sampling.

The sediment density was calculated from sediment weight and volume in a volumetric flask. For iron, humic acid and porewater analyses, sediment cores were processed anoxically in a modified glove box (N_2 atmosphere; 2–4% H_2), which allowed the cores to be introduced vertically through the bottom of the chamber. Cylindrical samples were taken from the cores at different depths using 1- or 5-ml plastic syringes cut at the front end. For porewater analyses, sediment samples (20 ml) were transferred into 50-ml stainless steel centrifugation tubes which were sealed in the glove box and centrifuged at $12\,000 \times g$ and 4°C for 30 min. Again inside the glove box, the supernatant was separated from the pellet and used for further analyses, thus ensuring anoxic conditions throughout the whole process.

For methane analysis, subsamples (5 ml) were taken from the sediment core with plastic syringes, transferred into 60-ml serum bottles containing glass beads and 20 ml NaOH (10 M) and sealed immediately with butyl rubber stoppers. After vigorous mixing, methane was measured in the headspace by gas chromatography.

2.2. Media

Most-probable-number (MPN) analyses were performed in anoxic, bicarbonate-buffered mineral medium [15] with 1 mM sulfate as sulfur source. After autoclaving and cooling under N_2/CO_2 (80:20, v/v), trace element solution SL9 (1 ml l^{-1}) [16] and vitamin solution were added (1 ml l^{-1}) [17], and the pH was adjusted to 7.2. No reducing agent was added.

Substrates were added from sterile stock solutions. Poorly crystalline ferric iron hydroxide was prepared as described by Schwertmann and Cornell [18]. Humic acids for microbiological experiments were isolated from the investigated sediment (0–10 cm depth) as described in Section 2.4, and purified after the IHSS protocol (International Humic Substance Society [19]) omitting the dialysis step. Humic acids were suspended (50 mg ml^{-1}) in 30 mM phosphate buffer, pH 7.0, stirred under vacuum in butyl-rubber-stoppered vials, repeatedly flushed with nitrogen, autoclaved at 121°C for 25 min, and added to the medium after cooling.

2.3. Enumeration of microorganisms

Serial dilutions of sediment samples were basically performed as previously described [20]. Initial dilution steps were carried out in butyl-rubber-stoppered glass vials; the first tube of each series contained glass beads and was shaken vigorously to homogenize the sediment and to release attached bacterial cells. To increase the accuracy of the results, further dilutions were carried out in microtiter plates using 3.3-fold dilutions in eight parallels. Wells were filled with 100 µl medium. The plates were incubated in anaerobic jars containing N_2/CO_2 (80:20, v/v) at 30°C in the dark under anoxic conditions. Samples were analyzed after 6 and again after 10 weeks. MPNs were calculated from the pattern of positive wells as described by Cochran [21].

Iron-reducing bacteria were enumerated in medium supplemented with acetate (5 mM) or lactate (10 mM) as substrate and poorly crystalline ferric iron hydroxide (40 mM) as electron acceptor. The wells in the microtiter plates were considered positive if the ferrozine assay for Fe(II) [22] was positive (determined visually by the presence of the purple color of the Fe(II)–ferrozine complex).

Humic-acid-reducing bacteria were enumerated in medium containing acetate (5 mM) or lactate (10 mM) as substrate and humic acids (0.5 mg ml^{-1}) as electron acceptor. Because of the low electron-accepting capacity of the humic acids we also added poorly crystalline ferric iron hydroxide (40 mM) using the chemical reduction of Fe(III) by the reduced form of humic acids [12] as an indicator for humic acid reduction. It has to be noted

that this strategy could be applied only because the number of humic-acid-reducing bacteria was higher than the number of iron-reducing bacteria in all samples.

Lactate-fermenting bacteria were enumerated in medium containing lactate (10 mM) and yeast extract (0.05%, w/v). Wells in the microtiter plates were considered positive if high-performance liquid chromatography (HPLC) analysis indicated that lactate was consumed and propionate and acetate were formed.

2.4. Humic acid analyses

For humic acid extraction, sediment samples (10 ml) taken in an anoxic glove box (see above) were transferred into stainless steel centrifugation tubes containing 20 ml anoxic 0.1 M NaOH [23]. Tubes were closed with stainless steel lids sealed with an O-ring and incubated at 30°C on a rotary shaker (90 rpm) for 24 h. Afterwards, the tubes were centrifuged at $12\,000 \times g$ for 30 min at 4°C; the supernatant was recovered in the glove box and acidified to pH < 2 with HCl (1 M) in polypropylene centrifuge tubes. Tubes were kept at 4°C for 24 h to allow the humic acids to precipitate. The precipitates obtained after centrifugation ($12\,000 \times g$ for 30 min at 4°C) were freeze-dried, weighed, and then stored under anoxic conditions at 4°C.

In order to determine the electron uptake capacity, humic acids were dissolved (1 mg ml^{-1}) in 50 mM anoxic phosphate buffer, pH 7.0. An aliquot was incubated under H_2 in the presence of a Pd catalyst (5% Pd on activated charcoal; 1 mg ml^{-1}) on a rotary shaker at 30°C for 24 h, and then repeatedly degassed and flushed with N_2 under stirring to remove excess H_2. Both the reduced and the untreated solutions were titrated with a potassium hexacyanoferrate(III) solution [24]. The amount of reducing equivalents released by the untreated preparation was defined as their reducing capacity; the value obtained for the reduced aliquot was defined as the total reducing capacity. The difference in reducing equivalents released by the reduced and the non-reduced sample was defined as the electron uptake capacity of the preparation [9].

2.5. Analytical procedures

Lactate and other organic acids were analyzed by HPLC as described previously [9]. Low concentrations of acetate and propionate (< 2 mM) were quantified by gas chromatography [9]. Methane was measured by gas chromatography with a flame ionization detector using a molesieve column (1.8 m × 1 mm; 60/80 mesh, 5 Å) at an oven temperature of 120°C.

Sulfate in the porewater was determined by ion chromatography (Sykam, Gilching, Germany) using an anion exchange column (LCA A03, Sykam) as the stationary phase and an isocratic eluent (5 mM Na_2CO_3, 0.005% (w/v) 4-hydroxybenzonitrile and 1% (v/v) acetonitrile) at a flow rate of 2 ml min^{-1}. The oven temperature was 30°C and anions were detected with a conductivity detector (S3110, Sykam). Sulfate was identified and quantified using external standards.

For extraction of HCl-extractable iron, sediment samples (0.1 ml) were transferred into serum bottles (60 ml) containing 10 ml 1 M HCl and sealed with butyl rubber stoppers inside the glove box. After incubating on a rotary shaker (90 rpm) at 30°C for 24 h, the bottles were centrifuged at $1500 \times g$ for 10 min. Ferrous iron and total iron were determined in the acidic supernatant using the ferrozine method [22]. To account for heterogeneity of the sediment, averages were calculated from three replicate samples taken at each depth of the sediment core.

Redox potential and pH were measured in the porewater with commercially available combination electrodes. The electrical circuit for the redox measurement was calibrated using saturated quinhydrone solutions in pH calibration buffers of known pH. Oxygen depth profiles were determined using subcores and micromanipulator-driven microelectrodes. Clark-type oxygen microelectrodes with guard cathodes [25] were constructed in our laboratory and calibrated as described [26].

3. Results

3.1. Physicochemical characterization of the sediment

Profundal sediment samples consisted of fine-grained material without any major macroscopic heterogeneity. Sediment color and concentration profiles of various physicochemical parameters allowed several layers (A–D) representing the major zones of microbial redox processes (Fig. 1; see also [20]) to be distinguished; subsamples from these layers were used for MPN estimation of various microbial metabolic groups (see Section 3.4).

The top layer of all cores showed a rusty-brown color, probably caused by freshly deposited organic material and iron hydroxides, which extended less than 1 cm below the sediment surface. Microelectrode measurements of oxygen indicated that the top millimeters of layer A represent the zone of oxygen respiration (Fig. 1a). The green-brown zone of iron respiration (layer B) extended to a depth of about 5 cm and was followed by a black layer (C) which extended down to 8 cm depth, indicating the presence of iron sulfides. The sulfate concentration profile in the porewater (Fig. 1a) confirmed that layer C represents the zone of sulfate reduction. Layer D, starting at 8 cm depth where all sulfate was depleted, was gray and consisted of very fine-grained material, probably including pyrite. This layer coincided with the zone of methane production, which usually extended down towards the end of the sediment core (about 20 cm) (Fig. 1a). The apparent redox potential (E_h) of the porewater decreased from positive values in layer A to negative values between −150 and

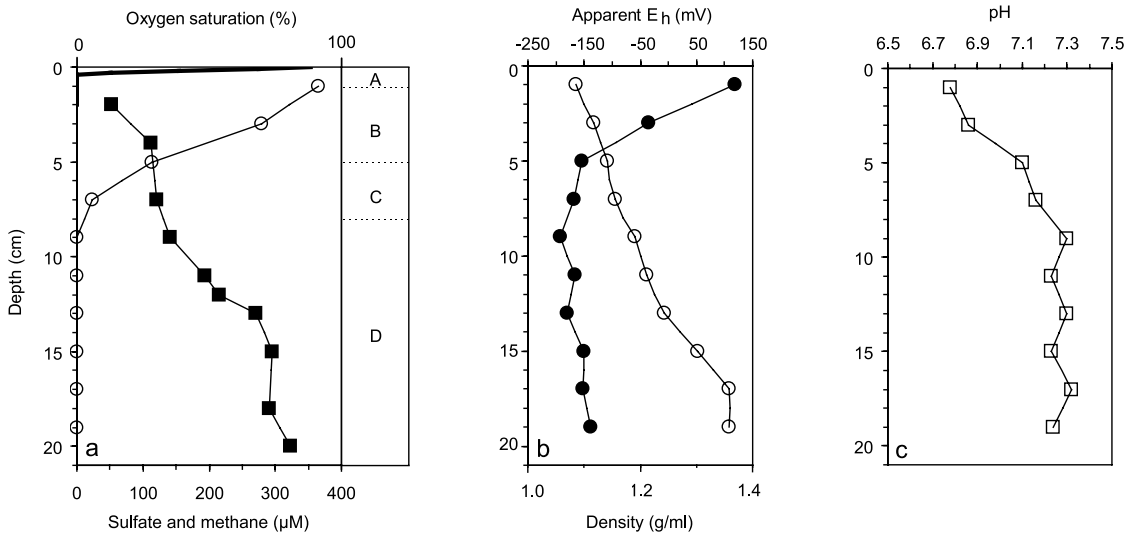

Fig. 1. Representative depth profiles of redox metabolites and physicochemical parameters in the sediment cores. a: Oxygen (thick line) was determined with microsensors, sulfate (○) was measured in the porewater, and methane (■) was determined with the headspace technique. b: Sediment density (○) and apparent redox potential (●), which was measured in the porewater. c: pH in the porewater (□). Layers A–D designate the zonation of the major microbial redox processes in the sediment, inferred from the depth profiles: A, oxygen (and probably nitrate) respiration; B, iron reduction; C, sulfate reduction; D, methanogenesis.

−200 mV in layers C and D. The pH in the porewater and the sediment density increased slightly with depth (Fig. 1b,c). These depth profiles were reproduced with at least four independent cores from the same site, with minor variations.

3.2. *Ferrous iron (Fe(II)) and ferric iron (Fe(III))*

The amount of total HCl-extractable iron per ml sediment increased with depth from 32 µmol in the surface layer to 68 µmol at 20 cm depth (Fig. 2a). In the top layer (0–1 cm), no Fe(II) was recovered and all iron was present in its oxidized state. However, the Fe(II) content of the sediment increased steadily with depth, and at 20 cm depth, 55 µmol Fe(II) per ml sediment was found. The ratio of Fe(II) to total Fe increased strongly with depth (Fig. 2b) and reached its maximum (80%) in the sulfate reduction zone (layer C). The amount of acid-extractable Fe(III) in each layer represents the (theoretical) electron uptake capacity of the Fe(III) fraction (Fig. 2b). It was highest in the sediment surface (31.7 µequiv (ml sediment)$^{-1}$), decreased with depth in layers A and B, and stayed constant between 10 and 12 µequiv (ml sediment)$^{-1}$ in the deeper layers C and D.

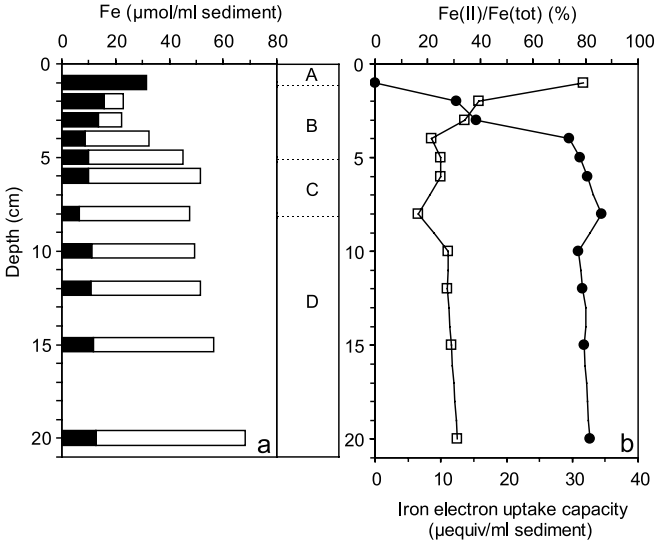

Fig. 2. a: Representative values for concentrations of HCl-extractable ferric iron (black bars) and ferrous iron (white bars) in different sediment layers. b: Relative proportion of iron(II) among the total acid-extractable iron in different sediment layers (●) and the electron uptake capacity given by the Fe(III) present in the sediment layers (□).

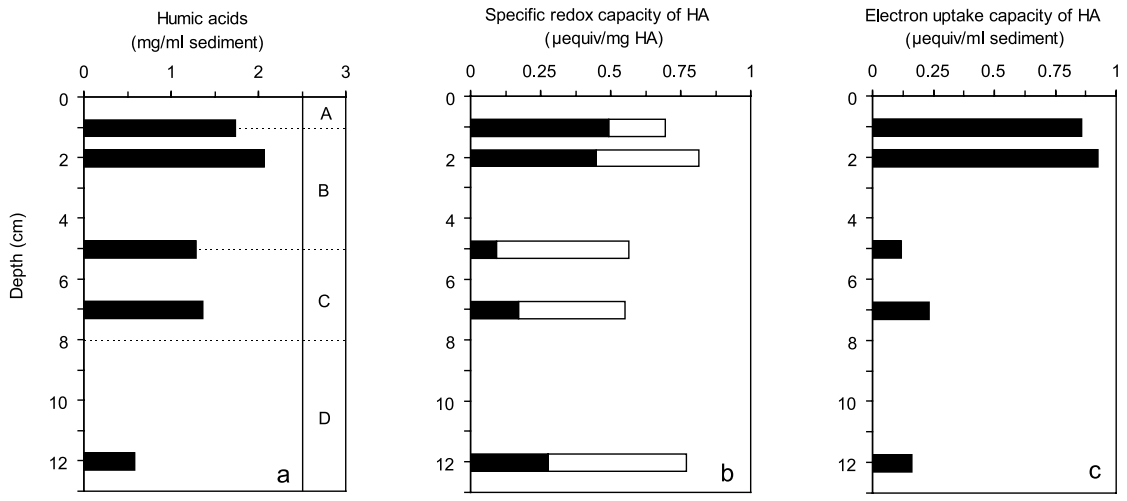

Fig. 3. Representative depth profiles of concentration and redox capacity of humic acids (HA) extracted from different sediment layers. a: Amount recovered from the sediment layers defined in Fig. 1. b: Specific redox capacity, consisting of oxidation capacity (black bars) and reducing capacity (white bars) of the preparations (for details, see Section 2). c: Total electron uptake capacity, calculated for each layer using the data in panels a and b.

3.3. *Humic acids*

Humic acids were recovered from each sediment layer by alkaline extraction under anoxic conditions and subsequent acid precipitation. The humic acid content decreased from 1.8 and 2.2 mg (ml sediment)$^{-1}$ in the surface layer to about 1.5 mg in the sulfate reduction zone, and finally to 0.7 mg (ml sediment)$^{-1}$ in the methanogenic zone (Fig. 3a).

The redox capacity of each humic acid preparation, i.e., the capacity to take up electrons (oxidation capacity) and to release electrons to Fe(III) (reducing capacity), was determined by redox titration with hexacyanoferrate (see Section 2). The specific oxidation capacity of humic acids (Fig. 3b) was highest in the top layer of the sediment and decreased to a minimum in the upper region of the sulfate reduction zone (layer C). Deeper down, the specific oxidation capacity increased again slightly. The specific reducing capacity of the humic acids (Fig. 3b) was lowest at the top of the sediment and increased with depth, with the highest values in the upper region of the sulfate reduction zone (layer C) and the methane production zone.

Using the amount of humic acids extracted from each sample (Fig. 3a) and the specific oxidation capacity (Fig. 3b), the total electron uptake capacity of the humic acids in each layer was calculated (Fig. 3c). The electron uptake capacity of the humic acids was maximal in the top sediment layers ($\sim$0.9 µequiv (ml sediment)$^{-1}$), and much lower in deeper sediment layers ($\sim$0.2 µequiv (ml sediment)$^{-1}$).

3.4. *Humic-acid-reducing bacteria and other microbial populations*

The population sizes of humic-acid-reducing and iron-reducing bacteria using lactate or acetate as electron donor were determined in sediment samples from the four different layers (A–D; Fig. 1) and were compared to each other and to the population sizes of lactate-fermenting bacteria (Table 1).

Generally, lactate-oxidizing humic-acid-reducing bacteria and lactate-fermenting bacteria represented the largest populations in all depth layers. Within each layer, their numbers did not differ significantly from each other. Lactate-oxidizing and acetate-oxidizing humic-acid-reducing bacteria showed similar population sizes in all layers ex-

Table 1
MPNs of microorganisms in different layers of profundal sediments of Lake Constance

Sediment layer	Sampling depth (cm)	Humic-acid-reducing bacteria[a]		Iron-reducing bacteria[b]		Lactate-fermenting bacteria
		Lactate	Acetate	Lactate	Acetate	
A	0–0.5	8.33 ± 0.42	4.60 ± 0.23	0.07 ± 0.00	0.08 ± 0.00	5.90 ± 0.30
B	1.5–2.5	4.60 ± 0.23	3.12 ± 0.16	0.56 ± 0.03	0.47 ± 0.02	3.74 ± 0.19
C	6.5–7.5	2.63 ± 0.13	4.60 ± 0.23	< 0.01	0.01 ± 0.00	3.21 ± 0.16
D	11.5–12.5	1.80 ± 0.09	0.60 ± 0.03	< 0.01	0.01 ± 0.00	2.76 ± 0.14

The population sizes of bacteria reducing humic acids or iron(III) were determined with lactate or acetate as electron donor and were compared to those of lactate-fermenting bacteria. All values are multiples of 10^6 cells (ml sediment)$^{-1}$.

[a]Humic acids (20 mg ml^{-1}).

[b]Poorly crystalline ferric iron hydroxide (40 mM).

cept layer D, with the corresponding MPNs decreasing with sediment depth.

Populations of acetate-oxidizing and lactate-oxidizing bacteria capable of reducing poorly crystalline ferric iron hydroxide, however, were considerably lower. The largest numbers were obtained for layer B, coinciding with the iron reduction zone. MPNs on acetate and lactate did not differ significantly in layers A and B; in layers C and D, lactate-oxidizing iron-reducing bacteria were below the detection limit. In all layers, the MPNs of bacteria reducing poorly crystalline ferric iron directly were considerably smaller than those obtained in the presence of humic acids.

4. Discussion

This study describes the depth distribution of humic-acid-reducing and iron-reducing bacteria in a freshwater sediment, and compares it to the general redox characteristics of humic acids and iron extracted from the corresponding sediment layers. The populations of humic-acid-reducing bacteria were found to be 10–100 times higher than the populations of iron-reducing bacteria in the respective sediment layers.

4.1. Microbial humic acid reduction

It is reasonable to assume that bacteria capable of oxidizing either lactate or acetate with humic acids as electron acceptor represent a subset of the total humic-acid-reducing community in the sediment. In a previous study, Coates et al. [3] have found that all humic-acid-reducing bacteria isolated from various sediments with acetate as electron donor and AQDS as electron acceptor were also able to reduce poorly crystalline ferric iron hydroxide [3]. At first glance, these findings seem to disagree with the results of the present study, where the number of humic-acid-reducing bacteria oxidizing lactate or acetate was much higher than that of bacteria reducing poorly crystalline ferric iron hydroxide with the same electron donors in all sediment layers. However, it must be considered that also pure cultures of fermenting and halorespiring bacteria, which are unable to reduce ferric iron hydroxide directly, are able to use humic acids as electron acceptor [9,11]. Moreover, in the present study, the number of lactate-fermenting bacteria was in the same range as that of lactate-oxidizing bacteria reducing humic acids in all sediment layers.

Therefore, even considering inaccuracies of MPN studies and keeping in mind that MPN counts regard only a fraction of unknown size of the total community, our results suggest that the community of humic-acid-reducing microorganisms in profundal sediments of Lake Constance consists largely of microorganisms that are unable to reduce ferric iron hydroxide directly – at least in the provided poorly soluble form – and at least some of them might represent fermenting bacteria. Unfortunately, our results do not allow us to differentiate between the lactate-oxidizing and acetate-oxidizing populations, since their respective numbers did not differ significantly in most sediment layers.

It has been shown recently that humic acids can also be reduced by sulfate-reducing, halorespiring, methanogenic, and hyperthermophilic microorganisms [10,11]. Apparently, the ability to reduce humic acids is widespread, and the spectrum of microorganisms capable of reducing iron(III) indirectly (see Section 4.2) is much larger than that of 'iron-reducing bacteria' sensu stricto. We conclude that humic acid reduction is a metabolic capability more common among bacteria than previously thought – a fact that should be considered in future models of electron flow in anoxic habitats.

4.2. Electron shuttling via humic acids to iron(III) (hydr)oxides

The depth profiles show a decrease in the concentration of humic acids and an increase of iron concentration with increasing depth, both probably due to degradation of organic matter, calcite dissolution, and sediment compression (Fig. 1, see also [23]). The resulting higher density and lower porewater content of the sediment lead to an increase of the relative iron content with depth. The electron uptake (oxidation) capacities of the humic acids were 30–80 times lower than those of Fe(III) in all sediment layers, and consequently, their importance as terminal redox acceptors appears small when compared to that of the Fe(III) fraction. However, due to the ability of humic acids to shuttle electrons to Fe(III) hydroxides [2,8,12,14,27], microbial reduction of humic acids is chemically coupled to the much larger pool of oxidized iron and manganese.

Owing to their low solubility, iron and manganese oxides accumulate in sediments [28,29], where they represent one of the dominating electron acceptors, albeit endowed with a strong kinetic limitation. In order to reduce insoluble Fe(III) (hydr)oxides in the absence of electron shuttles or chelating agents, Fe(III)-reducing bacteria need to be in direct contact with the minerals, which is considered to be a rate-limiting step in iron(III) reduction. Insoluble forms of Fe(III) are reduced more slowly than soluble forms of Fe(III) [30]. In the presence of humic acids as redox mediators, the coupled system could overcome the limitations imposed by the low electron uptake capacity of humic acids and the low solubility of the Fe(III) (hydr)oxides. As a consequence, the low electron uptake capacity does not limit the oxidation of organic substrates, and the kinetics of Fe(III) (hydr)oxide reduction can be enhanced.

It has to be mentioned, however, that humic acids extracted with NaOH at alkaline pH contain organic molecules that are already dissolved under in situ conditions

but also molecules that are not dissolved at circumneutral pH. The relative contribution of either fraction to the total amount of extracted humic acids is not known, and this is true also for the potential kinetic limitations on rates of enzymatic electron transfer to solid-phase humic acids and on rates of Fe(III) oxide reduction by reduced solid-phase humic acids.

In addition to their function as electron shuttles, humic acids could stimulate microbial iron(III) reduction also by complexation of either Fe(III) or Fe(II) [27]. The first process would render Fe(III) more accessible to the microorganisms, whereas the second would prevent adsorption of Fe(II) to the surfaces of minerals and microbial cells, thus providing sufficient 'free surface' for reduction [31]. In addition, Fe(II) complexation would lower the concentration of free Fe(II), thus increasing the thermodynamic driving force for Fe(III) reduction that decreases with increasing Fe(II) concentrations. However, the actual extent of these complexing mechanisms caused by humic acids during iron reduction in natural environments still has to be determined.

The actual rate and extent of microbial reduction of humic acids and iron(III) in each layer will depend not only on the presence of the appropriate microorganisms. Also the substrate concentration and the redox potential, which is determined by the presence and electron uptake capacities of the respective electron acceptors, viz., iron(III) or oxidized humic acids, control the extent of the reduction. The specific redox capacity of the humic acids recovered from profundal sediments of Lake Constance (0.5–0.7 μequiv (mg humic acids)$^{-1}$) as measured in our study corresponds to approx. 3–4% redox-active quinoid constituents in the humic acids, a value which is only slightly lower than values determined by Kappler and Haderlein [32] and Struyk and Sposito [6] for soil and aquatic humic acids, respectively.

In order to be a good redox mediator, an electron-shuttling compound must have a redox potential low enough to reduce Fe(III) and high enough to be re-reduced by the microorganisms after its chemical oxidation by Fe(III). If electrons come from the oxidation of glucose to CO_2 ($E^{\circ\prime} = -434$ mV) or lactate to CO_2 ($E^{\circ\prime} = -343$ mV) and are transferred to iron minerals with a potential between -300 and $+100$ mV depending on the mineral and Fe^{2+} concentration [33], a potential electron shuttle needs to have a relatively confined redox potential to fulfill this role. However, humic acids with a broad spectrum of different redox potentials indicated by nearly linear redox titration curves over a range from $E_h = -300$ mV to +400 mV (obtained in our lab with hexacyanoferrate as oxidant; not shown) could accomplish this demand.

Given the high apparent redox potential in the top layers (A and B) of the sediment and the large specific electron uptake capacities of the humic acids, microbial humic acid reduction is probably most favorable in these layers. However, the prevailing redox potential in the porewater (-150 to -200 mV, see Fig. 1) and the electron-accepting capacities of humic acids (0.12–0.23 μequiv (ml sediment)$^{-1}$, see Fig. 3) in deeper sediment layers are probably still in the appropriate range for humic acids to serve as electron shuttles between microorganisms and iron(III) hydroxides. Both the prevailing redox potential and the electron-accepting capacity of humic acids determined in this study are comparable to the conditions given in experiments in which AQDS was added to aquifer material ($E_{0,\,AQDS} = -184$ mV; $c_{AQDS} \leq 0.250$ μmol (g sediment)$^{-1}$): in these studies, AQDS was shown to be an excellent electron shuttle between bacteria and iron(III) hydroxides [14]. It should be emphasized, however, that the redox potentials of porewater, the humic acid pool, and the iron pool are not in equilibrium, but rather in a steady-state flow situation governed by the kinetics of specific rate-limiting reactions.

Due to the rapid chemical oxidation of Fe(II) by oxygen and the low solubility of Fe(III) in water, iron accumulates in upper sediment layers as Fe(III) (hydr)oxides [34]. As discussed above, Fe(III) is reduced to Fe(II) below the oxic sediment surface. However, chemical reoxidation (e.g., reoxidation of iron(II) by oxygen diffusing into the sediment) and iron(II) oxidation by aerobic [35,36] or nitrate-reducing bacteria [20] recycle the iron(II) and thus replenish the iron(III) pool. Even in the deeper sediment layers (C and D), a complete reduction of iron(III) was not observed although reduced humic acids and a significant humic-acid-reducing bacterial population were present. It appears likely that part of the iron(III) is physically not accessible (e.g., because of formation of aggregates of iron minerals where the iron(III) minerals are covered by iron(II) precipitates) or that thermodynamic constraints imposed by the low redox potential and lower electron-accepting capacity of humic acids and/or iron(III) are responsible for the observed incomplete reduction.

4.3. Environmental significance of humic acid reduction

From the results presented in this paper and the ubiquitous presence of humic acids, we conclude that the ability to reduce humic acids is probably a widespread microbial electron transfer mechanism that could represent an important path of electron flow in anoxic environments such as sediments. Microbial reduction of humic acids has consequences not only for iron cycling. Also the fate of inorganic and organic pollutants in the environment might be influenced by the reduction of humic acids. Reduced humic acids bind heavy metals more strongly than oxidized humic acids do, whereas the binding capacity of humic acids for naphthalene is decreased after their reduction [37]. Furthermore, reduced humic acids reductively transform different organic pollutants, e.g., chlorinated solvents [32,38] and nitroaromatic compounds [39]. These results suggest that microbial reduction of humic acids can play an important role in the mobilization and retention of

toxic metals and hydrocarbons and in the degradation of organic pollutants.

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