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The interplay of hydrological, chemical and microbial processes in the formation of iron-rich floating films in aquatic environments at a circumneutral pH

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ABSTRACT

The interplay of hydrological, chemical and microbial processes in the formation of iron-rich floating films in aquatic environments at a circumneutral pH

The direct contribution of microbial activity to the formation of iron-oxide minerals is difficult to prove in wetlands due to the high reactivity of solid iron phases with different compounds and the variety of redox processes that may occur at each oxic-anoxic boundary. Here, we propose an explanation for the formation of iron-oxide films in wetlands and groundwater seepage areas fed by sandy aquifers based on the interaction of hydrological, chemical and microbiological processes under circumneutral conditions. The presence of a floating iron-oxide film was found to create a boundary at the air-water interface that maintains a suboxic and slightly acidic environment below the film compared with the environments obtained in other free-film wetland areas. The water trapped below this film had an average pH of 6.1, was particularly poor in O_2, HCO_3^-, Na^+, Ca^{2+}, Mg^{2+}, K^+, and Tot-S, and has high concentrations of Tot-P, Tot-Fe, NH_4^+ and Zn. The formation of a floating iron-oxide film was reproduced under anaerobic conditions after progressive enrichment through the incubation of natural sediment samples in the laboratory. Heterotrophic bacteria belonging to the genus *Enterobacter* were the dominant bacteria in the enrichments that resulted in the formation of a floating iron-oxide film. The X-ray diffraction patterns showed that the presence of two-line ferrihydrite was common to the iron-oxide films collected in both the natural environment and the laboratory cultures, whereas other iron-oxides (goethite and low-crystalline lepidocrocite) were observed only in the natural environment. This study highlights the role of ubiquitous bacteria, which are generally considered unimportant participants in iron-transformation processes in the environment, and the contribution of both biological and non-biological processes to iron oxidation in natural systems under circumneutral conditions.

Key words: Iron surface complexes, heterotrophic bacteria, groundwater, Doñana.

RESUMEN

La interacción de procesos hidrológicos, químicos y microbiológicos en la formación de películas flotantes ricas en hierro en ambientes acuáticos de pH circumneutro

En los humedales, es difícil probar que la actividad microbiana sea la responsable de la formación de óxido de hierro mineral debido, tanto a la gran reactividad del hierro en fase sólida con diferentes sustancias, como a la variedad de procesos redox que pueden ocurrir en cada interfase óxica-anóxica. El presente trabajo propone una explicación, basada en la interacción de procesos hidrológicos, químicos y microbiológicos en condiciones circumneutras, para explicar la formación de un film de óxido de hierro en humedales y manaderos donde aflora agua subterránea proveniente de acuíferos de arenas silíceas. Además, la presencia de un film de óxido de hierro que flota sobre la interfase agua-aire genera condiciones subóxicas y ligeramente ácidas en el agua que queda atrapada debajo, y que son muy distintas a otras zonas libres de film en el mismo

humedal. Este agua atrapada bajo el film se caracterizó por presentar un pH medio de 6.1, una menor concentración de O_2, HCO_3^-, Na^+, Ca^{2+}, Mg^{2+}, K^+, y S total, pero una mayor riqueza en P total, Fe total, NH_4^+ y Zn. La formación de un film flotante de óxido de hierro se reprodujo en el laboratorio, en condiciones anaeróbicas, tras el enriquecimiento progresivo de las muestras del sedimento natural que habían sido incubadas. En dichos enriquecimientos, donde se produjeron films flotantes de óxido de hierro, la bacteria dominante perteneció al género Enterobacter. *Mediante difracción por rayos X, se encontró ferrihidrita con estructura en doble cadena, tanto en el film de muestras naturales como de cultivos de laboratorio. Además se encontraron otros tipos de óxidos minerales (goetita y lepidocrocita de pobre cristalización) sólo en las muestras naturales de film. El presente estudio muestra la relevancia de bacterias ubicuas, hasta ahora consideradas sin importancia en procesos naturales de transformación del hierro, y la participación tanto de procesos bióticos como abióticos en la oxidación del hierro en sistemas naturales sometidos a condiciones circumneutras.*

Palabras clave: *Hierro acomplejado en superficies, bacterias heterótrofas, agua subterránea, Doñana.*

INTRODUCTION

Iron-cycling is a central issue in aquatic systems. Iron not only is essential for living organisms but also plays an important role in the redox transformations of numerous compounds, the bioavailability of nutrients and trace metals, and the binding processes of organic and inorganic ligands. The rate of the oxidation of ferrous iron by O_2 as a function of pH and other solution variables is well known (Stumm & Sulzberger, 1992), although a large variety of iron oxides, hydroxides and oxyhydroxides can be formed due to the high reactivity of solid iron phases with different compounds at different oxic-anoxic boundaries. Hence, more studies are needed to understand the mechanism underlying the formation of iron-oxide films and their biogeochemical role (Kleja *et al.*, 2012). Despite the wide-spread presence of iron-oxide films floating on the water surface of wetlands all over the world, these films are often overlooked, mistaken for oil due to their oily appearance, or misidentified as biofilms strictly generated by biological activity (Grathoff *et al.*, 2007). Therefore, the relative contribution of microorganisms in the formation of ferrous/ferric iron complexes and minerals in non-acidic environments has not yet been quantified because the oxidation of ferrous iron can also proceed along purely chemical routes (Châtellier *et al.*, 2004; Trolard, 2006). In this sense, Rentz *et al.* (2007) used cyclic voltammetry in short experimental times (<30 min) to capture the rapid ki-

netics of the natural iron-oxidation rates under circumneutral conditions and evidenced that microaerobic conditions alone can account for the oxidation of ferrous iron even in the presence of iron-oxidizing bacteria. Nevertheless, bacteria can play both a metabolic and non-metabolic role in iron oxidation because ferrous iron can adsorb reversibly onto bacterial cell surfaces and reach an adsorption equilibrium within minutes under anoxic conditions at an effective pH range of 3 to 7 (Châtellier & Fortin, 2004). This type of biotic surface-mediated iron complexation will, in turn, promote abiotic iron oxidation on the cell surface in oxic systems. Furthermore, recent findings suggest that biotic and abiotic iron oxidation co-occur at the same temporal and spatial scales in natural systems and thus cannot be separated because even biotically oxidised ferrous iron will serve as a nucleation matrix for abiotic ferric iron precipitation at circumneutral pH (Ionescu *et al.*, 2015).

The significance of microbial metabolism in the weathering and formation of iron minerals is widely recognized. Specific microorganisms, such as iron-oxidizing bacteria, iron-reducing bacteria and bacteria forming iron-complexing agents, mediate major transformations that change iron from a soluble to an insoluble form under different redox and pH conditions (Berthelin *et al.*, 2006). Studies of 16S rRNA gene sequences have indicated that virtually every major group of prokaryotes can be associated with aerobic ferrous iron oxidation in natural

environments under both acidic and neutrophilic conditions (Neubauer *et al.*, 2002; James & Ferris, 2004, Sobolev & Roden, 2004).

Wetlands may provide a suitable environment for determining the extent to which microorganisms actively mediate the oxidation of dissolved ferrous iron at a variety of aerobic/anaerobic interfaces on the water surface, sediment layers and rhizosphere of aquatic macrophytes (Emerson & Weiss, 2004). Wetlands and bogs fed by the groundwater seepage of sandy aquifers provide the perfect neutrophilic environment for studying this process because the alternation of the redox potential due to fluctuations in the water table produces heterogeneous accumulations of iron oxides and hydroxides, which eventually crystallize due to further diagenetic processes (Knapp *et al.*, 1998). In this type of environment, iron can be transported several kilometres by the flow of groundwater through a combined sequence of biotic and abiotic processes that often leads to a modification of the mineralogical composition of the original iron oxides (Herbillon, 2006). The oxidation of dissolved ferrous iron, which occurs as soon as the seepage water reaches the air surface, is a common feature of these aquatic environments that generally leads to the precipitation of lepidocrocite at neutral pH and ferrihydrite in the presence of aqueous silica at redox boundaries and surfaces (Châtellier *et al.*, 2004), and the crystallization of these precipitates will lead to the formation of goethite and hematite (Cornell & Schwertmann, 1996).

The Iberian Pyrite Belt (SW Spain) is one of the largest massive sulphide deposits in the world. It was formed in the early Carboniferous and later transformed during the Hercynian Orogeny. As a consequence of pyrite oxidation since mining activity was initiated in this area approximately 5000 years ago, the Tinto and Odiel rivers are now acidic (pH < 3) and have drained large amounts of sulphate and dissolved metals (Fe, Al, Mn, Cu, Zn, Cd, Pb) to the Gulf of Cádiz (Davis *et al.*, 2000). The Doñana aquifer (~ 3400 km^2) borders the Tinto estuary and extends westward to the Guadiamar and Guadalquivir rivers (Llamas, 1990). The aquifer is bottomed by impermeable marine blue marls

of Miocene age upon which sands, silts, and silty-clay materials of estuarine, fluvial and eolian origins were later deposited. It holds an unconfined aquifer with a shallow water table and several flow systems that feeds hundreds of small ponds, wetlands and seepage areas (Serrano *et al.*, 2006). A discontinuous iron hardpan composed of goethite (Siljeström & Clemente 1990) is commonly found at a soil depth of less than 1.5 m, coincident with former groundwater seepage areas that have become buried by younger dune generations (Muñoz-Reinoso, 2001). Despite its complexity, the hydrology of some of these wetlands is well established (Sacks *et al.*, 1992), and given the presence of shallow iron oxide deposits, these wetlands form an ideal environment for studying the formation of iron-oxide films. The present work describes the environmental, microbiological and mineralogical features of floating iron-oxide films at a circumneutral pH in several groundwater seepage areas of the Doñana National Park (SW Spain). It also records the formation of a similar film in the laboratory after the enrichment and incubation of natural sediment samples, which raises several questions: a) whether the formation of this floating iron-oxide film is actively mediated by microorganisms, b) the identity of the microorganisms that are involved, and c) whether the process is mediated by light. Finally, a model that takes into account some of the hydrological, chemical and microbiological features of one of the study sites is proposed to explain both the formation of floating iron-oxide films on the SW shoreline of the study site of interest and the lack of a film on the opposite NE shore.

MATERIAL AND METHODS

Site description

The Doñana region (37°N, 6°W) extends along the coastal plain of the Gulf of Cádiz from the estuary of the Tinto River to the left bank of the Guadalquivir River and inland from the coastline to the northern uplands bordering the Iberian

Pyrite Belt (Fig. 1). This region has a Mediterranean climate with Atlantic influence and is generally classified as dry subhumid. Doñana includes several territories with different degrees of environmental protection covering more than 100 000 ha. The Doñana National Park covers half of this extension and is the most important wetland area in Spain. It exhibits an extraordinary variety of aquatic systems, which are classified into two broad groups according to the hydro-geomorphology of their basins: the sand dunes and the marsh floodplain (Serrano *et al.*, 2006).

Sample collection and environmental analyses

From October 2003 to June 2006, water and sediment samples were collected from several temporary ponds in the Doñana Biological Reserve where groundwater seepage areas are
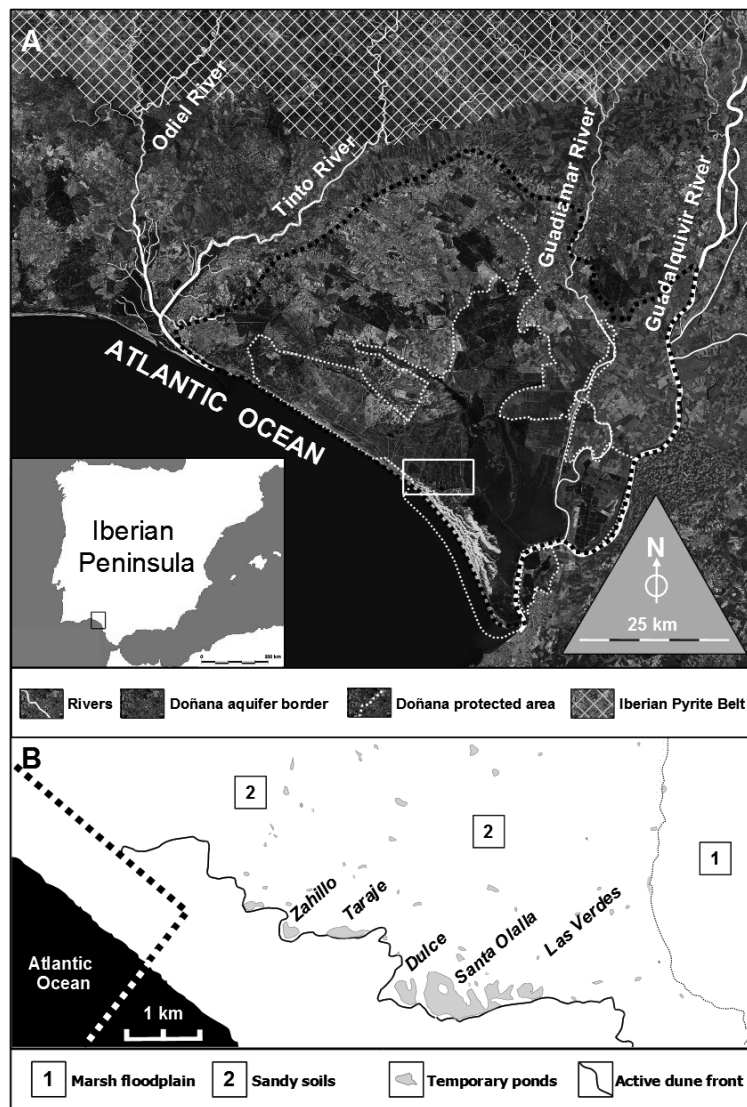

Figure 1. A: Map of the Doñana region within the Iberian Peninsula showing the location of the Iberian Pyrite Belt and the Doñana aquifer. B: Detailed map of the study area with the temporary ponds. *A: Mapa de Doñana en la Península Ibérica indicando las localizaciones de la Faja Pirítica Ibérica y el acuífero de Doñana. B: mapa detallado del área de estudio con las lagunas temporales.*

common (Fig. 1): Las Verdes, Dulce, Santa Olalla, Zahíllo, and Taraje ponds. Water was collected from the surface with a 1-litre sample jar, and the top sediment was sampled with a hand gauge. Additionally, Dulce pond was sampled at distances of 0, 1 and 3 m to the SW shoreline and to the NE shoreline (three replicates at each site) in June 2006. The electrical conductivity (compensated at $25\,^\circ$C), concentration of dissolved O_2 and pH in the water were measured *in situ* with the corresponding electrodes. Three replicate samples of the floating iron-oxide film covering the shoreline were also collected using a sterilized spatula, and each portion was placed in a sterile container. Pore-water samples were collected *in situ* to measure the sediment pH in the laboratory. These were collected anaerobically using Teflon rhizons (Eijkelkamp Aqrisearch Equipment, The Netherlands) connected to vacuum serum bottles (2-4 replicates). The surface- and pore-water samples were divided into two parts. One part was fixed with 1 % nitric acid and analysed for Ca, Mg, Fe, Al, P, S, Si, Na, K and Zn using an Inductively Coupled Plasma Mass Spectrophotometer (ICP-MS X-series, Thermo, Waltham, MA, USA). Quality assurance measures included blanks, replicate analyses and matrix spikes. The recoveries from matrix spikes ranged from 95 % to 107 %. The repeated analyses did not reveal differences greater than 5 %. The other part of the samples was fixed with 0.125 g/L citric acid and analysed for NO_3^- (Kamphake *et al.*, 1967) and NH_4^+ (Grasshoff & Johansen, 1972) using an Auto Analyser (AA 3, Bran + Luebbe, Norderstedt, Germany). The CO_2 and HCO_3^- concentrations were analysed using a 0525 HR infrared carbon analyser (Oceanography International). The concentration of organic matter in the top sediment samples was estimated in 3-4 replicates by loss on ignition ($450\,^\circ$C, 5 h). The samples of water and ignited sediment were digested with 0.5 M H_2SO_4 and $K_2S_2O_8$ at $120\,^\circ$C. Then, the Tot-P was analysed as i-P (Murphy & Riley, 1962), and the Tot-Fe was analysed as dissolved ferrous iron using o-phenanthroline and ascorbic acid as the reducing agent (Golterman, 2004). The P-fractionation of the sediment samples was per-

formed in 2-4 replicates with 10 mL of sediment suspension according to the EDTA method for sequential extraction (Golterman, 2004).

Mineralogical analysis

The mineralogical analysis was performed at the Research General Services of the University of Seville (CITIUS) through X-ray diffraction (XRD) using a Bruker D8 Advance powder device with CuKα radiation and a monochromator. Samples of the iron-oxide film collected in the natural environment and the laboratory cultures were analysed. Each film sample was powdered with an agate mortar and scanned from 3 to 80° 2θ (double theta) at a 0.02° step size and with a 10-s count time per step.

Sediment profiles with microelectrodes

Measurements of the oxygen and sulphide concentrations and pH within sediments of both sides of the studied pond were performed using microelectrodes attached to a motor-driven micromanipulator (Unisense, Århus, Denmark) following previously described methods (Revsbech, 2005). Measurements were collected every 200 μm for a depth of approximately 5 cm. Microelectrode calibrations were performed as previously described (Revsbech, 2005) following the manufacturer's recommendations.

Enrichments from natural communities

The upper layer of sediment (0-2 cm) and shallow water were sampled and incubated at $28\,^\circ$C under aerobic and anaerobic conditions with illumination. Sterilized controls were conducted. If an iron-oxide film was formed, an aliquot of the enrichment was transferred to G medium. This transfer to G medium resulted in bacterial growth, which persisted through repeated cultivations, always producing floating iron-oxide films on the surface of the cultures. G medium (Lovley *et al.*, 1993) was composed (per litre) of 13.7 g Fe(III) citrate, 2.5 g NaHCO$_3$, 1.5 g NH$_4$Cl, 0.6 g NaH$_2$PO$_4$, 0.1 g KCl, 2.5 g sodium acetate, and 0.25 g Na$_2$WO$_4 \times$ 2H$_2$O with 10 mL of trace el-

Table 1. Average dissolved oxygen (mg/L) and chemical concentrations (μM) of surface water samples ($n = 2 - 8$) collected at distances of 0, 1 and 3 m from the shoreline and of pore water samples collected at different depths in the sediment profiles at each shoreline (NE and SW). The significant differences (one-way ANOVA + Holm-Sidak test, $p < 0.05$) between the surface water samples collected from the SW shoreline are noted as follows: [ab] between the surface water samples collected at distances of 0 and 1 m, [ac] between the surface water samples collected at distances of 0 and 3 m, [ad] between the surface water sample collected at a distance of 0 m and the pore water samples collected at depths of 0-2 cm, [ae] between the surface water sample collected at a distance of 2-5 cm, [af] between the surface water samples collected at a distance of 0 m and the pore water samples collected at depths of 5-10 cm. In addition, significant differences between the different shorelines are noted as follows: [aa] between the samples collected at a distance of 0 m from the SW shoreline and the samples collected at a distance of 0 m from the NE shoreline. *Concentraciones promedio de oxígeno disuelto (mg/L) y de disitntas sustancias químicas (μM) en las muestras de agua superficial (*n = 2 – 4*) recogidas a 0, 1 y 3 m de distancia desde la orilla, y en el agua intersticial a diferentes profundidades en los perfiles de sedimento de cada orilla (SW y NE). Se indican las diferencias significativas (ANOVA de una vía + test Holm-Sidak,* p < 0.05*) entre distintas muestras de agua superficial de la orilla SW de la siguiente forma:* [ab] *entre 0 y 1 m en agua superficial,* [ac] *entre 0 y 3 m en agua superficial,* [ad] *entre el agua superficial a 0 m y el agua intersticial a 0-2 cm de profundidad,* [ae] *entre el agua superficial a 0 m y el agua intersticial a 2-5 cm,* [af] *entre el agua superficial a 0 m y el agua intersticial a 5-10 cm; así como* [aa] *entre el agua superficial de ambas orillas a 0 m.*

		surface water			pore water at 0 m		
		0 m	1 m	3 m	0-2 cm	2-5 cm	5-10 cm
O_2 (mg/L)	NE	6.9	8.1	10.9	—	—	—
	SW	0.2^{aa}	1.4	4.6	—	—	—
pH	NE	7.2	7.7	8.4	7.3	7.2	7.0
	SW	$6.1^{aa,ab,ac}$	6.6	8.3	6.1	6.1	6.2
Cl^-	NE	10712.1	9305.9	9623.6	10620.7	10824.4	10148.4
	SW	$1629.3^{aa,ac}$	2158.8	8064.2	1290.2	1235.5	1277.7
HCO_3^-	NE	2001.2	1614.2	1632.7	2701.9	2804.3	2261.0
	SW	$704.5^{aa,ab,ad,ae,af}$	1294.4	1354.7	1344.2	1307.5	1351.6
Tot-S	NE	981.0	939.2	942.9	723.3	594.0	496.0
	SW	$48.4^{aa,ac}$	132.6	918.8	38.4	34.0	33.5
NO_3^-	NE	22.8	20.2	22.0	22.8	20.5	20.2
	SW	$11.9^{aa,ac,af}$	12.5	18.1	14.7	13.5	15.7
Tot-P	NE	5.5	6.6	3.2	11.1	14.4	12.0
	SW	$9.5^{ad,ae,af}$	9.2	4.4	16.8	28.4	24.3
Tot-Na^+	NE	12156.0	11760.9	11304.3	12094.6	12137.5	11552.2
	SW	$1992.2^{aa,ab,ac}$	3065.4	11022.8	1229.2	1185.5	1260.1
Ca^{2+}	NE	930.1	848.6	828.0	977.0	951.1	959.3
	SW	$446.3^{aa,ab,ac,ad,ae,af}$	646.2	892.8	596.0	581.1	590.4
Mg^{2+}	NE	1693.3	1631.1	1591.0	1663.0	1649.3	1343.2
	SW	$249.6^{aa,ab,ac}$	531.0	1613.4	306.5	296.6	304.9
Tot-Fe	NE	3.7	2.7	3.0	19.1	44.9	654.8
	SW	$617.8^{aa,ac,ad,ae}$	462.3	13.3	964.5	1078.9	898.6
K^+	NE	210.7	196.1	201.0	224.7	249.8	244.7
	SW	$78.7^{aa,ac}$	113.6	165.8	83.5	80.8	86.2
NH_4^+	NE	16.5	14.9	13.2	20.0	19.5	14.9
	SW	$78.1^{ad,ae,af}$	64.2	14.3	340.1	290.7	249.6
Fe^{2+}	NE	0.0	0.0	0.0	—	—	—
	SW	$27.4^{aa,ac}$	29.2	0.0	—	—	—
Tot-Mn	NE	14.6	1.6	0.7	54.6	51.3	61.6
	SW	13.2	33.6	1.5	14.4	14.1	14.3
Zn	NE	0.1	0.0	0.0	0.3	0.3	0.5
	SW	$2.1^{aa,ab,ac}$	0.0	0.0	2.3	3.1	3.0

ement solution and 10 mL of vitamin solution (Balch *et al.*, 1979). The medium pH was adjusted to 6.0, and the culture was incubated anaerobically under 80 % N_2 + 20 % CO_2 gas. Bacterial films were collected using a sterile spatula and containers. Once dried (24 hours at 70 °C), the films were digested with 4 mL of nitric acid (65 %) and 1 mL of hydrogen peroxide (35 %) using an ETHOS-D microwave labstation (Milestone, Sorisole, Italy). The digests were diluted (25×) and analysed with an ICP-MS as above.

Molecular analyses

Natural samples of water and sediment from the SW and NE pond shores were analysed for RNA to determine the metabolically active fraction of the bacterial community. Laboratory enrichments were analysed based on their DNA to identify the bacteria forming these cultures. DNA and RNA were extracted using the Nucleospin Food DNA extraction kit (Macherey-Nagel, Düren, Germany) and the RNAqueous4PCR Total RNA extraction kit (Ambion Inc., Austin, USA), respectively. The protocol for total RNA extraction included DNaseI treatment (37 °C for 1 h) to remove any DNA remaining in the final RNA extract.

A reverse transcriptase reaction was performed to obtain the complementary DNA (cDNA) to the RNA corresponding to the 16S rRNA genes to be amplified. The ThermoScript reverse transcriptase (Invitrogen, Carlsbad, CA, USA) was used in this study with a 16S rRNA gene-specific primer, namely 518R (5′-ATT ACC GCG GCT GCT GG (Muyzer *et al.*, 1993)), at an annealing temperature of 55 °C for 1 h. Controls lacking reverse transcriptase were also performed to check for the presence of DNA traces in the extracted RNA. A standard amplification reaction by PCR was performed after cDNA preparation. Amplification of 16S rRNA fragments from the cDNA was performed by PCR using the forward primer 27bF (5′-AGA GTT TGA TYM TGG CTC AG (Portillo *et al.*, 2008)) and the reverse primer 518R. ExTaq (Takara, Shiga, Japan) was used as the DNA polymerase for PCR, following the manufacturer's recom-

mendations. The thermal conditions for the amplification reaction consisted of the following steps: 95 °C for 2 min; 30 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 1 min; and a final incubation at 72 °C for 10 min. Amplifications from DNA extracted from the enrichments were performed following a similar protocol using the primers 27bF and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T Lane, 1991).

DNA libraries were constructed from the amplification products obtained from the DNA extracted from the cultivated enrichments and from the cDNA prepared from reverse-transcribed RNA extracted from the natural samples. The DNA libraries were constructed from the PCR products using the TA-TOPO cloning kit (Invitrogen, CA, USA). The DNA library construction and screening was performed as described by González *et al.* (2003). Selected clones were sequenced. The sequences were manually edited and processed with the BLAST algorithm (Altschul *et al.*, 1990) to search for the closest relatives to the retrieved sequences using the GenBank database at NCBI (http://www.ncbi. nlm.nih.gov/blast). The sequences were checked for chimeras using the Ccode program as described by González *et al.* (2005). The bacteria identified based on RNA corresponded to the metabolically active fraction of the total microbial community because the RNA per bacterial cell is quantitatively proportional to cell growth (Mills *et al.*, 2004). The microbial communities from the natural samples and the enrichments were characterized by molecular fingerprinting. The fingerprints were prepared using DGGE (Denaturing Gradient Gel Electrophoresis) as previously described (Muyzer *et al.*, 1993) with the modifications introduced by Portillo and González (2008) to obtain relative quantitative fingerprints. To determine the grade of coverage of the detected sequences with respect to the bacterial community, rarefaction curves were constructed according to Hughes and Hellmann (2005). These curves represent the number of processed clones against both the number of detected OTUs (Operational Taxonomic Units) and the number of different phyla detected in this study. For practical purposes, we considered an

OTU as a set of apparently identical sequences showing higher than 97 % identity (Huber *et al*., 2007).

Statistical analyses

We used one-way ANOVA followed by the Holm-Sidak method to examine the differences in the concentration of the measured variables between the site with the floating iron-oxide film and the rest of the sites. The statistical analyses were conducted using the SigmaPlot for Windows package (v 11.0). Significant differences between the 16S rRNA gene fingerprints of the natural bacterial communities were estimated using the fingshuf software according to Portillo and González (2008).

RESULTS

The top sediment samples of the groundwater seepage areas analysed in this study were generally rich in Tot-Fe because concentrations higher than 180 μmol/g d.w. were recorded in 10 out of 12 sites. The organic matter and Tot-P concentrations in the sediment ranged widely (1.2-34.8 % and 2.3-121.7 μmol/g d.w., respectively) and were significantly correlated with each other ($r = 0.739$, $p < 0.01$). The concentrations of Tot-P and Tot-Fe in the sediment also showed a significant correlation ($r = 0.760$, $p < 0.01$). At the sites where the sediment organic matter concentration was higher than 9 %, dissolved ferrous iron (Fe^{2+}) was detected in the sediment pore water at a concentration ranging from 10.7 to 159.4 μM.

The highest concentration of sediment Tot-Fe (1420 μmol/g d.w.) was found on a narrow fringe (< 0.5 m) along the SW shoreline of Dulce pond covered by patches of a fine floating metallic film (with a thickness < 1 mm). Its chemical composition revealed a predominance of Fe (87.0 % of dry weight in the digested sample) with traces of other metals, such as Mn (0.32 %). The water collected at a distance of 1 m from the SW shoreline toward the centre of the pond did not contain this floating iron-oxide film, but the precipitation of colloidal Fe was visible in the water
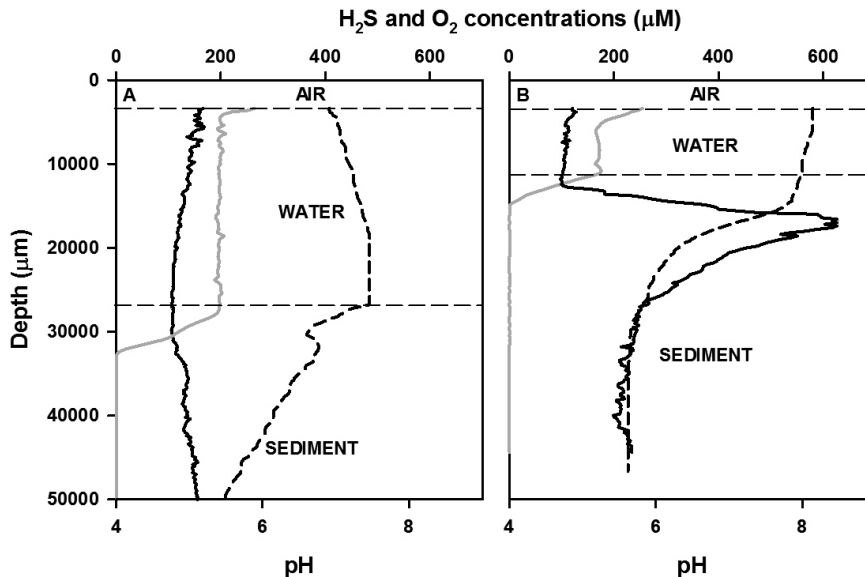

Figure 2. Profiles of H_2S (black line) and O_2 (grey line) concentrations and pH (short-dashed black line) in sediment cores collected from the SW and NE shores of Dulce pond (A and B, respectively). The air-water and water-sediment interfaces are indicated by long-dashed horizontal lines. *Perfiles de las concentraciones de H_2S (línea negra) y O_2 (línea gris), y el pH (línea negra punteada) en muestras de sedimento tomadas de las orillas SW y NE de la laguna Dulce (A y B, respectivamente). Las interfases aire-agua y agua-sedimento se indican mediante líneas de trazos horizontales.*

column. On the opposite NE shoreline, no iron-oxide film or colloidal Fe was visible, and the concentration of Tot-Fe on the top sediment was only 22 $\mu mol/g$ d.w.

A detailed characterization of several environmental features of both the SW and NE shorelines was then performed in June 2005 (Table 1). The surface water trapped below the floating iron-oxide film observed at a distance of 0 m from the SW shoreline exhibited several particular features compared with the other surface and pore water samples, and many of these differences were significant (Table 1). The water below this film showed the lowest pH and concentrations of O_2, HCO_3^-, Na^+, Ca^{2+}, Mg^{2+}, K^+, and Tot-S compared with all of the other surface sites and it presented the highest concentration of Tot-P, Tot-Fe, NH_4^+ and Zn. Additionally, the concentrations of HCO_3^-, NO_3^-, NH_4^+, Ca^{2+} and Tot-P were significantly lower in the surface water below this film than in the deepest pore water samples collected on the SW side (Table 1). The surface sediment below the film was relatively rich in organic matter (15.1 %), Tot-Fe and Tot-P (254.3 and 29.9 $\mu mol/g$ d.w., respectively).

The profiles of the O_2 concentration in a sediment core determined by microelectrodes showed an initial decline of O_2 just below the air-water interface and a second decline at the sediment-water interface that resulted in the rapid decrease in the concentration of O_2 with increasing depth (Fig. 2). The H_2S concentration showed a relatively constant profile with a low H_2S production at the SW shore and a sharp H_2S production layer at the NE shore just below the depth,

where O_2 was depleted. The depth profiles of pH showed relatively constant values at depths with measurable O_2 concentrations and a slight acidification at increasing depths in the anoxic zone of both the SW and NE shorelines (Fig. 2).

Anaerobic incubations of water and sediment samples collected from the natural environment resulted in visible bacterial growth, as judged by a turbidity analysis and phase-contrast microscopy. Floating iron-oxide films were observed in anaerobically incubated samples (Table 2). Aerobically incubated samples and any sample maintained in darkness did not result in iron-oxide film formation. Cultured enrichments obtained in G medium and incubated under anaerobic conditions (either in N_2 or $H_2:CO_2$, 80 %:20 %, atmosphere) showed both bacterial growth and formation of a floating iron-oxide film on the medium surface. During growth, a change in coloration of the medium could be observed. The original colour of the medium was dark brown, this colour cleared over the incubation to become transparent, and a black film was eventually observed floating on the surface of the cultures. After exposure to oxygen (air atmosphere), the colour of these floating films turned to ochre, and the films presented a similar appearance to natural iron-oxide films in the natural environment. Further incubation under illumination conditions with exposure to oxygen resulted in a thickening of the film, and anaerobic conditions persisted under the film (Table 2).

A molecular survey of the microorganisms present in the laboratory cultures was performed. The major bacterial components were detected by DGGE profiling and identified through clone

Table 2. Formation of a floating film in laboratory incubations of the original water/sediment samples under different conditions and their respective controls. Repeated G-medium enrichments where only performed for those incubations in which floating films formed. *Formación del film flotante en las incubaciones de laboratorio provenientes de las muestras de agua/sedimento bajo diversas condiciones, y sus respectivos controles.*

	Anaerobic conditions		Aerobic conditions		O_2 exposure after anaerobic conditions
	Light	Darkness	Light	Darkness	
Original sample incubations	YES	NO	NO	NO	YES[2]
G-medium enrichments	YES	NO[1]	—	—	YES[2]
Sterilized controls	NO	NO	NO	NO	NO

[1]Very poor film formation; [2]It turns to ochre and thickens.
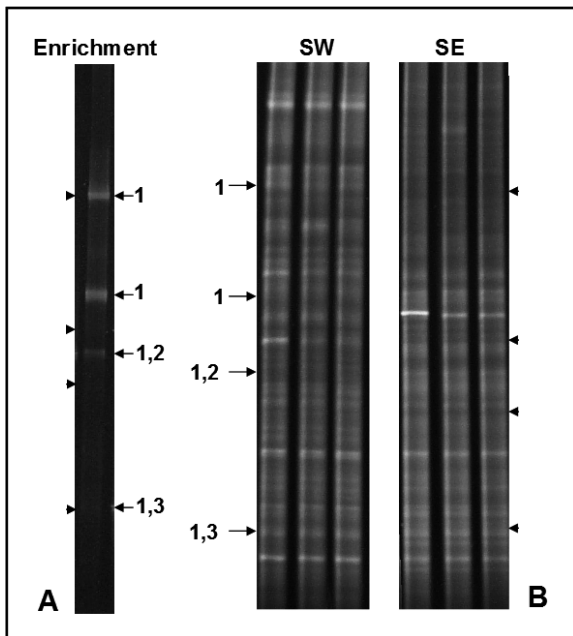
Figure 3. A: DGGE fingerprints based on DNA extracted from the Fe-film formed through cultivated enrichment. B: DGGE fingerprints based on RNA extracted from the natural communities of the SW and NE shores of Dulce pond. The numbered arrows indicate the migration of the different phylotypes identified in the cultivated enrichment: 1, *Enterobacter*; 2, *Clostridium*, and 3, *Anaeroarcus*. The unnumbered arrowheads indicate the location of migration markers corresponding to (from top to bottom) *Pseudomonas aeruginosa*, *Escherichia coli*, *Paenibacillus* sp., and *Streptomyces caviscabies*. *A: Perfil DGGE de los cultivos enriquecidos formadores de la película de Fe. B: Perfiles duplicados de las comunidades naturales de la orilla SW y NE de la laguna Dulce. Las flechas con número corresponden a la migración de los distintos filotipos identificados en los cultivos enriquecidos: 1,* Enterobacter*; 2,* Clostridium*, y 3,* Anaeroarcus*. Las puntas de flechas sin número corresponden a la migración de los siguientes marcadores (de arriba a abajo):* Pseudomonas aeruginosa, Escherichia coli, Paenibacillus sp., *y* Streptomyces caviscabies.

screening and sequencing from the constructed DNA libraries (Fig. 3). Bacteria belonging to the genus *Enterobacter* were the major representatives of the cultured enrichments forming the floating iron-oxide films and represented only a minor component of the natural communities. This genus was present as several phylotypes showing differential migration on the DGGE fingerprints. Firmicutes, which are closely related to the genera *Anaeroarcus* and *Clostridium,* were also detected at lower abundances in the DNA libraries and the DGGE analysis results.

The metabolically active microbial communities from the SW and NE shores were analysed through DGGE fingerprinting and construction of 16S rRNA gene libraries and sequencing. A total of 252 clones were analysed (131 and 121 clones from the SW and NE shores, respectively), and the results revealed 90 different OTUs (46 and 44 OTUs from the SW and NE shores, respectively). Among the microorganisms identified from these samples, typical iron-reducing and oxidizing specialist bacteria were scarce (1.6 % of the total number of clones) and thus represented a minor metabolically active component of the microbial communities from both of the two studied shores. These potentially iron-reducing bacteria were detected at both shores and were related to the genera *Leptothrix* and *Gallionella* (Betaproteobacteria) and *Geobacter* (Deltaproteobacteria). The phylum distribution of the metabolically active microorganisms detected by sequencing of the 16S rRNA gene libraries is shown in Table 3. The nucleotide sequences obtained in this study can be accessed under numbers from JN699913 to JN700019. The major functional component of these communities were phototrophic microorganisms (approximately 29 % of processed clones), including both eukaryotic algae (chlorophytes and diatoms) and cyanobacteria. Cyanobacteria were present at a lower proportion in the metabolically active community at the SW (10.7 %) compared with the NE (38.0 %) shore. The presence of Betaproteobacteria (*Leptothrix* and *Gallionella*) among the metabolically active microorganisms was detected at a higher proportion in the film-covered SW shore (35.9 %) than in the NE shore (17.4 %), and most of the clones showed highest homology to sequences from uncultured bacteria.

The level of coverage achieved during the analysis of the 16S rRNA gene libraries performed in this study represented a fraction of 0.75-0.79. The rarefaction curves presented for the SW and NE shores, which were estimated at 97 % similarity for OTU differentiation, indicated similar levels of diversity at both sides of the pond. When phyla were considered, the rarefaction curves showed that most of the major

Table 3. Proportion of metabolically active microorganisms classified into different phyla that were detected at the SW and NE shores of Dulce pond. *Proporción de microorganismos metabólicamente activos clasificados en distintos filos que fueron detectaron en las orillas SW y NE de la laguna Dulce.*

| Phylum | Percentage of total processed clones | | |
	SW	NE	Total
Eukaryotic algae	29.8	28.9	29.4
Betaproteobacteria	35.9	17.4	27.0
Cyanobacteria	10.7	38.0	23.8
Deltaproteobacteria	5.3	3.3	4.4
Bacteroidetes	3.0	5.8	4.4
Alphaproteobacteria	2.3	4.1	3.2
Verrucomicrobia	5.3	0.0	2.8
Fusobacteria	3.0	0.0	1.6
Chloroflexi	0.8	1.6	1.2
Spirochaete	1.5	0.0	0.8
Gammaproteobacteria	1.5	0.0	0.8
Chlorobi	0.8	0.0	0.4
Firmicutes	0.0	0.8	0.4

taxonomic categories present at these sites were detected. A higher number of bacterial phyla were detected in the film-covered side of the pond (12 phyla) than at the film-free side (9 phyla).

The chemical composition of the natural floating film revealed a dominance of Fe as the most abundant component (87 % of total). The proportion of Fe after digestion in the incubated film was very similar to that obtained in the natural film (Table 4). Si was the second most common element in the natural film, as expected by the quartzitic nature of these sandy soils (average concentration of Si in the water of the groundwater seepage area was 324 μM). In contrast, in the film obtained at the laboratory, Na^+ was the second most common element in abundance, probably due to the use of sodium salts in the medium composition. The X-ray diffraction patterns showed that the presence of two-line ferrihydrite was common to the iron-oxide films collected in both the natural environment and the laboratory cultures, whereas other oxides, such as goethite and low-crystalline lepidocrocite, were detected only in the natural environment.

Table 4. Concentrations of main elements after digestion of the film incubated in the laboratory and the natural film collected from the study pond. *Concentraciones de los principales elementos tras la digestión del film obtenido tras incubación en el laboratorio, y del film natural recogido en la laguna estudiada.*

| | incubated film | | natural film | |
	μmol/g d.w.	(%)	μmol/g d.w.	(%)
Fe	1476.8	79.24	6218.8	87.00
Na	178.3	9.56	129.1	1.81
P	65.4	3.51	67.4	0.94
Si	53.4	2.87	293.9	4.11
S	33.2	1.78	96.1	1.34
Ca	26.6	1.43	179.2	2.51
Al	10.3	0.56	20.1	0.28
K	6.5	0.35	33.1	0.46
Mg	3.9	0.21	74.6	1.04
$Zn-H_2$	3.5	0.19	4.9	0.07
W	2.2	0.12	0.3	<0.01
B	2.0	0.11	3.0	0.04
Mn	0.4	0.02	22.7	0.32

DISCUSSION

Dulce pond is a flow-through shallow system where dilute groundwater out-seepages into the pond along the western margin (upgradient end) and recharges the ground water by in-seepage at its downgradient end on the eastern shore (Sacks *et al.*, 1992). Groundwater feeding remains relatively constant throughout the year, creating an in-seepage zone along the western side of this pond, where the water is slightly acidic and isotopically lighter and has a more diluted concentration of major cations and anions than that at the eastern margin; evaporation on the pond surface and calcite dissolution as water seeps back into the shallow aquifer explain the differences in the concentration of these ions (Sacks *et al.*, 1992). Thus, this mechanism accounts for the higher concentration of ions in both the surface and pore water samples collected from the NE side in the present study (Table 1). The concentration of NH_4^+ was significantly higher on the SW side and was maximal in the sediment pore water compared with the surface water samples. In contrast, the concentration of NO_3^- in the surface and pore water samples was significantly lower compared with that detected in the NE side. The accumulation of reduced dissolved inorganic N in water pockets covered by the iron-oxide film may be due to poor nitrifying activity resulting from the suboxic conditions found along the SW shoreline (Table 1).

The accumulation of oxides on the sediment surface due to groundwater out-seepage resulted in the observed differences in Fe concentration between both sides of the study site. The concentration of Fe^{2+} in the water underneath this iron-oxide film has been reported to reach 18.8 mg/L (Portillo *et al.*, 2008). This concentration is high compared with those observed in other freshwater and brackish environments (Colliene, 1983; Luther *et al.*, 1992; Shaked *et al.*, 2004) but similar to the concentrations recorded in the Tinto River, an acidic river (mean pH = 2.5) that drains across the Iberian Pyrite Belt and reaches the Atlantic coast to the west of our study sites (López-Archilla & Amils, 1999). Because the pH in the study pond was never as low as in the Tinto

River, where sulphur- and iron-oxidizing bacteria are common (López-Archilla *et al.*, 2001), the concentration of Fe^{2+} in the SW margin of the study pond requires a different explanation. Additionally, typical iron-reducing and oxidizing specialist bacteria were not identified as the major metabolically active bacterial components at the study site (Table 3).

The relevance of light in the formation of iron-oxide films in the laboratory incubations of the water and sediment samples (Table 2) suggests that the light-induced redox cycling of iron may provide an explanation for the presence of this floating iron-oxide film in the natural environment. According to Sulzberger *et al.* (1989), a relatively high concentration of Fe^{2+} can be preserved at oxic-anoxic boundaries when the oxygenation rate is slow compared with the reduction of Fe^{3+} due to the presence of surface complexes of iron-oxyhydroxides (or FeOOH). This reductive dissolution of FeOOH surfaces is a photocatalytic process that is usually accompanied by the reoxidation of Fe^{2+} by O_2 and a further Fe-mediated oxidation of organic matter (Stumm & Sulzberger, 1992). As a result, a high concentration of insoluble FeOOH overlies a concentration peak of Fe^{2+} at the oxic-anoxic boundary (Sulzberger *et al.*, 1989). In the littoral of lakes, the oxic-anoxic interface is generally set at the sediment surface or at a depth of a few centimetres into the sediment (Gerhardt & Schink, 2005). This was the case at the NE shore of the study pond, where the concentration of Tot-Fe increased drastically in the sediment pore water samples collected at depths below 5 cm probably due to the reductive dissolution of iron-oxides. At the SW shoreline, in contrast, the oxic-anoxic boundary was positioned at the air-water interface likely due to the seepage of poorly oxygenated groundwater and the low impact of the dominant wind on the shoreline protected by the dune. The iron-oxide film did not remain intact at a distance of 1 m from the SW shoreline because the water surface was no longer protected from the wind action by the dune steep, which caused the film to start to break and dissolve. The direct mixing of groundwater seepage with pond open-water was not likely the cause of the disappear-

ance of the iron-oxide film at a distance of 1 m because the concentration of major conservative ions in the water (Cl^- and Na^+) was not significantly different between the samples collected at distances of 0 and 1 m. Therefore, the concentrations of Tot-Fe and Fe^{2+} in the water were still high at a distance of 1 m, where particles of colloidal iron were present, especially when the water pH was below 7.

Because the concentration of Tot-S and sulphide production were relatively low at the SW side, the concentration of sulphide was not sufficiently high to induce the abiotic removal of all the iron via the formation of FeS. The concentration of Tot-Fe in the surface and pore water samples was higher on the SW side, and Fe^{2+} was maintained in solution due to the existing reducing conditions. On the NE side, the elevated production of sulphide by sulphate-reducing bacteria at the upper sediment layers likely trapped iron, resulting in the formation of FeS precipitates. In fact, sulphate-reducing bacteria have been reported exhibit decreased activity at high iron concentrations (Lovley & Phillips, 1986; Chapelle & Lovley, 1992). Thus, the lower sulphate-reduction activity observed at high iron concentration suggests a larger availability of low-molecular-weight organic compounds (otherwise consumed by sulphate-reducing bacteria), which can become a nutrient resource for other heterotrophic bacteria, as was the case in these organic-rich environments. These results support the findings obtained in previous studies (Lovley & Phillips, 1986; Chapelle & Lovley, 1992), indicating the existence of competitive bacterial phenomena regulated by the iron concentration in groundwater and shallow marshes.

The inorganic matrix of this natural film was mainly composed of Fe (87 %), followed by Si, Ca^{2+}, Na^+, S, Mg^{2+} and traces ($< 1 \%$) of P, K, Mn, Al, Zn, B, As, Sr and Cu. Trace metals can be expected to be found due to the metal-binding capacity of iron-oxyhydroxides (Nelson et al., 1999). The composition of the iron-rich films obtained in this study was very similar to that found in the air-water interface of Fe^{2+}-rich groundwater seepage discharged at the base of Pleistocene sand dunes in Oregon (pH ~ 6.0).

These iron-rich films were composed of two-line ferrihydrite upon further abiotic oxidation, as is commonly the case in organic-rich environments with pH levels of ~ 5.0 (Grathoff et al., 2007). Organic matter, which reached 14 % based on the measurement of organic carbon, was a significant component of floating surface films in two Swedish groundwater discharge areas with pH values similar to those found in our study sites (Kleja et al., 2012). Ferrihydrite was likely present as small particles with humic material sorbed onto surfaces or included in the particles, making the particles sufficiently hydrophobic to avoid settling in the absence of significant physical disturbance (Kleja et al., 2012).

Using as an analogy the iron-oxide film produced by laboratory enrichment through anaerobic incubations and its thickening in the presence of light, we suggest that the formation of this floating film in nature is due to a combination of both biotic and abiotic processes. Common by-products of the metabolism of fermenting bacteria are effective anionic ligands for Fe^{3+} reduction, and bacteria (including non-metabolizing iron bacteria such as *Bacillus subtilis*) can adsorb Fe^{2+} onto their cell surfaces and facilitate iron-oxide formation (Châtellier & Fortin, 2004). Furthermore, both bacterial organic acids produced during anaerobic degradation and fermentation processes of complex organic matter and bacterial extracellular polymers can initiate the photocatalytic binding and oxidation of iron because they adsorb positively charged Fe-oxides (Sulzberger et al., 1989). An alternative cycling pathway may be the rapid oxidation of Fe^{2+} in the presence of moderate concentrations of both O_2 and H_2O_2 in sunlit surface waters and the subsequent reduction of Fe^{3+} by reductants, such as superoxide, formed upon solar irradiation at circumneutral pH (Emmenegger et al., 2001). The growth of facultative anaerobic bacteria, such as *Enterobacter*, may be an essential part of this redox cycling because these organisms produce H_2O_2 during heterotrophic metabolism under suboxic conditions and can use Fe^{2+} as an electron acceptor to neutralize this H_2O_2 production (Ghiorse, 1984; Sorokin, 1999). This iron-oxide film could bring

other advantages to the growth of facultative anaerobic bacteria as this floating film may provide bacteria a photoprotection mechanism because intense direct sunlight represents one of the major factors affecting the viability of Gammaproteobacteria and Firmicutes, among other bacteria (Barcina *et al.,* 1990).

In conclusion, a combination of environmental factors, such as protection against wind, groundwater seepage, and the existence of a sandy aquifer, and the activity of ubiquitous heterotrophic bacteria were likely responsible for the formation and prevalence of iron-oxide films floating on the water surface in the study site. Slightly acidic and suboxic conditions were maintained in areas where this iron-oxide film was intact, creating significant differences in the nutrient content, trace metal concentrations, and bacterial communities between different areas of the same water body. The accumulation of iron-oxides increased the pool of P bound to iron oxyhydroxides in the sediment, whereas the reducing conditions promoted the solubilization of Fe(II) and Zn and the accumulation of NH_4^+ in both surface and pore water. In contrast to other natural environments with iron-oxide depositions, our results describe the formation of floating iron-oxide films by microorganisms not belonging to the typical iron-related bacterial genera and their interaction with geohydrological and chemical phenomena in the iron redox cycle of a freshwater wetland. The finding of the formation of a thin iron-oxide layer in anaerobic laboratory incubations from the natural sediment samples indicates a greater contribution of the heterotrophic bacterial community to iron transformations than previously thought due to the ubiquity of these bacteria in natural environments rich in organic matter.

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