Neutrophilic iron-oxidizing bacteria: occurrence and relevance in biological drinking water treatment
A. Gülay, S. Musovic, H.-J. Albrechtsen and B. F. Smets

ABSTRACT
Rapid sand filtration (RSF) is an economical way to treat anoxic groundwater around the world. It consists of groundwater aeration followed by passage through a sand filter. The oxidation and removal of ferrous iron, which is commonly found in anoxic groundwaters, is often believed to be a fully physicochemical process. However, persistently low temperatures in RSF across Denmark may negatively affect the kinetics of chemical oxidation. The slower chemical oxidation of ferrous iron may increase the chances for iron bioconversion by neutrophilic iron-oxidizing bacteria (FeOB), which are found naturally in many environments. In this study, we used a combination of a cultivation-based opposing gradient enrichment technique and 16S rRNA gene targeted molecular tools to isolate, quantify and identify FeOB from a RSF. The microscopic quantification of selectively enriched FeOB cells revealed that in RSF, neutrophilic iron oxidizers were present at the level of up to $7 \times 10^5$ cells g$^{-1}$ sediment. The spatial abundance and diversity of FeOB inferred by denaturing gradient gel electrophoresis fingerprinting differed greatly both between and within individual sand filters. The results suggest a larger than assumed role of FeOB in iron removal at waterworks using RSF technologies.

Key words | diversity, FeOB, iron-oxidizing bacteria, neutrophilic, rapid sand filtration

INTRODUCTION
Rapid sand filtration (RSF) has a widespread application for drinking water (DW) treatment in Denmark and other European countries. In general, RSF consists of three steps: (i) aeration, (ii) pre-filtration, and (iii) post-filtration. The removal of surplus chemicals and gases (e.g. ammonium, iron, methane) from groundwater in RSF is either a physicochemical or biological process. The removal of naturally occurring ferrous iron from groundwater is an important process, as iron residues may cause clogging issues in water distribution systems, and change the taste of tap water. Ferrous iron bioconversion by environmental bacteria is a well recognized process. However, the extent of ferrous iron bioconversion in RSF under the circumneutral pH and low temperature of anoxic groundwater is still unknown. Under those conditions, ferrous iron can be transformed both via enzymatic- (Sobolev & Roden 2001) and autocatalytic- (Rentz et al. 2007) or self-accelerating oxidative mechanisms (Stumm & Morgan 1996).

The intensive studies on chemical ferrous iron conversion revealed that iron is highly unstable in oxic environments ($>200 \mu$M O$_2$) at neutral pH, and is rapidly auto-catalytically oxidized ($t_{1/2}$: 10–37 min) regardless of the presence of enzymatic catalysts (Stumm & Morgan 1996). The auto-catalytic oxidation together with a surface-catalyzed oxidation on microbial cell walls (Chan et al. 2010) are suggested to be the important obstacles that limit Fe$^{2+}$ accessibility for bacterial oxidation (Emerson & Moyer 1997). Nevertheless, under the conditions of low temperature (5–10 °C), typically found in groundwater, kinetic measurements indicate a greatly (>10-fold) reduced rate of chemical iron oxidation ($t_{1/2}$: 315 min) (Sung & Morgan 1980). The presumed slow chemical oxidation of Fe$^{2+}$ in RSF filter beds may, then, allow bacteria to biologically oxidize iron simultaneously with an on-going slow chemical oxidation, thereby contributing more to iron oxidation than believed.
The understanding of the iron bioconversion process and its contribution to the overall removal of iron from groundwater has been limited by our ability to isolate and culture the majority of iron oxidizers under laboratory conditions. Iron-oxidizing bacteria (FeOB) are poorly presented in nucleotide sequence databases (Hedrich et al. 2011). Moreover, functional gene documentation (Wang et al. 2009) is limited, emphasize the need for more intensive studies of FeOB, in order to predict their contribution to iron oxidation in RSF or similar environments.

Hence, the aim of this study was to investigate the abundance, diversity, and spatial distribution of FeOB in a highly oxic environment of RSF at waterworks. The neutrophilic FeOB were enriched and quantified by a standardized opposing-gradient technique. Morphologies and diversity were characterized by confocal microscopy (CLSM) and polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) techniques, respectively.

**MATERIALS AND METHODS**

**Water and sediment material sampling**

Water and filter material samples were collected at Islevbro, which is one of the largest waterworks in Denmark, supplying DW to the Copenhagen area. The ground water is subjected to RSF with the following treatment chain: aeration, prefiltration in a rock filter bed (particle diameter 10–20 cm), and after-filtration in 12 sand bed filters with coarse-grained sand (particle diameter 2–5 mm) overlaying a supporting gravel medium layer.

Samples were collected from all three units. Samples from the aeration chamber were collected by scalping the biomass from the mashed aeration tubes. Pre-filter samples were collected as sludge on the top of filter material. Sampling of the after filter sand beds was more extensive, and included water and sand material taken just above, or within, top 10 cm of filter bed, respectively. Samples from two independent filters were collected from three random positions (triplicates) 1 h before and after backwashing practice. The first sampling was performed half way in a filter run length, which corresponded to approximately 8 days since last backwashing practice. In addition, bulk water samples were collected from the main stream line before the aeration chamber (called raw water) and between pre-filter and afterfilter unit (called inlet water). Sampling was performed in August 2011 and samples transported on the ice, kept refrigerated and processed or permanently stored at −20 °C within 7 h. Microbial biomass in bulk water was harvested by filtration of 250 ml through a 0.2 μm pore size filter (Millipore) and stored at −20 °C.

**Most probable number counts**

Most probable number (MPN) was used to estimate the amount of metabolically active FeOB in sediment samples from pre- and after filters. Ten-fold dilution series of samples were made in sterile demineralized water and inoculated in opposing-gradient enrichment media (see below). All MPN media were run in triplicates, incubated at 10 °C for 20 days prior to visual inspection of growth. The number of FeOB in samples was estimated according to MPN tables in Man (1985).

**Enrichment and isolation of autotrophic FeOB**

Inoculation of growth media specific for FeOB was made by samples from an after filter. These samples were (a) regular bulk water, (b) pore water obtained by slow centrifugation (1,800g, 10 min) of the drained sand grains (Gobet et al. 2012) and (c) residual sand material suspended in 5 ml sterile water. Furthermore, from the analysed pre- and after filters, sludge or sand material were directly suspended in 5 ml sterile water and used as inoculum for enrichment experiments.

The cultivation media were made according to Emerson & Floyd (2005). Briefly, the upper part of a twolayered growth medium in a gradient tube (volume 6.75 ml) contained 0.15% (wt/vol) agarose (low melt agarose; Sigma-Aldrich), 0.5 mM sodium bicarbonate and pH was adjusted to 7.10. Wolfe’s vitamin and trace element solution (http://www.lgcstandards-atcc.org, MD-VS, MD-TMS) was added to a final concentration of 1 ml/l media. The lower part of growth medium (volume 1.25 ml) contained equal amount of fresh FeS and MilliQ water (vol/vol), added 1% (wt/vol) agarose. The gradient tubes were kept at +10 °C for 24 h prior to
microbial inoculation (see above), in order to allow formation of Fe$^{2+}$ and O$_2$ gradient. The inoculation of culture media was performed by slowly expelling the inoculum suspension while vertically moving the micropipette from the bottom to the top of media in a gradient tube. Control experiments consisted of gradient media inoculated with (i) a heterotrophic strain isolated on R2A agar media, (ii) gradient medium without the FeS plug, and (iii) abiotic controls prepared without bacteria. All enrichments were incubated at $+10$ °C to mimic the water temperature at waterworks.

The growth of FeOB in gradient media was identified by formation of brownish iron oxides band between the oxic and anoxic interface. The bands were visually compared to the more diffuse bands, which typically develop in control tubes not containing bacteria due to chemical oxidation of ferrous iron. The presence of microbes in brownish bands from gradient media was confirmed by microscopic visualization and molecular analysis (see below).

Bacterial cells from enrichment tubes at highest dilutions that showed microbial growth in bands were collected. Two subsequent serial dilutions cycles were repeated to obtain enrichments with uniform cell morphologies. The cell homogeneity was verified by epifluorescence microscopy and DGGE technique.

**Cell enumeration and morphological analysis**

The total number of bacterial cells isolated from gradient tubes and liquid cultures was estimated by direct microscopic counting (TDC) of (Syto-9, Molecular Probes, Inc., Eugene, OR) stained cells using Confocal Scanner Laser Microscopy (SP5; Leica). The acquired CSLM images were analysed with image analysis software (Image Pro Plus 4.1). Confocal microscopy with depth scanning allowed a better quantification of bacterial cells enmeshed in a matrix of iron oxides.

**Fluorescence in situ hybridization**

The bacterial cells from enrichments were also harvested, resuspended in phosphate buffered saline and fixed with 4% paraformaldehyde on ice. The fixed cells were hybridized with a mixture of eubacterial EUB338 (I,II,III) oligonucleotide probes (Amann et al. 1990; Daims et al. 1999), labelled at the 5’th end with FLUOS or Cy5 fluorochromes. Stained cells were visualized by CLSM.

**Nucleic acid extraction and PCR amplification of 16S rDNA**

Genomic DNA from the filter material and the enriched cultures was extracted using MP FastDNA™ SPIN Kit (MP Biomedicals LLC, Solon, USA) according to manufacturer’s instructions, and DNA in Tris–EDTA buffer was stored at $-20$ °C. The concentration and purity of extracted DNA was checked by NanoDrop (ThermoFisher Scientific). The highly variable V3 region of the bacterial 16S rRNA gene was amplified using the bacterial-specific primer set, 541F-GC (5’-GC-clamp-CCTACGGGAGGCAGCAG) and 518R (CCGTCAATTCMTTTGAGTTT) (Muyzer et al. 1993). PCR reactions (25 μl) contained 1 ng of template DNA, 0.2 U Tag polymerase, 12.5 pmol of each primer, 50 mM KCl, 30 mM Tris/HCl, 1.5 mM MgCl$_2$, and 12.5 mM dNTPs (Sigma Aldrich) and molecular biology grade water (5-Prime) in a reaction volume of 25 μl. Amplification involved an initial denaturation at 92 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 30 s at 72 °C, and a final extension reaction for 5 min at 72 °C.

**Denaturing gradient gel electrophoresis**

PCR products were loaded on a DGGE gel with a 35–53% denaturation gradient (100% denaturant solution contains 7M urea and 40% (vol/vol) formamide) and run for 16 h at 85 V and 60 °C. DGGE gels were stained with SYBR Gold stain (Invitrogen) in 1× TAE buffer for 50 min in the dark, DNA bands visualized and images recorded by the GelDoc (BioRad). DGGE bands were excised from the gels with a sterile blade, transferred to a sterile 1.5 ml tube containing 45 μl water and incubated overnight to allow DNA elution. Aliquots were used as template for PCR re-amplification using same primer set without GC clamp. The new PCR products were purified using a Qiaquick PCR purification kit (Qiagen Inc) and used for sequencing reaction with 341F primer. The partial DGGE sequences (170 bp) were analysed by MEGA5 software and compared against the NCBI nucleotide databases (http://www.ncbi.
Comparative sequence principal component analysis

Principal component analysis (PCA) involved a mutual comparison of DGGE bands obtained in different experiments, where each band corresponded was defined as an operational taxonomic unit (OTU). Briefly, DGGE bands on each gel were digitally normalized to a well-known reference heterotrophic isolate, using BioNumerics v4.0 software (Applied Maths). Bands were subsequently defined for each sample using the band search algorithm, and matrixes relying on band presence were generated.

RESULTS AND DISCUSSION

The presence, density, and diversity of FeOB across a water works was examined with a specific emphasis on their occurrence in the rapid sand filters. In general, the prevalence of chemical iron oxidation might be expected, if only pH and dissolved oxygen values were taken into account (Stumm & Morgan 1996), but the low groundwater temperatures (5–10°C) would reduce chemical oxidation rates, increasing the opportunities for microbial iron oxidation.

The possibility for biological iron oxidation was confirmed by Gibbs free energy calculations (at 0–20°C). Estimations showed that thermodynamically, biological iron oxidation in RSF is possible (−7.32 to −6.03 kCal/mol) at neutral pH, even at ferrous iron concentrations as low as 0.015 mg/L. In addition, the microscopic analysis of bacterial cells enriched from pre- and after filters at Islevbro waterworks confirmed the presence of cell morphologies similar to the well-studied iron oxidizer *Leptothrix* sp. (Figure 1).

The formation of brownish bands containing bacteria in gradient tubes with microbial inoculation was observed starting at Day 4. A different positioning of bands in gradient media containing bacterial cells in comparison to amorphous bands in control tubes, was an good indicator for specific bacterial growth in these bands (Figure 2) (Emerson & Moyer 2002). To verify whether heterotrophic bacteria were responsible for band formation in FeOB selective gradient media, a heterotrophic isolate from R2A plates was used. The tubes with gradient media were inoculated with heterotrophic bacterial solutions ranging from 10⁴ to 10⁹ cells per inoculated tube. Interestingly, the positioning of produced brownish bands in heterotrophic tubes was identical to the positioning of brownish bands in control (abiotic) tubes, but different from bands produced in gradient tubes inoculated with microbes from RSF. The observation suggests that heterotrophic biomass from RSF did not contribute to formation of the bands of interest in FeOB selective gradient media.

The density of FeOB ranged from 1.9×10⁴, 10⁴ to 10⁵ and 1×10⁴ to 7×10⁵ cells g⁻¹ sand (wet weight) in a pre-filter and two replicate after filter sand beds, respectively. The variation in FeOB abundance was up to 10-fold different between different sampling points within after filter beds.

Figure 1 | Surface of a sand grain from an after filter (green fluorescence due to Syto9 stain) (A), bacteria (Syto9 stain) enmeshed in iron oxides (red autofluorescence) on iron sludge from pre-filter (B), and *Leptothrix*-like bacterial morphologies from pre-filter (FISH with EUB 388 FLUOS probe) (C). The full colour version of this figure is available online at http://www.iwaponline.com/ws/toc.htm.
sand beds, but similar across filters. We speculated that the heterogeneity of filter material in the form of sand grain size, biofilms, precipitates, etc. could create large spots within a filter with a higher than average microbial abundance, or create micro-niches on sand grain particles with favourable conditions for FeOB. More FeOB cells were retrieved from macro- and medium pores by slow centrifugation in comparison with

Figure 2 | Opposing gradient enrichment tubes for FeOB selective inoculated with suspensions obtained from RSF (tubes 1–4, left to right) and abiotic controls without bacterial inoculums (tubes 5–10, left to right).

Figure 3 | (A) DGGE fingerprints of FeOB enrichments initiated with different inoculums obtained from different sampling locations at Islevbro waterworks. The labelled bands were subsequently sequenced (see Table 1). (B) Principal component analysis plot based on of DGGE profiles shown in Panel A.
the sand surface (data not shown). These observations suggest that the pores are not more favourable niches for FeOB than the sand surface, and a larger Fe-oxidizing potential may be located on the surface of sand grains. However, we do recognize the fact that efficiency of cell extraction from pores by slow centrifugation might not be complete.

The structure of microbial enrichments in the FeOB selective gradient media was characterized by DGGE fingerprinting (de Vet et al. 2009). DGGE fingerprints showed up to 22 dominant OTUs (Figure 3). A sequence analysis of representative bands showed a high similarity (up to 100%) to formerly deposited sequences in GenBank (Table 1), with most sequences belonging to the Proteobacteria. All currently identified oxygen-dependent neutrophilic iron oxidizers are Proteobacteria (Emerson et al. 2010). The short sequence length of obtained sequences (150 bp) limited the phylogenetic analysis at species level, however, allowed us to work at higher taxonomic levels (Schloss & Westcott 2011).

Comparison of DGGE profiles from different enrichments by PCA revealed that very few bands were present in more than a single enrichment from a RSF. The spatial heterogeneity of FeOB enrichment composition along with the spatial heterogeneity FeOB density (inferred from MPN), strongly suggest spatial variations within a single RSF which affects FeOB.

**CONCLUSIONS**

In summary, the present study showed that FeOB were abundant in the examined rapid sand filters (up to ca. 10^5 cells g^{-1})

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sand) suggesting a significant bioconversion potential for ferrous iron removal. The surprising heterogeneous spatial distribution of FeOB density and FeOB enrichment structure indicates that RSF cannot be considered as well mixed homogeneous environments. To what extent the FeOB contribute to ferrous iron oxidation and removal awaits further investigations.

REFERENCES


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